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PURIFICATION AND CHARACTERIZATION OF EEL WHITE MUSCLE PYRUVATE KINASE

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DEDICATION

To my parents,
family and friends

SUMMARY

Pyruvate kinase (ATP:pyruvate 2-O-phosphotransferase, EC 2.7.1.40) has been purified from the white muscle of the American eel, *Anguilla rostrata*, in good yield and its kinetic and structural properties have been compared to those of other pyruvate kinase isozymes. The enzyme was purified by ammonium sulphate precipitation, batch-wise treatment with CM-cellulose followed by DEAE-cellulose chromatography and gel filtration chromatography on Sephacryl S-300. The preparation was at least 95% pure as judged by electrophoresis on polyacrylamide gels. In the latter steps of the purification, 30% (v/v) glycerol was added to the buffers in order to stabilize the enzyme.

The enzyme activity was found to be maximal between pH 6.0 and pH 6.5 in three different buffers at 30°C. At the pH optimum and 30°C, the eel muscle enzyme exhibited Michaelis-Menten kinetics with respect to both the substrates adenosine 5'-diphosphate (ADP) and phospho-enol-pyruvate (PEP). However eel muscle pyruvate kinase, unlike the mammalian muscle M_1 isozyme, is activated by fructose-1,6-bisphosphate (FDP) in a complex and pH-dependent manner. At the pH optimum, the enzyme's affinity for PEP increased 1.5 fold when it was assayed in the presence of FDP. In addition to increasing the enzyme's affinity for PEP, FDP could also increase the maximal velocity for pH values greater than 6.3. This was particularly evident at pH 8.0 where the enzyme exhibited 2 apparent K_m values for

PEP of 0.66 mM and 2.40 mM in the absence of FDP but in its presence only one apparent K_m value of 0.33 mM was observed and there was a 1.9 fold increase in the maximal velocity. A Hill plot of the data obtained at pH 8.0 indicated that, in the absence of FDP, the enzyme exhibited no co-operativity in the binding of PEP for concentrations of the substrate less than 0.5 mM while slightly co-operative binding kinetics prevailed for PEP concentrations over 1.5 mM.

Eel muscle pyruvate kinase exhibited an absolute requirement for both a monovalent and a divalent cation for catalytic activity. While the eel enzyme was relatively insensitive to inhibition by ATP, it was very sensitive to inhibition by the amino acids alanine and phenylalanine. Phenylalanine was the more potent inhibitor of the 2 amino acids and it could reduce the enzyme's affinity for the substrate PEP. Of the mammalian pyruvate kinase isozymes, eel muscle pyruvate kinase most closely resembles the M_2 type enzyme kinetically.

As the kinetic properties of the eel enzyme were found to be substantially different from those of a mammalian muscle pyruvate kinase, the structural features of eel and rabbit muscle pyruvate kinases were determined and compared to those of other pyruvate kinase isozymes. Both enzymes were determined to be tetramers composed of 4 identical subunits of 59,000 molecular weight. The amino acid compositions of the 2 proteins were very similar with the greatest differences being in the smaller number of alanine and greater number of threonine residues in the eel enzyme. Cyanogen bromide peptide mapping on polyacrylamide gels indicated that eel and rabbit muscle

pyruvate kinases were likely to share some common cyanogen bromide peptides. Peptide mapping studies of ^{14}C-carboxymethylated eel and rabbit pyruvate kinases confirmed that although there were many structural differences between the 2 muscle isozymes, they shared several common peptides. The ^{14}C-carboxymethylcysteine-containing peptides of the eel and rabbit enzymes were purified and their primary structures were examined. Four of the 6 ^{14}C-carboxymethylcysteine-containing peptides derived from eel pyruvate kinase were found to be homologous to 4 of the 9 ^{14}C-carboxymethylcysteine-containing peptides derived from the rabbit enzyme. The fact that these particular structural features have been conserved in these evolutionary distant species suggests that they are of functional importance.

In spite of their similar subunit sizes and structures, eel pyruvate kinase exhibited a distinctly different isoelectric point than rabbit pyruvate kinase. In the absence of FDP, eel pyruvate kinase gave 2 major bands with pI values of 5.92 and 6.35 on isoelectric focusing gels while in the presence of FDP, only one band with a pI value of 5.90 was observed. On the other hand, rabbit muscle pyruvate kinase gave only one band on isoelectric focusing gels in the presence or absence of FDP with a pI value of 8.83. Of the mammalian pyruvate kinase isozymes, eel muscle pyruvate kinase most closely resembles the M_2 type isozyme.

A comparative study was conducted in order to examine the number, reactivity and location of the sulfhydryl groups of eel and rabbit muscle pyruvate kinases. Since eel muscle

pyruvate kinase is both kinetically and structurally similar to a mammalian M_2 isozyme, this study was undertaken in order to provide some information concerning the structure-function relationships of the mammalian M_1 and M_2 isozymes of pyruvate kinase. Eel and rabbit pyruvate kinases could be irreversibly inactivated by chemical modification by iodoacetamide at comparatively moderate inhibitor to enzyme subunit mole ratios. However under identical conditions, eel pyruvate kinase was found to be 6.4 times more sensitive than the rabbit enzyme to inhibition by iodoacetamide. Various substrate and cation combinations were tested for their ability to protect against enzyme inactivation. The cations K^+ and Mg^{+2}, either alone or in combination, and the substrate ADP could provide no protection against inactivation. While the substrate PEP could partially protect both enzymes, the combination of PEP and Mg^{+2} could completely protect both enzymes. FDP could partially protect the eel enzyme, but not the rabbit enzyme, from inactivation due to chemical modification by iodoacetamide.

Double labelling experiments provided information on the location and reactivity of the sulfhydryl groups of eel and rabbit muscle pyruvate kinases. A number of observations support the conclusion that the cysteine residues contained in the homologous cyanogen bromide-tryptic peptides EN2 and RN2, derived from eel and rabbit muscle pyruvate kinases respectively, are located either near or within the active sites of these enzymes and are functionally important. The results of the chemical modification studies reported here are discussed in terms of the findings of previous chemical modification studies. A

model is presented in order to account for the different inactivation kinetics exhibited by eel and rabbit muscle pyruvate kinases.

LIST OF SYMBOLS AND ABBREVIATIONS

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
β-NADH	β-nicotinamide adenine dinucleotide (reduced form)
bis-acrylamide	N,N^1-methylene-bisacrylamide
^{14}C-IAN	iodo[1-^{14}C]acetamide
^{14}C-IAA	iodo[2-^{14}C]acetic acid
CNBr	cyanogen bromide
C_i	curie (2.22×10^{12} d.p.m.)
CM-	carboxymethyl-
DEAE-	diethylaminoethyl-
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTT	dithiothreitol
EDTA	disodium ethylene diaminetetraacetic acid
FDP	fructose-1,6-bisphosphate
g	acceleration due to gravity
g	grams
^{3}H-IAA	iodo[2-^{3}H]acetic acid
IAN	iodoacetamide
IAA	iodoacetic acid
I.D.	internal diameter
K_m	Michaelis-Menten constant for a substrate
K_a	Michaelis-Menten constant for an activator
K_d	distribution coefficient
l	liters
min	minutes

M	molar
M.W.	molecular weight
m	milli (10^{-3})
Mes	2-(N-morpholino)ethanesulfonic acid
n_H	Hill coefficient
nm	nanometers
PEP	phospho-enol-pyruvate
pI	isoelectric point
PK	pyruvate kinase
SDS	sodium dodecyl sulphate
$S_{0.5}$	substrate concentration required to achieve one-half maximal velocity
Tris	2-amino-2(hydroxymethyl)-1,3-propanediol
TEMED	N,N,N',N'-tetramethylethylenediamine
μ	micro (10^{-6})
v/v	by volume
$V_{max.}$	maximum velocity
V_e	column elution volume
V_o	column void volume
V_s	column bed volume
w/v	weight by volume

Addendum

BAWP	solvent for descending paper chromatography consisting of butanol-acetic acid- water-pyridine (15:3:12:10, v/v/v/v)

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INTRODUCTION

A) LITERATURE REVIEW

The penultimate reaction in the glycolytic pathway in mammalian tissues involves the enzyme-mediated transfer of a phosphoryl group from phospho-enol-pyruvate (PEP) to adenosine 5'-diphosphate (ADP) in the presence of Mg^{+2} and K^{+} to produce pyruvate and adenosine 5'-triphosphate (ATP) respectively (1).

$$PEP + ADP + H^{+} \xrightarrow{Mg^{+2},\ K^{+}} Pyruvate + ATP \quad (1)$$

Pyruvate kinase (ATP:pyruvate 2-O-phosphotransferase, EC 2.7.1.40) is the enzyme which catalyzes this reaction. Because glycolysis is a ubiquitous process and its regulation is vital in energy metabolism, the enzymes which are involved in this process have been studied extensively. In particular, pyruvate kinase has received considerable attention because it is thought to be a key regulatory glycolytic enzyme. The reaction product, pyruvate can feed into a number of metabolic pathways via the actions of several enzymes including lactic dehydrogenase, pyruvate carboxylase, pyruvate dehydrogenase and alanine transaminase. Hence pyruvate kinase is located at a key metabolic crossroad. In addition, pyruvate kinase catalyzes a non-equilibrium reaction which proceeds overwhelmingly in favour of the production of ATP and pyruvate. Therefore it catalyzes a key reaction which can alter glycolytic flux and its regulation can be used to adjust to varying energy requirements of the cell. Much of the large amount of literature reporting studies of pyruvate kinase

is concerned with the examination of the kinetic properties of the enzyme from mammalian sources. Primarily on the basis of such studies, 4 isozymes have been identified in mammalian tissues.

1. MAMMALIAN PYRUVATE KINASE ISOZYMES

The M_1 type is the only pyruvate kinase isozyme present in adult skeletal muscle (Imamura & Tanaka, 1972). Electrophoretically similar pyruvate kinases have been found in adult cardiac muscle and brain. The L type is the major isozyme of the adult liver and it is also found in kidney (Imamura & Tanaka, 1972). The M_1 and L type pyruvate kinases are immunologically and kinetically distinct (Tanaka et al., 1967). In addition, the L but not the M_1 type is subject to dietary and hormonal regulation (Weber et al., 1965; Tanaka et al., 1967). It is not surprising therefore that the M_1 and L type isozymes have also been found to be distinctly different structurally (Saheki et al., 1978; Saheki et al., 1982a).

The M_2 type is the major isozyme of the adult kidney and it is also found in liver (Imamura & Tanaka, 1972). Electrophoretically similar pyruvate kinases have been found in adult testis, adipose tissue, lung, spleen and intestine. Purified M_2 isozymes have been shown to be immunologically and structurally similar to the M_1 isozyme (Imamura et al., 1972 ; Saheki et al., 1982a, b) while they are kinetically similar to the L isozyme (Hall & Cottam, 1978). The M_2 isozyme has been reported to be the predominant pyruvate kinase in all fetal tissues and in dedifferentiating cancer tissues. It has been proposed that this isozyme may represent the proto-

type of the mammalian pyruvate kinases (Imamura et al., 1972).

Erythrocytes contain a pyruvate kinase isozyme which is immunologically (Tanaka et al., 1967), kinetically (Koler & Vanbellinghen, 1968) and stucturally (Marie et al., 1977a, b) very similar to the L type isozyme.

The following section describes the physical and chemical properties of each of the mammalian pyruvate kinase isozymes and their inter-relationships. A bewildering number of systems of nomenclature have been employed to describe the different pyruvate kinase isozymes (Ibsen, 1977). For the purposes of the following discussion, the nomenclature of Imamura & Tanaka (1972) will be employed.

(i) COMPARISON OF THE M_1 AND M_2 TYPE PYRUVATE KINASES

The M_1 type is the best characterized pyruvate kinase isozyme because of its relative ease of purification, stability and abundance. By contrast, the characterization of the M_2 isozyme has been hampered by the fact that it is much less abundant than the M_1 type isozyme and it is often unstable. Table 1 summarizes the physical and chemical properties of some M_1 and M_2 type pyruvate kinases. While the M_1 isozymes are remarkably similar from species to species, the M_2 type isozymes are a heterogeneous group. The M_1 isozymes exhibit specific activities in the range of 350-450 units/mg of purified protein and their pH optima are in the range of 7.0-7.5. The M_2 isozymes exhibit specific activities which range from 274 to 1341 units/mg of purified protein and their pH optima range from 6.5 to 7.5.

TABLE 1

Comparison of the Physical and Chemical Properties of the Mammalian M_1 and M_2 Isozymes

	(1)	(2)	(3)	(4)	(5)	(6)
PROPERTY	MOUSE M_1	MOUSE SPLEEN M_2	PIG M_1	PIG KIDNEY M_2	HUMAN M_1	HUMAN LUNG M_2
Specific Activity units/mg	370	398	340	522	245	274
Assay Temperature ^oC	—	30	30	30	25	30
K_m(PEP) mM -FDP	0.035	0.19	0.02	0.25	0.035	0.30
n_H -FDP	—	—	—	0.8	—	1.06
K_m(PEP) mM +FDP	—	0.03	—	0.06	—	0.25
n_H +FDP	—	—	—	—	—	—
K_m(ADP) mM	0.28	0.27	0.3	0.3	—	0.38
Optimum pH	7.5	7.5	7.5	7.5	7.5	7.1
pI -FDP	7.25	6.22	7.2	5.6	—	6.2
pI +FDP	—	—	—	—	—	—
Molecular Weight	234,400	230,000	—	249,000	220,000	250,000
Subunit Molecular Weight	58,600	59,500	—	60,000	60,000	62,000

REFERENCES

(1) Ibsen et al. (1981)

(2) Ibid

(3) Berglund et al. (1977); Berglund & Humble (1979)

(4) Berglund et al. (1977); Berglund & Humble (1979)

(5) Baranowska & Baranowski (1975)

(6) Corcoran et al. (1976)

TABLE 1 (continued)

Comparison of the Physical and Chemical Properties of the Mammalian M_1 and M_2 Isozymes

	(1)	(2)	(3)	(4)	(5)
PROPERTY	RABBIT M_1	RAT M_1	RAT HEPATOMA M_2	RAT LUNG M_2	RAT SARCOMA M_2
Specific Activity units/mg	350	780	770	1341	200
Assay Temperature ^oC	30	37	37	37	24
K_m(PEP) mM -FDP	0.086	0.08	0.45	0.26	0.3
n_H -FDP	—	—	1.45	1.81	—
K_m(PEP) mM +FDP	—	—	0.10	0.056	0.04
n_H +FDP	—	—	1.0	1.06	—
K_m(ADP) mM	0.3	0.3	—	0.4	—
Optimum pH	7.5	7.5	7.2	6.5	7.5
pI -FDP	8.2-8.6	—	—	5.8	7.8
pI +FDP	—	—	—	6.4	6.6,6.2
Molecular Weight	230,000	250,000	240,000	224,000	240,000
Subunit Molecular Weight	59,000	60,000	60,000	62,000	60,000

REFERENCES

(1) Reynard et al. (1961); Cottam et al. (1969)

(2) Tanaka et al. (1967); Imamura et al. (1972)

(3) Imamura et al. (1972); Imamura & Tanaka (1982)

(4) Schering et al. (1982)

(5) Nagao et al. (1977); Nagao et al. (1982)

Both the M_1 and M_2 type pyruvate kinases are tetrameric enzymes consisting of 4 identical subunits of 58,000 to 62,000 molecular weight. The amino acid compositions of some M_1 and M_2 type isozymes appear in Table 2. The data indicates that the compositions of these isozymes are very similar from one species to another. In spite of their similar subunit sizes and amino acid compositions, the isoelectric points of the M_1 and M_2 type isozymes isolated from the same species are markedly different. The isoelectric points of the M_1 isozymes vary but it appears that they can be divided into 2 groups; those with a high pI around 8.8 and those with a lower pI value around 7.5. The isoelectric points of the M_2 type isozymes also vary but generally they are in the range of 5.5-6.5. FDP can alter the isoelectric points of the M_2 type isozymes but it has no apparent effect on the isoelectric points of the M_1 type isozymes.

The M_1 isozymes exhibit Michaelis-Menten kinetics with respect to both the substrates ADP and PEP and the concentration of one substrate does not appear to influence the enzyme's affinity for the second substrate. The M_2 type isozyme exhibits Michaelis-Menten kinetics with respect to the substrate ADP but slightly co-operative binding kinetics with respect to PEP. It has been reported for some M_2 type isozymes that the concentration of one substrate does not influence the enzyme's affinity for the second substrate. While a detailed kinetic analysis of an M_2 type isozyme is lacking, the M_1 type isozyme from rabbit muscle has been studied extensively. Reynard et al. (1961) proposed that a rapid equilibrium model could be used

TABLE 2

Comparison of the Amino Acid Compositions of the Mammalian M_1 and M_2 Isozymes

	(1)	(2)	(3)	(4)	(5)	(6)
AMINO ACID	MOUSE M_1	MOUSE SPLEEN M_2	PIG M_1	PIG KIDNEY M_2	HUMAN M_1	HUMAN LUNG M_2
CYS	9	--	9	10	15	--
ASP	49	51	51	51	50	48
THR	25	29	28	29	28	25
SER	28	32	29	28	30	17
GLU	52	47	53	53	59	58
PRO	24	23	26	25	22	43
GLY	41	43	43	44	41	30
ALA	58	64	59	59	55	66
VAL	46	51	50	50	46	60
MET	19	12	19	16	20	12
ILE	36	36	32	36	35	36
LEU	41	45	42	42	40	41
TYR	9	9	9	10	10	10
PHE	18	15	17	17	18	22
HIS	15	18	15	13	13	16
TRP	3	--	3	4	3	--
LYS	39	38	38	36	36	34
ARG	34	32	33	32	30	42
Subunit M.W.	60,000	59,500	60,000	60,000	60,175	62,000

REFERENCES

(1) Saheki et al. (1982b)

(2) Ibsen et al. (1981)

(3) Berglund et al. (1977)

(4) Ibid

(5) Harkins et al. (1977b)

(6) Corcoran et al. (1976)

TABLE 2 (continued)

Comparison of the Amino Acid Compositions of the Mammalian M_1 and M_2 Isozymes

	(1)	(2)	(3)	(4)	(5)	(6)
AMINO ACID	RABBIT M_1	RABBIT KIDNEY M_2	RAT M_1	RAT HEPATOMA M_2	RAT LUNG M_2	RAT SARCOMA M_2
CYS	9	10	9	10	8	10
ASP	51	50	49	49	50	49
THR	26	26	23	27	25	25
SER	30	24	27	27	30	26
GLU	52	51	51	52	56	51
PRO	23	26	29	22	28	24
GLY	42	43	41	42	50	42
ALA	61	58	61	60	48	59
VAL	46	49	46	48	37	49
MET	18	15	18	16	9	16
ILE	34	40	36	39	26	37
LEU	41	41	45	43	43	40
TYR	10	9	10	10	13	10
PHE	16	17	16	16	19	15
HIS	16	12	12	12	22	12
TRP	3	3	2	3	--	5
LYS	37	39	39	39	35	39
ARG	28	34	33	32	27	32
Subunit M.W.	59,250	60,000	60,000	60,000	55,000	59,000

REFERENCES

(1) Cottam et al. (1969)

(2) Saheki et al. (1982b)

(3) Ibid

(4) Ibid

(5) Schering et al. (1982)

(6) Nagao et al. (1982)

to describe the kinetic behaviour of rabbit muscle pyruvate kinase. However, Giles et al. (1976a) reported that while rapid equilibrium kinetics were applicable to the forward reaction leading to the production of pyruvate and ATP, such was not the case for the reverse reaction. Dann & Britton (1978) reported that rapid equilibrium kinetics were applicable to the reverse reaction but the binding of ADP and PEP was mutually co-operative at low substrate concentrations. Recently Ainsworth et al. (1983) proposed that an exponential model for a regulatory enzyme could be used to describe the complex kinetics exhibited by rabbit muscle pyruvate kinase at low substrate and cation concentrations. Evidently further characterization of the complex kinetic behaviour of rabbit muscle pyruvate kinase is required.

The M_2 isozyme is activated by FDP in a pH-dependent manner. At pH values in the range of 6.0-6.5, the enzyme exhibits hyperbolic PEP substrate saturation curves and FDP can slightly increase the enzyme's affinity for PEP. At pH values in the range 8.0-8.5, the M_2 type isozyme exhibits co-operativity in the binding of PEP and FDP can induce activation by increasing the maximal velocity and decreasing the apparent K_m (PEP) (Van Berkel et al., 1973; Van Berkel, 1974; Berglund & Humble, 1979). Hence with increasing pH, the M_2 isozyme becomes increasingly dependent on FDP for activation. The M_1 type isozyme is not activated by FDP at saturating levels of metal ions but Phillips & Ainsworth (1977) reported that at low Mg^{+2} and substrate concentrations, rabbit muscle pyruvate kinase exhibited co-operativity in the binding of both ADP and Mg^{+2}. At present, the co-operative behaviour of the M_1 isozyme is too ill-defined to permit detailed

comparison with other pyruvate kinase isozymes.

The reaction product pyruvate is a poor inhibitor of the M_1 type isozyme and the other mammalian pyruvate kinases have not been tested extensively to determine their sensitivities to inhibition by pyruvate. On the other hand, the reaction product ATP is a strong inhibitor of both the M_1 and M_2 type isozymes. Part of this inhibitory effect can be attributed to the fact that ATP can chelate with the divalent cation required for catalytic activity. But in addition, ATP is a competitive inhibitor of pyruvate kinase with respect to the substrate ADP. The M_2 type isozyme is more sensitive to inhibition by physiological concentrations of ATP than the M_1 isozyme (Imamura et al., 1972).

(ii) REGULATION OF THE M_1 AND M_2 TYPE ISOZYMES

Little is known about how the activity of the M_1 type isozyme is regulated in vivo. It has been suggested that ATP is the primary modulator of the M_1 type isozyme's activity (Dyson et al., 1975; Giles et al., 1976a) since the physiological concentration of ATP in resting mammalian muscle is several fold higher than the enzyme's affinity for ATP in vitro. High concentrations of phenylalanine can inhibit the M_1 isozyme (Carminatti et al., 1971) while high concentrations of FDP can activate the enzyme (Phillips & Ainsworth, 1977) but these effects are unlikely to be of physiological importance (Munday et al., 1980; Palmer & Odedra, 1982). Reports of inhibition of muscle pyruvate kinase by the phosphagen phosphocreatine have appeared (Kemp, 1973) but this has been attributed to the

presence of a contaminant in commercial preparations of this compound (Fitch et al., 1979).

The M_2 type isozyme by contrast exhibits the classical kinetic properties of a regulatory enzyme. The activity of the M_2 isozyme is thought to be regulated in vivo by ATP, alanine, phenylalanine, PEP and FDP since physiological concentrations of ATP, alanine and phenylalanine can inhibit the M_2 isozyme in vitro while physiological concentrations of PEP and FDP can activate the M_2 isozyme in vitro (Imamura et al., 1972; Van Berkel et al., 1973; Van Berkel, 1974; Berglund & Humble, 1979; Schering et al., 1982). This is in keeping with central role this enzyme plays in balancing the glycolytic and gluconeogenic activities of the kidney. In gluconeogenic tissues, the pyruvate kinase reaction is bypassed and the enzymes pyruvate carboxylase (2) and phospho-enol-pyruvate carboxykinase (3) are responsible for the conversion of pyruvate to PEP.

$$\text{Pyruvate} + CO_2 + \text{ATP} \longrightarrow \text{Oxaloacetate} + \text{ADP} + P_i \qquad (2)$$

$$\text{Oxaloacetate} + \text{GTP} \longrightarrow \text{PEP} + CO_2 + \text{GDP} \qquad (3)$$

One might expect that the activity of the key glycolytic enzyme pyruvate kinase would be regulated to avoid wasteful substrate cycling between PEP and pyruvate in kidney.

In addition, the activity of the M_2 isozyme may be regulated by a phosphorylation-dephosphorylation mechanism. The predominant pyruvate kinase isozyme of chicken liver is the M_2 type and it has been reported to be regulated by a cyclic-AMP-independent phosphorylation mechanism (Eigenbrodt et al., 1977 ;

Brunn et al., 1979). By contrast, the M_2 isozyme of pig kidney can not be phosphorylated by a cAMP-dependent protein kinase in vitro (Humble et al., 1975; Berglund et al., 1977) and the M_2 isozyme of rat lung can not be phosphorylated by either a cAMP-dependent or cAMP-independent protein kinase in vitro (Schering et al., 1982). Further studies will be required to determine whether tissue and species-specific M_2 type pyruvate kinases are regulated by a phosphorylation-dephosphorylation mechanism.

(iii) INTER-RELATIONSHIP OF THE M_1 AND M_2 TYPE PYRUVATE KINASES

On the basis of amino acid composition similarities alone, Harkins et al.(1977a) proposed that the M_1 and M_2 type isozymes were the products of two separate but related genes. Structural studies indicated that the M_2 isozyme's subunit was larger than that of the M_1 isozyme. Peptide mapping studies indicated that the M_1 and M_2 type pyruvate kinases shared several common peptides but each isozyme contained unique peptides which were not present in the other (Saheki et al., 1978). Ibsen et al. (1981) reported that mild treatment of the M_2 isozyme subunit with trypsin could convert it into a form which was electrophoretically indistinguishable from the M_1 subunit. However, the proteolytically modified M_2 isozyme was not kinetically equivalent to the M_1 isozyme. Ibsen et al. (1981) proposed that the M_1 isozyme subunit arose from post-translational modification of the C-terminal portion of a proteolytically modified M_2 isozyme polypeptide chain.

The question of whether M_1 and M_2 type pyruvate kinases

were encoded by different mRNAs was addressed by Noguchi & Tanaka (1982). Total RNA was extracted from rat muscle and ascites hepatoma cells and translated in vitro. Ascites hepatoma RNA directed the synthesis of a protein which was immunologically and electrophoretically indistinguishable from the M_2 type pyruvate kinase purified from ascites hepatoma cells. Similarly rat muscle RNA directed the synthesis of a protein which was indistinguishable from the M_1 type pyruvate kinase purified from rat muscle. The same results were obtained when protease inhibitors were added to the translation system. This data strongly indicated that M_1 and M_2 type pyruvate kinases were encoded by different mRNAs and a precursor-product relationship did not exist between them.

However it was not clear whether the 2 different mRNAs arose from the transcription of 2 distinct but closely related genes or from the same gene from which 2 mature mRNAs could be produced. This question was dealt with by 2 independent groups simultaneously (Hance et al., 1982; Saheki et al., 1982b). If the M_1 and M_2 type isozymes were the products of 2 separate but related genes then inter-species differences might occur in one isozyme which would not be found in the other. However if the M_1 and M_2 isozymes were products of the same gene then inter-species differences would be common to both isozymes. Hance et al. (1982) was able to show that inter-species differences between the M_2 isozymes were always accompanied by similar inter-species differences in the M_1 isozymes. Peptide mapping studies performed by Saheki et al. (1982b) confirmed the findings of Hance et al. (1982). In addition,

sequencing studies indicated that the M_1 and M_2 type pyruvate kinases were identical in the C-terminal portions of their subunits. This ruled out the possibility that the M_1 isozyme arose from the proteolytic cleavage and modification of the C-terminal portion of the M_2 type polypeptide chain (Ibsen et al., 1981).

At present, the M_1 and M_2 isozymes are thought to be the products of a single gene from which 2 distinct mRNAs can be produced. This suggests that the initial transcript from this gene can be spliced in two different ways to produce 2 mRNA molecules which encode the M_1 and M_2 isozymes. The fact that the M_2 isozyme predominates in fetal tissues and the M_1 isozyme only appears in adult muscle, heart and brain suggests that a switching over of the splicing pathway occurs after birth. The mRNA encoding the M_1 type isozyme may therefore be preferentially synthesized at the expense of the mRNA encoding the M_2 isozyme.

(iv) COMPARISON OF THE L AND ERYTHROCYTE TYPE PYRUVATE KINASES

Relatively few L and erythrocyte type isozymes have been purified and characterized. This is due to the complexities of purification procedures which result in low enzyme yields and the inherent instabilities of these isozymes. Table 3 summarizes the kinetic and physical properties of some L and erythrocyte isozymes. A wide range of specific activities have been reported for the L isozyme and this may be due to the fact that adequate precautions to ensure against enzyme denaturation during purification were not taken. The L isozymes exhibit pH optima

TABLE 3

Comparison of the Physical and Chemical Properties of the Mammalian Liver and Erythrocyte Isozymes

PROPERTY	(1) RAT LIVER	(2) PIG LIVER	(3) BOVINE LIVER	(4) HUMAN LIVER	(5) HUMAN R.B.C.	(6) RAT R.B.C.
Specific Activity units/mg	520	300	75	190	290	307
Assay Temperature ^{o}C	25	37	25	30	30	25
K_m(PEP) mM -FDP	0.3-0.9	0.3-0.6	0.5	0.4	0.6-0.8	1.0-1.4
n_H -FDP	1.6-2.0	2.3	2.5	1.7	1.0-1.4	1.2-1.6
K_m(PEP) mM +FDP	0.10	0.03	0.08	0.04	0.10	—
n_H +FDP	1.0	1.1	1.0	1.0	0.8-1.1	—
K_m(ADP) mM	0.1-0.4	0.3	0.18	—	0.4-0.6	0.4-0.6
Optimum pH	6.5	6.5	—	—	—	—
pI -FDP	—	6.1	5.1	5.85	5.8-6.7	—
pI +FDP	—	5.3	—	—	—	—
Molecular Weight	220,000	230,000	215,000	230,000	230,000	220,000
Subunit Molecular Weight	58,000	58,000	52,000	60,000	59,000	58,000

REFERENCES

(1) Tanaka et al. (1967); Imamura & Tanaka (1982)

(2) Kutzbach et al. (1973); Ljungström et al. (1976)

(3) Cardenas & Dyson (1973); Cardenas (1982)

(4) Kahn & Marie (1982)

(5) Ibid

(6) Imamura & Tanaka (1982)

in the range of 6.0-6.5 which is a full pH unit lower than the pH optima determined for the M_1 isozymes.

The L isozyme is a tetrameric enzyme consisting of 4 identical subunits of 58,000-60,000 molecular weight. The characterization of the erythrocyte isozyme has been hampered by the fact that it is susceptible to proteolysis. Hence 2 types of pyruvate kinase have been purified from human erythrocytes (Kahn & Marie, 1982). PK(R_1) is a homotetramer consisting of 4 L' subunits (M.W. 63,000) while PK(R_2) is a heterotetramer consisting of 2 L' subunits and 2 L_c subunits (M.W. 57-58,000). In addition, a third subunit called the L_b subunit (M.W. 60,000) is found in some erythrocyte pyruvate kinase preparations. It appears that the erythrocyte enzyme is synthesized as the $(L')_4$ tetramer but during red blood cell aging or purification, it is converted to the $(L')_2(L_c)_2$ heterotetramer and subsequently to the $(L_c)_4$ homotetramer. The proteolytic susceptibility of the erythrocyte enzyme may account for the multiple forms which appear upon isoelectric focusing of erythrocyte pyruvate kinase preparations. The isoelectric points of the L isozymes are distinctly lower than those of the M_1 and M_2 type isozymes and the pI value of the L isozyme is altered when isoelectric focusing is conducted in the presence of FDP. Peptide mapping studies have indicated that the liver and erythrocyte isozymes are almost structurally identical with the erythrocyte isozyme containing a few extra peptides (Saheki et al., 1978; Saheki et al., 1982a). As might be expected, the amino acid compositions of the liver and erythrocyte isozymes from the rat are almost identical (Harada et al.,

TABLE 4

Comparison of the Amino Acid Compositions of the Mammalian Liver and Erythrocyte Isozymes

AMINO ACID	(1) RAT LIVER	(2) PIG LIVER	(3) MOUSE LIVER	(4) HUMAN R.B.C.	(5) RAT R.B.C.
CYS	6	6	6	6	--
ASP	37	32	39	33	46
THR	25	23	26	20	29
SER	35	27	31	31	35
GLU	59	55	60	56	69
PRO	27	19	29	23	31
GLY	44	42	45	41	46
ALA	58	52	59	50	59
VAL	51	42	52	46	53
MET	14	9	13	11	11
ILE	40	36	36	32	42
LEU	50	39	45	40	46
TYR	9	7	9	7	9
PHE	17	14	18	14	17
HIS	11	9	11	10	10
TRP	4	3	4	8	--
LYS	22	18	23	20	21
ARG	40	31	41	50	34
Subunit M.W.	60,000	50,000	60,000	55,000	60,000

REFERENCES

(1) Saheki et al. (1982b)
(2) Kutzbach et al. (1973)
(3) Saheki et al. (1982b)
(4) Chern et al. (1972)
(5) Harada et al. (1978)

1978) (Table 4).

The L type isozyme exhibits Michaelis-Menten kinetics with respect to ADP and pronounced co-operativity in the binding of PEP at physiological pH. Like the M_2 isozyme, the L isozyme is activated by FDP in a pH-dependent manner such that the enzyme's affinity for PEP and the maximal velocity are increased. The kinetic parameters of the erythrocyte $(L')_4$ isozyme have been determined and found to be very similar to those of the L isozyme (Kahn & Marie, 1982).

Little detailed kinetic data is available on the L isozyme, however Macfarlane & Ainsworth (1974) reported that the pig liver enzyme was different from the M_1 and M_2 isozymes with respect to its binding kinetics. A decrease in the concentration of one substrate was found to increase the enzyme's affinity for the second substrate. This suggested that the mechanism of the L type pyruvate kinase from pig liver was of the ping-pong type (Macfarlane & Ainsworth, 1974). However, isotopic exchange studies of rabbit (Dann & Britton, 1977) and pig (Giles et al., 1976b) liver pyruvate kinases have indicated that the reactions do not proceed via a phospho-enzyme intermediate.

The L and erythrocyte type isozymes are the most sensitive pyruvate kinases to inhibition by ATP. At physiological concentrations of ADP and PEP, the L isozyme can be completely inhibited by physiological concentrations of ATP *in vitro* in the absence of FDP but FDP can completely overcome the inhibitory effect of ATP (Imamura et al., 1972). The amino acids alanine and phenylalanine have been tested for their

ability to inhibit the L isozymes and variable results have been obtained. Rat liver pyruvate kinase was found to be as sensitive as the M_2 isozyme from rat ascites hepatoma cells to inhibition by alanine but it was substantially less sensitive to inhibition by phenylalanine (Imamura et al., 1972). On the other hand, bovine liver pyruvate kinase has been reported to be insensitive to inhibition by alanine but sensitive to inhibition by phenylalanine (Cardenas et al., 1975b). Further characterization of the kinetic properties of the L and erythrocyte type pyruvate kinases is required in order to identify the functional differences between the mammalian pyruvate kinase isozymes.

(v) REGULATION OF THE L AND ERYTHROCYTE TYPE ISOZYMES

The L type, like the M_2 type, is the predominant pyruvate kinase isozyme of a gluconeogenic organ and as such it exhibits the classical kinetic properties of a regulatory enzyme. Like the M_2 type, the activity of the L isozyme is thought to be regulated *in vivo* by the inhibitors ATP, alanine and phenylalanine and by the activators PEP and FDP. In addition, the L type isozyme has been shown to be under hormonal and dietary control. Taunton et al. (1972) reported that liver pyruvate kinase activity was reduced within minutes of injecting rats with glucagon and this was co-incident with an increase in intracellular cyclic-AMP concentration. Short-term alterations of liver pyruvate kinase activity appear to be due to a cAMP-dependent phosphorylation-dephosphorylation mechanism. Rat and pig liver pyruvate kinases can be phosphorylated *in vitro*

by a cAMP-dependent protein kinase (Edlund et al., 1975; Hjelmqvist et al., 1974) and this involves the modification of one specific serine residue per enzyme subunit. The kinetic properties of the in vitro phosphorylated enzymes are similar to those of the liver isozymes from glucagon or cAMP-treated hepatocytes or glucagon-treated rat liver (Ekman et al., 1976; Ljungström et al., 1976). The phosphorylated enzyme is characterized by a decreased affinity for PEP, a decreased sensitivity to activation by FDP and an increased sensitivity to inhibition by ATP and alanine.

Titanji et al. (1976) showed that the effects of phosphorylation were reversible and the dephosphorylation of the phosphorylated liver isozyme resulted in enzyme re-activation. Glucagon can stimulate the phosphorylation of the liver isozyme in both liver slices (Ljungström & Ekman, 1977) and in the whole animal (Riou et al., 1978). It appears that glucagon can effect the rapid reduction of mammalian liver pyruvate kinase activity via a cascade mechanism. Glucagon can increase the intracellular cAMP level in the liver and thereby activate a cAMP-dependent protein kinase which phosphorylates the L isozyme resulting in enzyme inactivation. Little is known about the events of dephosphorylation or the protein phosphatase which presumably catalyzes this reaction. Claus et al. (1979) reported that insulin could promote the dephosphorylation and simultaneous re-activation of the phosphorylated liver isozyme. However, it has been reported that insulin can not counter-act the inhibitory effects induced by glucagon (Blair et al., 1976; Felíu et al., 1976). Although the cAMP-depend-

ent phosphorylation of the L isozyme has been studied extensively, little is known about the regulation of the phosphorylation-dephosphorylation mechanism.

A long term reduction of liver pyruvate kinase activity has been noted in fasting and alloxan-diabetic rats and rats fed a high protein diet (Tanaka et al., 1967). Hall et al. (1979) proposed that long term control of liver pyruvate kinase activity could be due to proteolytic degradation of the phosphorylated enzyme. They reported that phosphorylated liver pyruvate kinase was more susceptible to proteolysis in vivo than the dephosphorylated enzyme and this resulted in an irreversible reduction in the specific activity of the enzyme. Johnson & Veneziale (1980) reported that in fasted rats, the specific activity of the liver isozyme was decreased while the total amount of pyruvate kinase protein was unchanged. Hence glucagon may regulate the activity of the L isozyme via a dual mechanism. In the short term, glucagon may stimulate the cAMP-dependent phosphorylation and inactivation of the enzyme. In the long term, glucagon may promote the proteolytic degradation and irreversible inactivation of the enzyme.

Administration of insulin to diabetic rats or the administration of a high carbohydrate diet to fasted rats or rats fed a high protein diet can result in a long term increase in liver pyruvate kinase activity (Tanaka et al., 1967). The insulin-induced long term activation of L isozyme activity can be blocked by inhibitors of translation and transcription (Weber et al., 1965). Parks & Drake (1982) reported that insulin could increase the quantity and activity of liver pyruvate

kinase in diabetic rats. Recently, Noguchi et al. (1982) reported that the insulin-induced increase in the activity of the L isozyme could be attributed to an increase in the amount of translatable mRNA coding for the protein. Hence insulin, like glucagon, may regulate the activity of the L isozyme via a dual mechanism. In the short term, insulin may stimulate the dephosphorylation and simultaneous re-activation of the enzyme. In the long term, insulin may induce the synthesis of new L type pyruvate kinase protein.

At present it is not clear how substrate, product and inhibitor concentrations interact with phosphorylation-dephosphorylation mechanisms and proteolytic and synthetic events to regulate the activity of the L isozyme *in vivo*.

Little is known about how the activity of the erythrocyte type isozyme is regulated *in vivo*. Like the L type, the activity of the erythrocyte isozyme can potentially be regulated by the concentrations of the inhibitors ATP, alanine and phenylalanine and the activators PEP and FDP. The erythrocyte isozyme can also be phosphorylated *in vivo* (Marie et al., 1979) and *in vitro* (Boivin , 1980) via a cAMP-dependent mechanism. However, only the larger L' subunit can be phosphorylated and the smaller L_c subunit, which appears to be the proteolytic product of the L' subunit, seems to have lost the phosphorylation site. The phosphorylated erythrocyte enzyme is characterized by a decreased affinity for PEP , a decreased sensitivity to activation by FDP and an increased sensitivity to inhibition by ATP and alanine (Kahn & Marie, 1982). While the phosphorylation of the liver isozyme probably plays an important role in

the regulation of the balance between glycolysis and gluconeogenesis in the liver, it is not clear whether phosphorylation of the erythrocyte isozyme plays any regulatory function.

(vi) INTER-RELATIONSHIP BETWEEN THE L AND ERYTHROCYTE TYPE ISOZYMES

The fact that the L and erythrocyte type pyruvate kinases exhibit similar amino acid compositions, peptide maps and immunological properties suggests that these 2 isozymes are the products of the same gene or two very similar genes. The question of whether the L and erythrocyte type isozymes are encoded by different mRNAs was addressed by Marie et al. (1981). Total RNA was extracted from rat hepatocytes and erythroid cells and translated in a cell free system. Rat liver RNA directed the synthesis of a protein which was immunologically and electrophoretically indistinguishable from the L isozyme purified from rat liver. Similarly rat erythroid cell RNA directed the synthesis of a protein which was indistinguishable from the $(L')_4$ type isozyme purified from rat erythroid cells. The same results were obtained when protease inhibitors were added to the translation system. This data strongly indicated that liver and erythrocyte type pyruvate kinases are encoded by different mRNAs. Recently Simon et al. (1983) reported that the structural differences between the L' and L subunits were confined to the C-terminal ends of the polypeptide chains. Hence the mRNAs coding for the L' and L subunits should differ in their 3'-end coding sequences.

However, these 2 mRNA molecules could be derived from the same gene or from 2 different but structurally related genes.

It has been reported that patients who suffer from hereditary red blood cell pyruvate kinase deficiency also have a defective liver pyruvate kinase (Kahn et al., 1976; Shinohara et al., 1976; Nakashima et al., 1977). This would suggest that the genes coding for the L and erythrocyte type isozymes are genetically linked in man. Also the fact that the L and erythrocyte type isozymes appear to be nearly identical structurally in spite of the fact that they are encoded by 2 different mRNA molecules would suggest that these 2 isozymes are encoded by the same gene.

At present, it is not clear how these 2 different pyruvate kinase mRNAs are derived from the same gene. One explanation invokes the presence of 2 different mRNA splicing pathways in the erythroid and liver cells. This model has been used previously to describe the production of the M_1 and M_2 type isozymes from the same gene (Hance et al., 1982). However, because the L and erythrocyte type isozymes are synthesized in different tissues, there is an alternative to the differential splicing model. In the fetus, reticulocytes and hepatocytes may contain the same structural gene but during development gene rearrangement could give rise to 2 structurally similar genes. In order to distinguish between these 2 possibilities, it will be necessary to construct a cDNA to pyruvate kinase mRNA and probe for genomic rearrangement in hepatocytes and reticulocytes during development (Marie et al., 1982).

(vii) HYBRIDS

Although 4 principal isozymes of pyruvate kinase have been

found in mammals, studies have revealed the presence of minor forms which are electrophoretically distinct from the M_1, M_2, L and erythrocyte type pyruvate kinases (Imamura & Tanaka, 1972). Some authors have proposed that these minor pyruvate kinase forms are hybrids of the 4 principal isozymes (Ibsen, 1977). This is certainly not out of the question since it has been shown that inter-subunit features are sufficiently complimentary to permit the hybridization of the M_1 and L type isozyme subunits (Cardenas & Dyson, 1973; Dyson & Cardenas, 1973; Cardenas et al., 1975c). However there is no evidence for the existence of hybrid forms of pyruvate kinase in vivo and purified hybrid forms may be artifacts of purification procedures. The possibility that these minor pyruvate kinase forms are simply the proteolytic products of the 4 principal pyruvate kinase isozymes also exists.

2. NON-MAMMALIAN VERTEBRATE PYRUVATE KINASES

Very few pyruvate kinase isozymes have been purified from the tissues of non-mammalian vertebrates. The enzyme has been obtained from the skeletal muscle of the frog (Flanders et al., 1971), the chicken (Cardenas et al., 1975a), the sturgeon (Randall & Anderson, 1975) and the salmon (Guderley & Cardenas, 1980a) while cardiac muscle pyruvate kinases have been obtained from the frog (Flanders et al., 1971) and the turtle (Storey & Hochachka, 1974). One isozyme was purified from salmon liver (Guderley & Cardenas, 1980a) while 2 forms described as L and M_2 types were obtained from chicken liver (Eigenbrodt & Schoner, 1977) . The kinetic and physical properties of these enzymes

TABLE 5

Comparison of the Physical and Chemical Properties of the Pyruvate Kinases Isolated from Some Non-Mammalian Vertebrates

	(1)	(2)	(3)	(4)
PROPERTY	CHICKEN MUSCLE	CHICKEN LIVER M_2	CHICKEN LIVER L	* FROG MUSCLE
Specific Activity units/mg	236	520	320	133×10^4
Assay Temperature ^oC	25	37	37	25
K_m(PEP) mM -FDP	0.07	0.2	0.79	0.06
n_H -FDP	1.0	1.3	2.9	—
K_m(PEP) mM +FDP	—	0.11	0.15	—
n_H +FDP	—	—	—	—
K_m(ADP) mM	0.3	0.21	0.26	—
Optimum pH	7.0	7.25	7.25	—
pI -FDP	8.5-8.8	8.3	6.3	—
pI +FDP				
Molecular Weight	215,000	188,000	193,000	220,000
Subunit Molecular Weight	54,000	50,000	52,000	55,000

REFERENCES

(1) Cardenas et al. (1975a)

(2) Eigenbrodt & Schoner (1977)

(3) Ibid

(4) Flanders et al. (1971)

* One unit of activity is ordinarily defined as the amount of enzyme which can produce one micromole of pyruvate per minute. In this case, the authors (Flanders et al., 1971) define one unit of activity as the amount of enzyme which can decrease the O.D. by 0.001 per minute.

TABLE 5 (continued)

Comparison of the Physical and Chemical Proerties of the Pyruvate Kinases Isolated from Some Non-Mammalian Vertebrates

	(1)	(2)	(3)	(4)
PROPERTY	TURTLE HEART	SALMON MUSCLE	SALMON LIVER	STURGEON MUSCLE
Specific Activity units/mg	210	450	45	281
Assay Temperature ^{o}C	25	15	15	30
K_m(PEP) mM -FDP	0.117	0.28	0.23	0.6
n_H -FDP	—	1.2	1.0	2.45
K_m(PEP) mM +FDP	0.021	—	—	0.04
n_H +FDP	—	—	—	—
K_m(ADP) mM	0.227	0.42	0.30	—
Optimum pH	6.4	7.0	7.0	7.5
pI -FDP	6.05	7.8	5.35	—
pI +FDP	—	—	—	—
Molecular Weight	—	—	—	—
Subunit Molecular Weight	—	57,000	57,000	56,000

REFERENCES

(1) Storey & Hochachka (1974)

(2) Guderley & Cardenas (1980a,b)

(3) Ibid

(4) Randall & Anderson (1975); Anderson & Randall (1975)

TABLE 6

Comparison of the Amino Acid Compositions of the Pyruvate Kinases Isolated from Some Non-Mammalian Vertebrates

	(1)	(2)	(3)	(4)
AMINO ACID	CHICKEN MUSCLE	CHICKEN LIVER M_2	FROG MUSCLE	STURGEON MUSCLE
CYS	8	--	10	8
ASP	48	35	44	47
THR	26	32	26	28
SER	24	47	29	32
GLU	47	36	53	54
PRO	20	29	24	24
GLY	41	61	44	43
ALA	55	58	59	52
VAL	42	28	49	36
MET	18	6	16	14
ILE	32	23	35	32
LEU	35	16	44	42
TYR	7	5	10	10
PHE	16	11	19	18
HIS	18	8	14	10
TRP	4	--	4	--
LYS	37	23	33	31
ARG	31	25	34	28
Subunit M.W.	53,000	62,000	60,000	56,000

REFERENCES

(1) Cardenas et al. (1975a)

(2) Brunn et al. (1979)

(3) Saheki et al. (1982b)

(4) Anderson & Randall (1975)

are summarized in Tables 5 and 6. The data indicates that the mammalian L, M_1 and M_2 type isozymes have their counter-parts in the tissues of the chicken. The frog skeletal muscle enzyme is also very similar to a mammalian M_1 isozyme as it exhibits hyperbolic binding kinetics with respect to both PEP and ADP and it is not activated by FDP or inhibited by the amino acids alanine and phenylalanine. However, the muscle pyruvate kinases of the salmon, sturgeon and turtle are clearly different from the mammalian M_1 type isozyme. All these enzymes exhibit co-operativity in the binding of PEP and are activated by FDP. With respect to their binding of PEP and activation by FDP, the salmon and turtle muscle enzymes are similar to the mammalian M_2 isozyme. However turtle muscle pyruvate kinase, like the mammalian M_2 isozyme, is inhibited by both alanine and phenylalanine whereas the salmon muscle enzyme is insensitive to these effectors. Sturgeon muscle pyruvate kinase is similar to the mammalian L isozyme with respect to its binding of PEP but its pH optimum is closer to that of the M_1 and M_2 types. Clearly the relationship that exists between these pyruvate kinases and the mammalian isozymes is not apparent.

This is also the case for fish liver pyruvate kinase. The enzyme purified from salmon liver is not like the mammalian L or M_2 types because it exhibits hyperbolic binding kinetics with respect to PEP, is relatively insensitive to activation by FDP and is not inhibited by amino acids. The relative insensitivity of this fish liver pyruvate kinase

to modulators of mammalian pyruvate kinase activity is surprising and it has been postulated that the metabolic capacities of the fish liver may be fundamentally different from those of the mammalian liver

3. INVERTEBRATE PYRUVATE KINASES

Since only a handful of invertebrate isozymes have been purified it is difficult to make a comparison of invertebrate and vertebrate pyruvate kinases. Pyruvate kinase has been purified from two types of yeast (Yun et al., 1976; Bishofberger et al., 1970) and from *Streptococcus lactis* (Crow & Pritchard, 1976), *Pseudomonas citronellolis* (Chuang & Utter, 1979), *Halobacterium cutirubrum* (De Médicis et al., 1982), *Neurospora crassa* (Kapoor, 1975), sea mollusc muscle (Leon et al., 1982), shore crab hepatopancreas (Giles et al., 1976c) and house cricket muscle (Hoffmann, 1975). In addition, 2 types of pyruvate kinase, one activated by FDP and one activated by AMP, have been purified from *E. coli* (Waygood & Sanwal, 1974; Somani et al., 1977). These invertebrate pyruvate kinases generally consist of 4 subunits which have molecular weights which range between 50,000 and 73,000. Specific activities as low as 75 units/mg have been reported for the enzyme from *Streptococcus lactis* (Crow & Pritchard, 1976) while a high specific activity of 935 units/mg was reported for the enzyme from *Neurospora crassa* (Kapoor, 1975). The pH optima of these invertebrate pyruvate kinases vary from 6.0 (Bishofberger et al., 1970) to 7.5 (Crow & Pritchard, 1976). Isoelectric points have been determined for a few invertebrate pyruvate

kinases and they are in the range 6.0 to 6.5 (Yun et al., 1976; Bishofberger et al., 1970; Kapoor, 1975). On the basis of subunit molecular weights and amino acid compositions, the pyruvate kinases purified from 2 types of yeast are quite different structurally (Yun et al., 1976; Bishofberger et al., 1970) as are the AMP and FDP-activated pyruvate kinases from *E. coli* (Valentini et al., 1979).

Invertebrate pyruvate kinases have been reported to exhibit a wide range of affinities for PEP. Apparent K_m values as high as 4.0 mM have been reported for the FDP-activated enzyme from *E. coli* (Waygood & Sanwal, 1974) while values as low as 0.12 mM have been reported for the enzyme from shore crab hepatopancreas (Giles et al., 1976c). Almost all invertebrate pyruvate kinases, with a few exceptions (Munday et al., 1980), exhibit co-operative binding kinetics with respect to PEP and can be activated by an allosteric effector. In most cases, FDP is the most effective activator but some invertebrate enzymes can be activated by a wide range of sugar mono- and diphosphates (Crow & Pritchard, 1976) while others are preferentially activated by AMP (Somani et al., 1977). Some invertebrate pyruvate kinases exhibit co-operativity in the binding of ADP and the allosteric activator can increase the enzyme's affinity for this substrate (Yun et al., 1976; Crow & Pritchard, 1976). One invertebrate enzyme does not absolutely require a monovalent cation for catalytic activity (Chuang & Utter, 1979) while the enzyme from *Halobacterium cutirubrum* is highly unstable at less than 3 M NaCl or KCl (De Médicis et al., 1982).

Very little detailed kinetic data is available for the invertebrate isozymes. Macfarlane & Ainsworth (1972) reported that the reaction catalyzed by the isozyme from Baker's yeast proceeded via an ordered mechanism but a rapid equilibrium model appears to be more appropriate for the muscle and hepatopancreas enzymes of the shore crab (Munday et al., 1980).

Evidently there is considerable variation in the physical and chemical properties of the invertebrate pyruvate kinases studied to date. Further purification and characterization of other invertebrate isozymes will be required in order to gain a better understanding of the roles and inter-relationships of these enzymes.

4. STRUCTURE OF PYRUVATE KINASE

The three-dimensional structure of cat muscle pyruvate kinase has been studied in detail (Stuart et al., 1979; Levine et al., 1978). Cat muscle pyruvate kinase, like all other vertebrate pyruvate kinases studied to date, is a tetrameric enzyme consisting of 4 identical subunits. Each subunit is composed of approximately 530 amino acids and the polypeptide chain is folded into three domains which are called A, B and C. Domain A is the largest composed of about 220 residues and contains a cylindrical β-sheet of 8 parallel strands. Adjacent strands are connected by α-helices which form an outer cylinder coaxial with the first. Between the third strand of β-sheet and the third α-helix of Domain A, the polypeptide chain folds up into Domain B which contains at least 100 amino acids. The main secondary structural feature of Domain B is an anti-parallel

β-sheet. After the last α-helix in Domain A, proceeding in the direction of the C-terminal, the chain folds up into Domain C which contains the C-terminus and is composed of 120 amino acid residues. The first section of Domain C comprises two anti-parallel α-helices and the rest of the chain is folded up into a five stranded β-sheet interconnected by 2 α-helices. This latter feature is similar to the first mononucleotide binding fold of lactate dehydrogenase (Levine et al., 1978) and could serve as a binding site for an adenine nucleotide.

The active site of cat muscle pyruvate kinase appears to be located at the C-terminal end of the barrel of β-sheet in Domain A. PEP binds to a site located in the middle of the end of the barrel while ATP is situated so that the γ-phosphate group overlaps with the PEP binding site and the nucleoside portion is close to the C-terminal end of the fourth strand of β-sheet in Domain A.

Recently, Burke et al. (1983) isolated and sequenced the pyruvate kinase gene of Baker's yeast. The gene encodes a protein of 499 amino acids in a single open reading frame for a subunit molecular weight of 54,608. The sequence located at amino acids 170-205 is highly homologous to the N-terminal sequence of a large polypeptide fragment derived from the hydroxylamine cleavage of cat muscle pyruvate kinase (McAleese et al., 1982). The sequence located at amino acids 283-300 is highly homologous to the sequence of a cyanogen bromide peptide derived from sturgeon and rabbit muscle pyruvate kinases (Anderson & Randall, 1975). The sequence located at amino acids 313-346 is highly homologous to the sequence of a tryp-

tic peptide derived from bovine muscle pyruvate kinase (Johnson et al., 1979). However, there do not appear to be homologous regions in the yeast enzyme to the phosphorylation site sequences of rat and pig liver pyruvate kinases (Humble, 1980) or chicken liver M_2 type pyruvate kinase (Brunn et al., 1979). In addition, there does not appear to be a homologous region in the yeast enzyme to a tryptic pentapeptide, isolated from rabbit muscle pyruvate kinase, which has been reported to contain a catalytically-important cysteine residue (Chalkley & Bloxham, 1976; Bloxham et al., 1978). These observations indicate that specific sequences within the structure of pyruvate kinase have been conserved during the course of evolution and these structures are probably of functional importance. The protein sequence derived from the yeast gene should aid in an analysis of the X-ray data obtained for the enzyme purified from cat muscle and facilitate the elucidation of the catalytic mechanism of pyruvate kinase.

5. NUMBER OF ACTIVE SITES

Rabbit muscle pyruvate kinase is a tetrameric protein consisting of 4 identical subunits (Cottam et al., 1969). Electron spin resonance studies have indicated that there are 4 binding sites for a divalent cation per enzyme tetramer (Cottam & Mildvan, 1971) while equilibrium dialysis studies have indicated there are 4 binding sites for PEP and a monovalent cation (Kayne, 1971). Therefore, it follows that each subunit of rabbit muscle pyruvate kinase contains one active site. This has been confirmed by extensive crystallographic studies (Stuart et al., 1979; Levine et al., 1978). Cat muscle pyruvate kinase contains a second ADP binding site located in Domain C of each subunit (Stammers & Muirhead, 1975). Whether other pyruvate kinase isozymes contain a second ADP binding site is not known. L, M_2 and erythrocyte type pyruvate kinases have been shown to contain one FDP binding site per subunit (Hess & Kutzbach, 1971; Nagao et al., 1982; Garreau et al., 1977). The fact that the M_1 isozyme can under certain conditions, be activated by FDP suggests that it contains a low-affinity FDP binding site (Phillips & Ainsworth, 1977).

6. IDENTIFICATION OF CATALYTICALLY-IMPORTANT FUNCTIONAL GROUPS

A number of studies have sought to determine the molecular basis for the mechanism of action of pyruvate kinase. Several authors have attempted to identify the catalytically important functional groups of rabbit muscle pyruvate kinase because the kinetic behavior of this particular isozyme has been studied extensively.

(i) CYSTEINE

Flashner et al.(1972) reported that chemical modification of rabbit pyruvate kinase by the sulfhydryl reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) resulted in enzyme inactivation. The authors noted that a lag phase was present between the commencement of sulfhydryl group modification and the loss of catalytic activity. The substrate PEP in combination with the divalent cation Mg^{+2} could completely protect against enzyme inactivation and the modification of all but one sulfhydryl group per enzyme subunit. DTNB, when present at an inhibitor to enzyme subunit mole ratio of 1:1, could completely inactivate the rabbit enzyme and this was accompanied by the modification of 2 sulfhydryl groups per enzyme subunit. The authors proposed that DTNB reacted with a catalytically unimportant cysteine residue but disulfide interchange with a second neighbouring cysteine residue resulted in the formation of a disulfide bond within the enzyme and complete inactivation. In a subsequent report, Flashner et al. (1973) showed that neither of the two reactive cysteine residues were required for catalysis. Therefore DTNB catalyzed the formation of a disulfide bond between these 2 cysteine residues and this induced an unfavourable conformational change which rendered the enzyme inactive.

Bloxham & Chalkley (1976) reported that rabbit muscle pyruvate kinase could be inactivated due to chemical modification by 5-chloro-4-oxopentanoic acid. Enzyme inactivation was attributed to the alkylation of a specific cysteine residue. In a subsequent paper, Chalkley & Bloxham (1976) isolated

a tryptic pentapeptide from rabbit pyruvate kinase which contained a chemical reactive and presumably catalytically essential cysteine residue. The authors concluded that this cysteine residue was located in the active site of rabbit muscle pyruvate kinase and the alkylation of this group by 5-chloro-4-oxopentanoic acid lead to complete enzyme inactivation. However, the authors could not exclude the possibility that at their reaction conditions, enzyme inactivation was in fact due to the modification of a chemically reactive lysine residue participating in Schiff's base formation with the keto group of 5-chloro-4-oxopentanoic acid. Also, due to the poor recovery of their radioactively labelled tryptic pentapeptide containing the "reactive" cysteine residue, the authors could not clearly identify that particular cysteine residue as being catalytically important.

Harkins & Fothergill (1977) reported that a cysteine residue of cat muscle pyruvate kinase was preferentially alkylated by iodoacetic acid. This cysteine residue was found to be contained within a specific cyanogen bromide peptide which was homologous to the cyanogen bromide peptides isolated previously from rabbit and sturgeon muscle pyruvate kinases (Anderson & Randall, 1975). Harkins & Fothergill (1977) concluded that this cysteine residue was equivalent to the chemically reactive but catalytically unimportant cysteine residue of rabbit muscle pyruvate kinase (Flashner et al., 1972). However, the authors failed to determine whether the alkylation of this cysteine residue of cat muscle pyruvate

kinase resulted in enzyme inactivation.

In an effort to verify their previous findings, Bloxham et al. (1978) used the reagent methyl methanethiosulphonate to investigate the role of the sulfhydryl groups of rabbit muscle pyruvate kinase. The authors reported that the modification of a specific cysteine residue, located either near or within the active site of the enzyme, resulted in complete enzyme inactivation. This reactive cysteine residue was found to be contained within a specific tryptic pentapeptide which was identical to the tryptic peptide isolated previously by Chalkley & Bloxham (1976). However, the authors could not identify this reactive cysteine residue as being catalytically important. Previous to this report, Flashner et al. (1972) had indicated that sulfhydryl reagents, such as methyl methanethiosulphonate, were susceptible to displacement from enzyme thiol groups due to disulfide bond interchange with neighbouring cysteine residues. Therefore, the authors could only identify the chemically reactive cysteine residues which retained the radioactively labelled sulfhydryl reagent.

It is apparent that in spite of numerous investigations, our understanding of the role, location and reactivity of the cysteine residues of pyruvate kinase is poor.

(ii) HISTIDINE AND ARGININE

Mildvan & Cohn (1965) suggested a histidine residue might be present at the active site of rabbit muscle pyruvate kinase since a pK_a of 6.8 was determined for the binding of Mn^{+2} to the enzyme. Dann & Britton (1974) conducted chemical

modification studies using diethylpyrocarbonate and provided evidence to support this hypothesis. Meshitsuka et al. (1981) examined the role of the histidine residues of rabbit muscle pyruvate kinase by proton NMR spectroscopy. The authors were able to examine the resonance lines of 6 of the 14 histidine residues of the rabbit enzyme subunit. One of these 6 histidine residues was reported to be close to the active site because the binding of the substrate PEP to the enzyme resulted in a 0.4 unit decrease in the pK_a of this particular functional group. However, the authors could not rule out the possibility that this effect was due to a conformational change induced by the substrate. In addition, they were unable to comment on the state of the remaining 8 histidine residues known to be present in each subunit of this enzyme.

Cardemil & Eyzaguirre (1979) examined the susceptibility of rabbit muscle pyruvate kinase to inactivation by 2,3-butadione. The enzyme could be inactivated by this reagent but only at high inhibitor to enzyme subunit mole ratios. Also there was no apparent stoichiometry between the modification of arginine residues and the inactivation of the protein. At the present time, no direct evidence is available to indicate that arginine or histidine residues are involved in the catalytic mechanism of pyruvate kinase.

(iii) LYSINE

The chemical modification of the lysine residues of rabbit muscle pyruvate kinase by pyridoxal 5'-phosphate has

been reported to lead to a loss of catalytic activity (Johnson & Deal, 1970). Hollenberg et al. (1971) reported that the inactivation of rabbit muscle pyruvate kinase was accompanied by the incorporation of one mole of 2,4,6-trinitro-benzene-1-sulfonate (TNBS) per mole of enzyme subunit. The modification of one specific lysine residue was indicated by the finding that ϵ-TNP-lysine was the only modified amino acid present in a hydrolysate of the chemically modified protein. ADP but not PEP was able to protect against enzyme inactivation therefore Flashner et al. (1973) suggested that TNBS modified a specific lysine residue located in the vicinity of the nucleotide binding site of the active site. Chemical modification of bovine muscle pyruvate kinase by TNBS yielded similar observations (Hubbard & Cardenas, 1975). Johnson et al. (1979) isolated and sequenced a tryptic peptide, composed of 34 amino acids, from bovine muscle pyruvate kinase which contained the chemically reactive lysine residue. The role this reactive lysine residue plays in the catalytic mechanism of pyruvate kinase has not been determined.

(iv) AFFINITY LABELS

A variety of affinity labels have been employed in order to attempt to identify the reactive functional groups at the active site of pyruvate kinase. Wyatt & Colman (1977) reported that 5'-p-fluorosulfonyladenosine (FSBA), a structural analogue of ADP, could completely inactivate rabbit muscle pyruvate kinase. The chemical modification of 2 tyrosine residues

located at 2 separate sites of the rabbit enzyme subunit was responsible for the loss of catalytic activity (Annamalai et al., 1979). However, Likos & Colman (1981) reported that 5'-p-(flourosulfonyl)benzoyl-1,N^6-ethenoadenosine(FSBEA), another analogue of ADP, could also completely inactivate rabbit muscle pyruvate kinase by reacting with a catalytically important cysteine residue at one site and a catalytically unimportant functional group, presumably a tyrosine residue, at a second site of the enzyme subunit. Tomich et al. (1981) reported that 5'-p-(fluorosulfonyl)benzoyl guanosine (FSBG), an analogue of GDP, could completely inactivate rabbit pyruvate kinase by reacting with a catalytically important cysteine residue and a catalytically unimportant functional group. However, the chemically modified cysteine residue did not retain the affinity label because it was displaced due to nucleophilic attack by a neighbouring cysteine residue. In view of the fact that the affinity labels FSBEA and FSBG appear to inactivate rabbit pyruvate kinase via a similar mechanism, it is not clear why FSBG and not FSBEA is displaced from the chemically reactive cysteine residue of the rabbit enzyme subunit.

Annamalai & Colman (1981) re-investigated the inactivation of rabbit muscle pyruvate kinase due to chemical modification by FSBA. The authors reported that FSBA reacted with a catalytically important cysteine residue, but like the affinity label FSBG, it was displaced from the chemically modified cysteine residue due to nucleophilic attack by a neighbouring cysteine residue. Like the affinity label FSBG,

FSBA also reacted with a tyrosine residue but whereas the tyrosine residue chemically modified by FSBG was reported to be catalytically unimportant (Tomich et al., 1981), the tyrosine residue chemically modified by FSBA was reported to be important for catalysis (Annamalai & Colman, 1981).

Little can be concluded from these series of studies. The studies employing FSBG and FSBEA indicated that the modification of one tyrosine residue per subunit of rabbit muscle pyruvate kinase did not result in enzyme inactivation but this is not the case with FSBA. FSBEA could completely inactivate the enzyme by modifying one essential cysteine residue per enzyme subunit but unlike the affinity labels FSBG and FSBA, this reagent was not displaced from the enzyme due to disulfide interchange. Clearly, these different affinity labels , which are all structural analogues of ADP, inactivate rabbit pyruvate kinase by different mechanisms. Why this should be the case is not clear.

The authors frequently assume that these affinity labels react only at the 2 known nucleotide binding sites of the rabbit muscle pyruvate kinase subunit (Stammers & Muirhead, 1975). However, Hinrichs & Eyaguirre (1982) reported that dialdehyde-ADP, yet another structural analogue of ADP, reacted with 6 to 7 functional groups of the rabbit enzyme subunit. Therefore the specificity of these substrate analogue affinity labels is open to question.

7. SUBSTRATE SPECIFICITY

Rabbit muscle pyruvate kinase exhibits a rather broad spec-

ificity for the dinucleotide substrate of the reaction. Plowman and Krall (1965) determined that ADP and GDP were the best substrates followed by UDP and lastly CDP. The enzyme's affinity for ADP is however at least 4 times greater than its affinity for any other nucleotide diphosphate.

By contrast, rabbit muscle pyruvate kinase exhibits a very high specificity for the phosphoryl group donor. A number of PEP analogues have been synthesized including phospho-enol-∝-ketobutyrate (Woods et al., 1972), phospho-enol-∝-ketovalerate (Woods et al., 1972) and phospho-enol-bromopyruvate (Stubbe & Kenyon, 1972). In the presence of saturating concentrations of these PEP analogues, the rabbit enzyme exhibited a maximal velocity which was less than 1% of the maximal velocity observed in the presence of PEP. In spite of this fact, the enzyme's affinity for these PEP analogues was comparable to its affinity for PEP.

A comparison of the substrate specificities of several pyruvate kinase isozymes could provide some information regarding the active site structures of these different enzymes. Unfortunately, the substrate specificity of only the M_1 isozyme from rabbit muscle has been studied in detail.

8. COFACTORS

Pyruvate kinase was the first enzyme for which an absolute requirement for a monovalent cation was demonstrated (Boyer, 1962). Potassium ion was found to be the best activator of rabbit muscle pyruvate kinase followed by NH_4^+, Rb^+, Tl^+, Na^+ and Li^+ in order of decreasing effect (Kayne, 1971). The catalytic role of the

monovalent cation is not clear. Kayne (1971) showed that the monovalent cation was present at the active site when it was bound to the enzyme. Reuben & Kayne (1971) were able to estimate the distance between the monovalent and divalent cation binding sites of the rabbit enzyme. This distance decreased from 8.2 Å to 4.7 Å upon the addition of the substrate PEP to the enzyme. It was proposed that the monovalent cation played a permissive role by facilitating substrate-induced conformational changes in the enzyme. Potassium ion could also increase the affinity of the binary enzyme-Mn^{+2} complex for PEP except in cases where the carboxyl group of the substrate was blocked (Nowak & Mildvan, 1972). Thus the monovalent cation might be required for the coordination of the substrate PEP at the carboxyl group. Presently, there is no direct evidence for the participation of the monovalent cation in facilitating the proper binding of PEP by the enzyme (Villafranca & Raushel, 1982).

Pyruvate kinase also exhibits an absolute requirement for a divalent cation. Magnesium ion is the best activator of rabbit muscle pyruvate kinase followed by Co^{+2}, Mn^{+2} and lastly Ni^{+2} (Kwan et al., 1980). However the enzyme's affinity for Co^{+2} and Mn^{+2} is 5 times higher than its affinity for Mg^{+2} and Ni^{+2}. The divalent cation, when bound to the rabbit enzyme, has been shown to be present at the active site close to the dinucleotide substrate binding site (Mildvan & Cohn, 1965; Mildvan & Cohn, 1966). It was proposed that the divalent cation participated in a tertiary enzyme-metal-ADP complex with the cation acting as a bridge. However Reuben & Cohn (1970) reported that while the enzyme-bound divalent cation was not

directly co-ordinated to the dinucleotide substrate it was located in the vicinity of the β-phosphate moiety of the enzyme-bound ADP. Nowak & Mildvan (1972) proposed that if the divalent cation was not required for the correct orientation of ADP at the active site, perhaps it was directly co-ordinated to the phosphoryl group undergoing transfer. However, Kayne (1973) has pointed out that while this possibility seems likely, there is no direct proof for it at present.

Gupta et al. (1976) provided evidence that rabbit muscle pyruvate kinase required a second divalent cation for catalytic activity in addition to the enzyme-bound cation. The second divalent cation appears to be co-ordinated to the α and β-phosphate moieties of ADP and it is required for the proper orientation of the phosphoryl group acceptor (Baek & Nowak, 1982).

9. THERMODYNAMICS

The pyruvate kinase reaction proceeds overwhelmingly in favour of the production of ATP and pyruvate and hence, this reaction is regarded as being irreversible *in vivo*. Nageswara Rao et al. (1979) calculated a value of 3.33×10^3 at 15°C and pH 8.0 for the equilibrium constant of the pyruvate kinase reaction. Rabbit muscle pyruvate kinase was employed for this study and similar calculations have not been performed for other pyruvate kinase isozymes.

10. ACTIVE SITE STRUCTURE OF PYRUVATE KINASE

The active site of rabbit muscle pyruvate kinase can be divided into 4 parts (Reynard et al., 1961). There is one

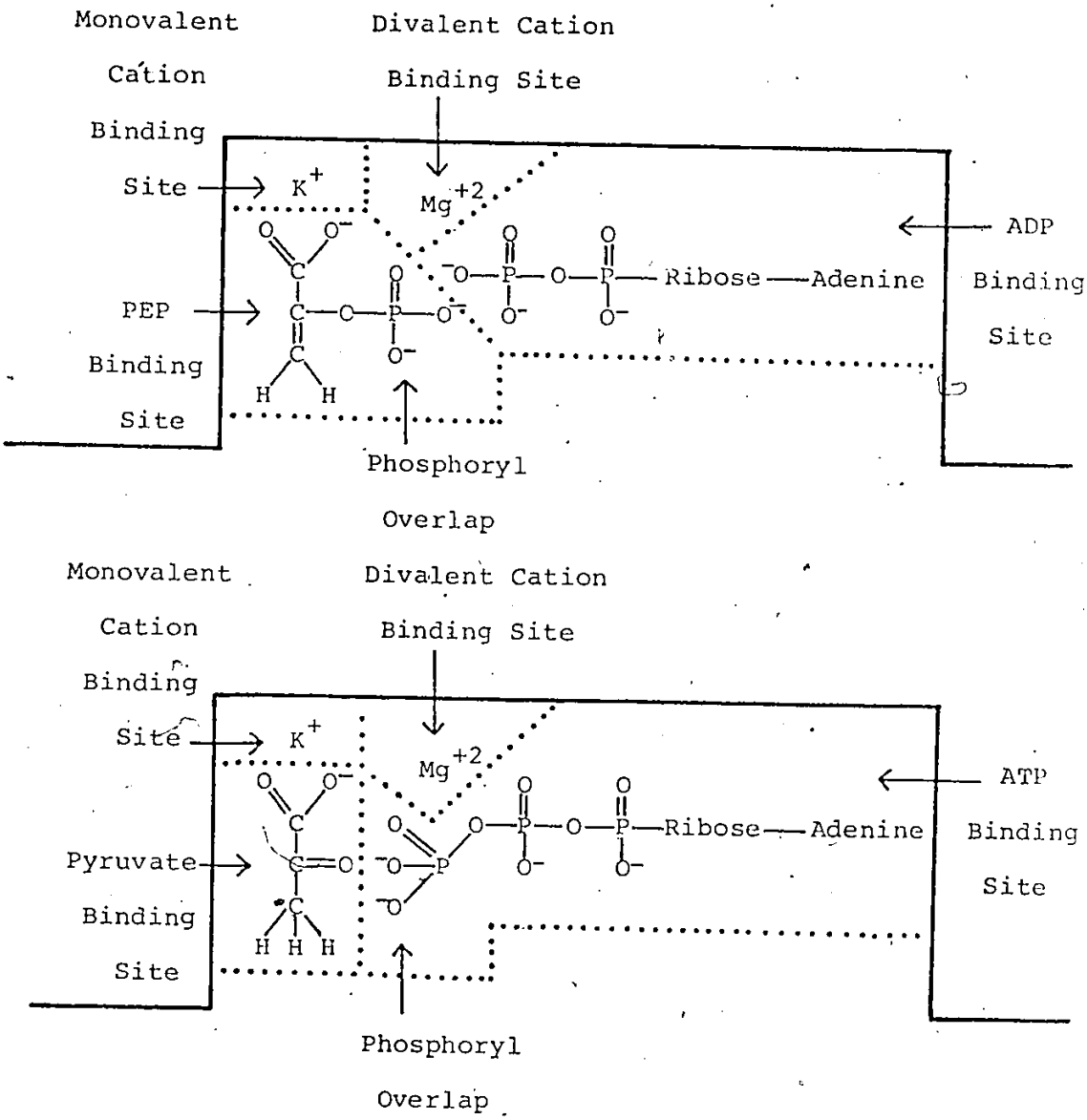


FIGURE 1. Active Site Structure of Pyruvate Kinase

binding site for a monovalent and a divalent cation and a binding site for each of the substrates of the pyruvate kinase reaction, PEP and ADP (Fig. 1). The reaction product pyruvate can also occupy the PEP binding site while the product ATP can occupy the ADP binding site. Reynard et al. (1961) recognized that ATP could inhibit the pyruvate kinase reaction by competing with PEP and ADP for binding sites at the enzyme ac-

tive site. Based on this observation, the authors concluded that the binding site for the γ-phosphate group of ATP overlapped with the binding site for the phosphoryl group of PEP. This phosphoryl overlap region is therefore located between the pyruvate and ADP binding sites (Fig. 1).

11. MECHANISM OF ACTION

A proposed scheme for the mechanism of action of rabbit muscle pyruvate kinase has been described (Nowak, 1978) (Fig. 2). The pyruvate kinase reaction can be divided into 4 parts; 1) the binding of substrates and metal ions to the active site, 2) the transfer of a phosphoryl group from PEP to ADP resulting in the generation of an enolate intermediate, 3) the collapse of the enolate intermediate with the subsequent protonation of the C-3 carbon resulting in the formation of pyruvate and 4) the departure of products.

Binding of Substrates

According to this model, no substrates are present at the active site initially and the monovalent and divalent cation sites are occupied. The distance between these two sites has been estimated to be 8.7 Å (Reuben & Kayne, 1971). The substrate PEP would bind to the active site such that its carboxyl group would be co-ordinated to the monovalent cation (Nowak & Mildvan, 1972) and its phosphoryl group would be co-ordinated to the divalent cation (Mildvan & Cohn, 1965). These cations are required for the proper orientation of the substrate PEP and for the neutralization of the charges of the carboxyl and phosphoryl groups. In addition, these cations

FIGURE 2

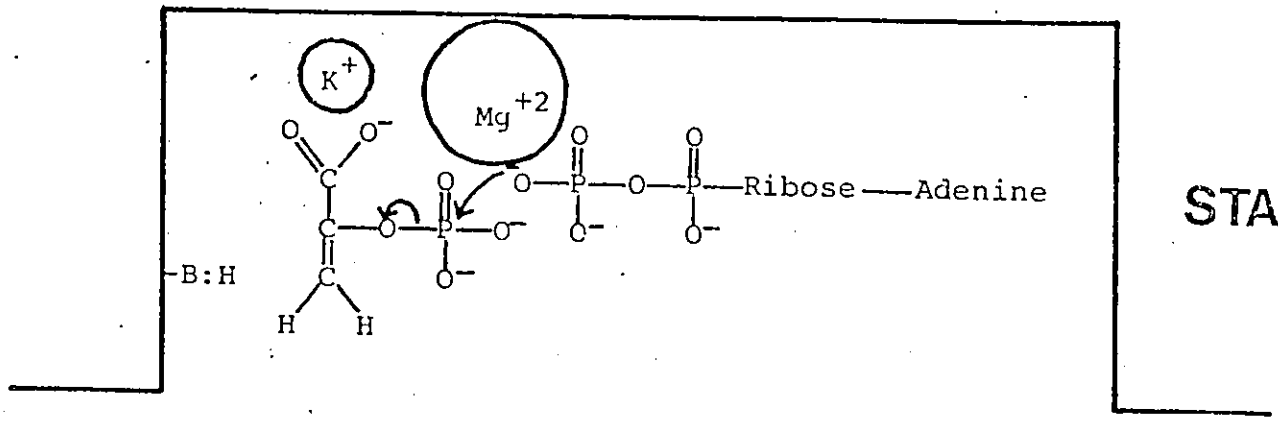
Proposed Scheme for the Mechanism
of Action of Rabbit Muscle Pyruvate Kinase

A simplified version of a scheme for the mechanism of action of rabbit muscle pyruvate kinase (Nowak, 1978) is shown here.

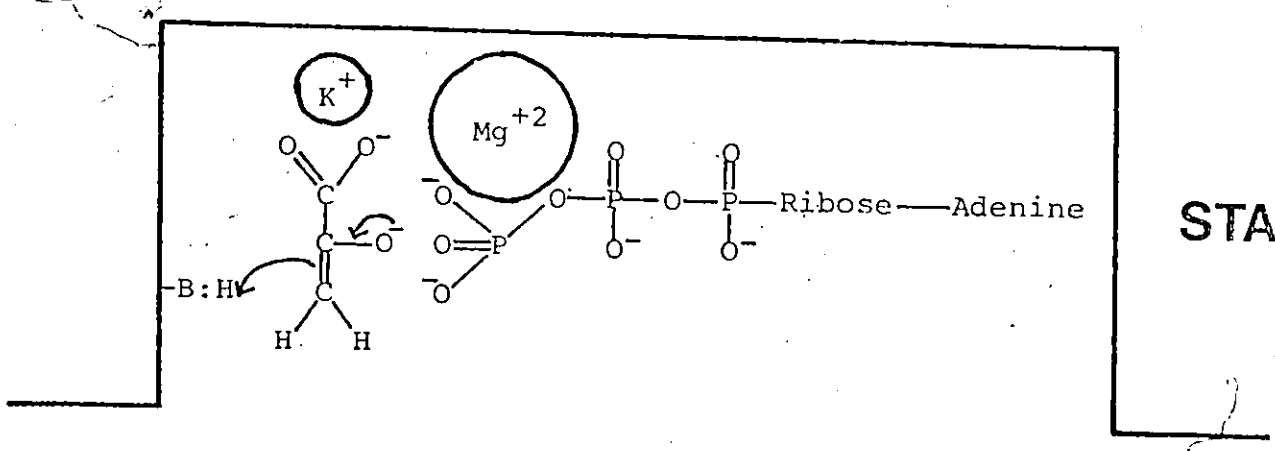
Stage 1: The pyruvate kinase reaction involves the nucleophilic attack of the phosphoryl group of PEP by the β-phosphate group of ADP in the presence of K^+ and Mg^{+2}. If an associative mechanism of phosphoryl group transfer is involved, then phosphoryl group departure from PEP occurs simultaneously with the formation of the oxygen bridge between the β and γ-phosphate groups of ATP as shown here.

Stage 2: Following phosphoryl group transfer, enol-pyruvate and ATP are present at the active site. Protonation of the C-3 carbon of the enolate intermediate results in the formation of pyruvate. A basic functional group of the enzyme (-B:H) may participate in this reaction.

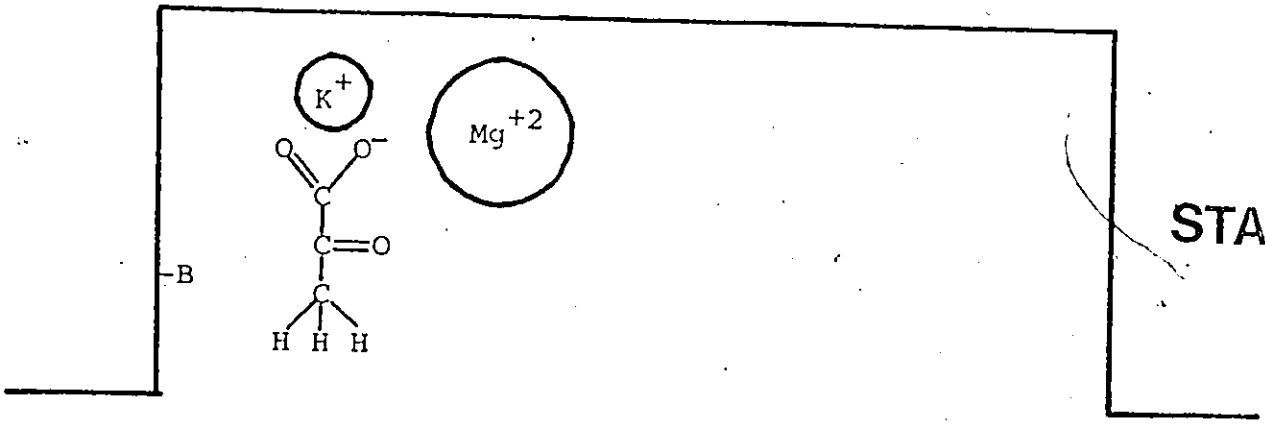
Stage 3: Following the departure of ATP, pyruvate remains at the active site. The release of pyruvate from the enzyme may represent the rate-limiting step in the pyruvate kinase reaction.



STAGE 1



STAGE 2



STAGE 3

greatly increase the enzyme's affinity for PEP and ADP. Rabbit pyruvate kinase has been shown to exhibit mutually co-operative effects in the binding of Mn^{+2} and ADP (Mildvan & Cohn, 1966), Mn^{+2} and PEP (Mildvan & Cohn, 1966) and K^{+} and Mg^{+2} (Nowak, 1973). The binding of PEP results in a substantial conformational change which is manifested by a decrease in the inter-cationic distance to 6.5 Å (Nowak, 1978). Pyruvate kinase may bind the substrates PEP and ADP in a random order but for the purposes of this scheme, PEP binds before ADP. If ADP were to bind to the enzyme before PEP only a slight conformational change would be observed and the inter-cationic distance would narrow to 8.2 Å (Nowak, 1978). ADP is not directly co-ordinated to either of the bound cations at the active site. A basic functional group at the ADP binding site may be responsible for the proper alignment of the β-phosphate group of ADP (Flashner et al., 1973; Hollenberg et al., 1971). Recent evidence suggests that a second divalent cation may be required at the active site and it would chelate to the α and β-phosphate groups of ADP (Gupta et al., 1976; Baek & Nowak, 1982). The addition of ADP to the quaternary enzyme-K^{+}-Mg^{+2}-PEP complex would be accompanied by a conformational change at the active site which would result in the squeezing together of the substrates and the inter-cationic distance would close to less than 6.1 Å (Nowak, 1978). Water molecules present at the active site would be expelled during this process.

Transfer of Phosphoryl Group

Reynard et al. (1961) proposed that the pyruvate kinase reaction involved the direct transfer of a phosphoryl group from PEP to ADP. Lowe & Sproat (1979) reported that pyruvate kinase-catalyzed phosphoryl group transfer occurred via a dissociative mechanism. This implied that phosphoryl group departure from PEP preceded the formation of the oxygen bridge between the β and γ-phosphate groups of ATP. However, Hassett et al. (1982) recently reported that pyruvate kinase-catalyzed phosphoryl group transfer occurred via an associative and not a dissociative mechanism. If an associative mechanism is involved then phosphoryl group departure from PEP would occur simultaneously with the formation of the oxygen bridge between the β and γ-phosphate groups of ATP. Hassett et al. (1982) has argued that an associative mechanism of phosphoryl group transfer would appear to be more logical since it would avoid the possibility of the fruitless reaction of the highly reactive metaphosphate ion (PO_3^{-2}) with water molecules trapped within the active site (Hassett et al., 1982).

Protonation of the Enolate Intermediate

Following phosphoryl group transfer, enol-pyruvate and ATP are present at the active site and protonation of the enolate intermediate gives rise to the reaction product pyruvate. A basic functional group located within the active site close

to the C-3 position of the enzyme-bound intermediate may participate in this protonation reaction (Robinson & Rose, 1972). Rabbit muscle pyruvate kinase can catalyze the detritiation of pyruvate labelled with ^{3}H in the C-3 position, in the presence of phosphate buffer or ATP (Rose, 1960). This suggests that the phosphoryl overlap region of the active site must be occupied in order for the detritiation reaction to occur. Robinson & Rose (1972) showed that tritium was lost from the C-3 position of ^{3}H-PEP during the pyruvate kinase reaction prior to the release of pyruvate. On the basis of these observations, it has been proposed that the rate-limiting step in the pyruvate kinase reaction is the release of pyruvate from the enzyme. Since the loss of tritium from ^{3}H-PEP only occurs during the course of the net reaction, the protonation of the enolate intermediate must follow phosphoryl group transfer.

Departure of Products

Just as there are conflicting reports regarding the order of substrate combination with the enzyme, the order of product departure has not been determined. However, Nowak (1978) reported that the departure of ATP from the active site resulted in a relaxation of the closed conformation and the intercationic distance increased to 6.9 Å. The departure of the second product, pyruvate would restore the active site to its original conformation.

Further studies are required to determine the mechanism of action of pyruvate kinase. Much of our knowledge concerning

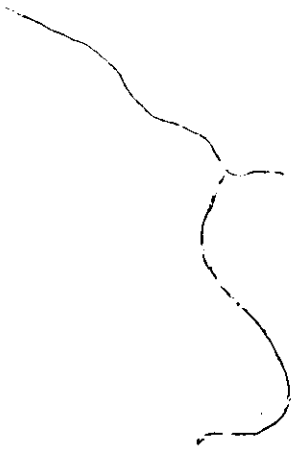
the location and role of substrates and metal ions at the active site is based on NMR studies employing paramagnetic cationic probes. By necessity, these studies have been unable to use K^{+} and Mg^{+2} which are the usual cations employed in kinetic studies. It will be necessary to determine the spatial relationships at the active site when these cations are present.

Current models suggest that substantial conformational changes occur at the active site during catalysis but the nature of these changes has not been determined. In addition, the temporal relationships of catalysis are unknown. The order of substrate and cation binding to the enzyme and the order of product and cation release from the enzyme is not known. Some studies have reported that rapid random equilibrium conditions apply hence there is no order of ligand binding to the enzyme (Reynard et al., 1961; Ainsworth & Macfarlane, 1973). However, it has also been reported that substrate binding is ordered (Dann & Britton, 1978) while other groups contend that substrate binding is random but product departure is ordered (Giles et al., 1976a). Evidently the events of catalysis are largely a mystery.

Although catalytically essential lysine (Hollenberg et al., 1971) and cysteine residues (Annamalai & Colman, 1981; Flashner et al., 1972) have been reported to be present at the active site, little is known about the participation of any functional group of the protein in the events of catalysis. A basic functional group may be involved in the catalysis of the enolization of pyruvate but this residue has not been identified

(Robinson & Rose, 1972).

Essentially all of the kinetic and spectroscopic studies of pyruvate kinase have been conducted with the enzyme from rabbit muscle. The mechanism of action of other pyruvate kinase isozymes has not been investigated.



B) RESEARCH OBJECTIVES

Further studies of purified pyruvate kinases from various sources are required to define the structural features and physiological role of pyruvate kinases. A potentially useful way to help determine the origin and role of pyruvate kinases in mammals is to examine the enzymes in the tissues of more primitive species where the purified enzyme can be obtained in good yield. Examination of the enzyme from the muscle of such an animal can help determine if the kinetic and structural properties of the M_1 isozyme of mammals are unique to mammals or are general features of pyruvate kinase from skeletal muscle.

Previously it was shown that sturgeon muscle pyruvate kinase, unlike the M_1 isozyme from rabbit muscle, exhibited pronounced co-operativity in the binding of the substrate PEP and was activated by FDP (Randall & Anderson, 1975). Subsequent studies, designed to investigate the structural basis for these kinetic differences, revealed that sturgeon and rabbit muscle pyruvate kinases shared similar subunit structures (Anderson & Randall, 1975). It would be interesting therefore to examine the structural and kinetic properties of a muscle pyruvate kinase of a fish which has evolved more recently than the sturgeon. Comparison of the properties of this enzyme to those of other isozymes might be expected to shed some light on the evolution of pyruvate kinase isozymes.

Partially purified pyruvate kinase from the white muscle of the American eel, *Anguilla rostrata*, has been reported to exhibit different kinetic properties from the mammalian M_1-

type enzyme (Moon & Hulbert, 1980a,b). The white muscle of the American eel is characterized by its low oxidative capacity, large fiber diameter, poor vascularization and high glycolytic potential (Hulbert & Moon, 1978a,b). It has been proposed that the energy for white muscle contraction is derived primarily from anaerobic glycolysis (Boström & Johansson, 1972). Therefore one might expect that the manner in which the activity of the key glycolytic enzyme pyruvate kinase is regulated in eel white muscle might be substantially different from the manner in which the enzyme is regulated in mammalian muscle.

The life cycle of the American eel is such that it inhabits the freshwater rivers and lakes of North-Eastern America for 7 to 12 years. At the end of this time, sexual differentiation and maturation begin and these events are accompanied by many metabolic and physiological changes as the eel develops to the Silver or Bronze stage (Tesch, 1977). These changes are associated with the seaward migration of the eels which return to the Sargasso sea for spawning. During the spawning migration, the eel experiences a natural starvation period during which the energy for locomotion and gonadal development must be derived from stored reserves. Therefore one might expect that in the white muscle of the mature, bronze eel, the activity of pyruvate kinase would be regulated in order to adjust to the varying energy requirements of the tissue.

Pyruvate kinase has been purified from the white muscle of the mature, bronze American eel, *Anguilla rostrata*, in order

to further define the kinetic properties of the enzyme and to attempt to correlate them with structural features and physiological role. Chapter 3 of this thesis describes the purification of eel white muscle pyruvate kinase in sufficient yield so as to permit structural studies. The kinetic properties of the purified enzyme were determined and compared to those of other pyruvate kinase isozymes. As the kinetic properties of eel white muscle pyruvate kinase were found to be substantially different from those of the M_1 isozyme from rabbit muscle, the subunit and primary structures of these two different muscle isozymes were examined and compared to those of other pyruvate kinase isozymes (Chapter 4). Finally Chapter 5 describes chemical modification studies which were conducted in order to determine the number, role and reactivity of the sulfhydryl groups of eel and rabbit muscle pyruvate kinases. As numerous authors have indicated that rabbit muscle pyruvate kinase contains at least one catalytically important cysteine residue, this study was undertaken in order to shed some light on the structural basis for the kinetic differences exhibited by eel and rabbit muscle pyruvate kinases.

- CHAPTER 2 -

MATERIALS AND METHODS

A) MATERIALS

1. Chemicals

The following chemicals were obtained from Sigma Chemical Co., St. Louis, Missouri.

Acrylamide, N,N^1-methylenebisacrylamide, the di(cyclohexylammonium) salt of adenosine 5'-diphosphate, the disodium salt of adenosine 5'-triphosphate, alanine, bovine serum albumin, dansyl chloride, 5, 5'-dithiobis(2-nitrobenzoic acid), 1-ethyl-3(3-dimethyl-amino-propyl)-carbodiimide-HCl, the tetracyclohexylammonium salt of D-fructose-1,6-bisphosphate, imidazole, rabbit muscle lactic dehydrogenase Type V, lysozyme, $MgCl_2 \cdot 6H_2O$, 2(N-morpholino)ethane-sulfonic acid, the cyclohexylamine salt of the reduced form of β-nicotinamide adenine dinucleotide, the tri-monocyclohexylammonium salt of phospho-enol-pyruvate, phenylalanine, KCl, sodium dodecyl sulphate, bovine thyroglobulin, Trizma base, TPCK-treated trypsin and soybean trypsin inhibitor.

The following chemicals were obtained from Pierce Chemical Co., Rockford, Illinois.

Citraconic anhydride, cyanogen bromide, dithiothreitol, ethyl acetate, N-ethyl morpholine, glycine ethyl ester hydrochloride, guanidine hydrochloride, iodoacetamide, ninhydrin, phenyl isothiocyanate, trifluoroacetic acid and urea.

Rabbit muscle pyruvate kinase and rabbit muscle creatine kinase were obtained from Boehringer-Mannheim Co., Montreal,

Canada. The rabbit muscle pyruvate kinase used in the structural and kinetic studies reported here was judged to be pure as determined by SDS and 8 M urea-polyacrylamide gel electrophoresis. The specific activity of this commercial preparation was between 350 and 380 units/mg of protein.

Sephadex G-25 (coarse), Sephadex G-75 (superfine) and Sephacryl S-300 were products from Pharmacia Fine Chemicals, Montreal, Canada.

Whatman DE-32 and CM-23 advanced ion exchange celluloses were obtained from Mandel Scientific Co., Montreal, Canada.

Iodo[2-^{14}C]acetic acid and iodo[1-^{14}C]acetamide were obtained from Amersham-Searle Co., Oakville, Ontario.

Iodo[2-^{3}H]acetic acid and Aquasol were obtained from New England Nuclear, Lachine, Quebec.

Bio-lyte 3/10 and Bio-lyte 5/7 ampholyte solutions were obtained from Bio-Rad Labs, Richmond, California.

Iodoacetic acid, glycerol and 2-mercaptoethanol were obtained from Baker Chemicals Co., Phillipsburg, New Jersey.

N,N,N',N'-Tetramethylethylenediamine was obtained from Eastman Kodak Co., Rochester, New York.

2. The Animal

The white muscle of mature bronze American eels, *Anguilla rostrata*, was used as the source of pyruvate kinase. The eels were captured during their seaward migration and maintained as described previously (Hulbert & Moon, 1978a). The animals were approximately 60 cm in length and were healthy and demonstrated large fat reserves at the time of sacrifice.

B) METHODS

1. Enzyme Assays

Pyruvate kinase activity was determined using a modification of a coupled assay (Bücher & Pleiderer, 1955). Eel white muscle pyruvate kinase activity was routinely assayed using 50 mM imidazole-HCl buffer, pH 6.3 containing 2.0 mM ADP, 2.5 mM PEP, 45 mM KCl, 8.0 mM $MgCl_2$, 0.15 mM β-NADH and 3 units of dialyzed lactic dehydrogenase in a cuvette volume of 2.5 ml. Concentrated solutions of eel pyruvate kinase were assayed by diluting an aliquot of the solution in 50 mM imidazole-HCl buffer, pH 6.3 containing 30% (v/v) glycerol. Rabbit muscle pyruvate kinase activity was routinely assayed using 50 mM Tris-HCl buffer, pH 7.5 containing 2.0 mM ADP, 2.5 mM PEP, 100 mM KCl, 8.0 mM $MgCl_2$, 0.15 mM β-NADH and 3 units of dialyzed lactic dehydrogenase in a cuvette volume of 2.5 ml. Concentrated solutions of rabbit pyruvate kinase were assayed by diluting an aliquot of the solution in 50 mM Tris-HCl buffer, pH 7.5 containing 30% (v/v) glycerol. All enzyme solutions and assay buffers were freshly prepared for each day's experiment.

In order to determine reaction rates, the change in $O.D._{340}$ was recorded as a function of time. By application of the Lambert-Beer law, the change in the $O.D._{340}$ could be related to a change in the concentration of β-NADH in the assay solution as follows:

$$\Delta c = \Delta O.D._{340} \div (\epsilon \times l)$$

where Δc = the change in β-NADH concentration per minute

$\Delta O.D._{340}$ = the change in the $O.D._{340}$ per minute

ϵ = the molar extinction coefficient for β-NADH, i.e. 6.22×10^3 l/mole·cm (Bücher & Pleiderer, 1955)

l = cuvette path length, i.e. 1.0 cm

The value for Δc was multiplied by the volume of the assay solution (0.0025 l) in order to calculate the change in the number of moles of β-NADH per minute. In the presence of an excess of lactic dehydrogenase, the production of 1 micromole of pyruvate per minute, catalyzed by pyruvate kinase, leads to the conversion of 1 micromole of β-NADH to β-NAD per minute. One unit of pyruvate kinase activity is defined as the amount of enzyme which produced one micromole of pyruvate per minute.

Cuvette temperatures were controlled by coupling the jacketed cuvette holder to a Neslab refrigerated water bath. Assays were conducted at 30°C unless otherwise indicated and the initial reaction rates were linear for at least 2 minutes. To determine the effects of various modifiers of pyruvate kinase activity, concentrated enzyme solutions were preincubated with these substances at 4°C for 5 minutes prior to dilution into the assay buffer containing the modifiers. Double reciprocal plots were used to determine maximum velocities ($V_{max.}$) and substrate or activator concentrations required to achieve one-half maximum velocity (apparent K_m and apparent K_a respectively). Where Michaelis-Menten kinetics were not applicable, substrate concentrations required to achieve one-half maximum velocity were determined directly from substrate saturation plots ($S_{0.5}$). Hill plots were used to determine Hill coefficients. Each kinetic experiment was performed in triplicate with a very small variation in the kinetic parameters obtained (less than 5%). In addition, experiments were performed with enzymes representing different purification batches and identical results were obtained.

2. Protein Determination

Protein was measured by a modified Biuret procedure using bovine serum albumin as the protein standard (Itzhaki & Gill, 1964). For solutions of the purified eel muscle enzyme, protein concentration, expressed in mg/ml, was determined using an absorbance coefficient value ($A_{280}^{0.1\%}$) of 0.69 cm^{-1} at 280 nm. The absorbance coefficient value was determined as follows: a 5.0 mg/ml solution of purified eel pyruvate kinase was dialyzed exhaustively against water at 4°C and the dialysate was centrifuged at 15,000*g* for 20 minutes. The absorbance of the clear supernatant solution at 280 nm was recorded. In order to determine the protein concentration of the clear supernatant solution, an aliquot of a known volume was removed, hydrolyzed with a known amount of a norleucine standard and amino acid analysis was conducted. The number of moles of glycine, alanine and valine in the sample were determined. The protein content could then be determined based on the fact that eel pyruvate kinase contains a total of 141 moles of glycine, alanine and valine per mole of enzyme subunit. In addition, the protein concentration of the clear supernatant solution was determined by adding 2.0 ml of the solution to 1.0 ml of 30% (w/v) aqueous NaOH. The absorbance of this solution was recorded at 280 nm and compared to that of a solution consisting of a known weight of lyophilized pyruvate kinase dissolved in a known volume of 10% (w/v) aqueous NaOH. For solutions of rabbit muscle pyruvate kinase, protein concentration was determined in a similar manner using an absorbance coefficient of 0.54 cm^{-1} (Hall & Cottam, 1978).

3. Enzyme Purification

All procedures were carried out at 4°C. Frozen eel white muscle (approx. 500g) was ground in a meat grinder and extracted for 30 min with two volumes of a solution containing 10 mM potassium phosphate-2 mM EDTA, pH 5.8, 10% (v/v) glycerol and 0.1 mM DTT. The suspension was then strained through cheesecloth and the muscle was re-extracted with two volumes of 5 mM EDTA buffer, pH 5.8 containing 0.15 M KCl and 0.1 mM DTT. The second suspension was strained through cheesecloth and the two extracts were pooled and strained again. After adjustment of the pH of the extract to pH 5.4 by the dropwise addition of 5 M acetic acid, the extract was centrifuged at 9,000*g* for 15 min in a Beckman JA-21 centrifuge.

Ammonium sulphate fractionation.

The supernatant of the pH 5.4 treatment was brought to 40% saturation by the gradual addition of solid $(NH_4)_2SO_4$ (243 g/litre of initial solution) and after stirring for 30 min the solution was centrifuged at 15,000*g* for 15 min. The supernatant was brought to 75% saturation by the addition of solid $(NH_4)_2SO_4$ (245 g/litre of supernatant) and after 30 min of stirring the solution was centrifuged at 15,000*g* for 15 min. The precipitate was dissolved in 150 ml of 5 mM potassium phosphate buffer, pH 5.8 containing 30% (v/v) glycerol and 0.1 mM DTT (Buffer A) and desalted by passage through a column (5 cm x 80 cm) of Sephadex G-25 equilibrated with this buffer.

ion-exchange treatments.

CM-23 cellulose, equilibrated with Buffer A, was added batch-wise to the protein solution (approx. 650 ml). After each addition of CM-23 cellulose (approx. 20 ml of swollen ion exchanger per addition), the solution was stirred and the ion exchanger was allowed to settle before the supernatant fraction was assayed for pyruvate kinase activity. The procedure was stopped when the pyruvate kinase activity in the supernatant fraction was reduced to 95% of the initial activity observed prior to the addition of the ion exchanger (the total volume of CM-23 cellulose added was approx. 150 ml). The mixture was then centrifuged at 9,000*g* for 15 min and the supernatant fraction containing the pyruvate kinase activity was collected. The CM-23 cellulose was washed with an equal volume of Buffer A and the washings were combined with the supernatant fraction. After the pH was adjusted to pH 7.8 by the dropwise addition of 5 M KOH, the solution was applied to a column (2.5 cm x 40 cm) of DE-32 cellulose equilibrated with 5 mM potassium phosphate buffer, pH 7.8 containing 30% (v/v) glycerol and 0.1 mM DTT (Buffer B). The column was washed with two volumes of Buffer B and then with two volumes of Buffer B containing 0.04 M KCl. The pyruvate kinase activity was eluted with a linear gradient formed from 400 ml of Buffer B containing 0.04 M KCl and 400 ml of Buffer B containing 0.12 M KCl. Pyruvate kinase activity was eluted as a single peak. The fractions containing this activity were concentrated by ultrafiltration in an Amicon cell equipped with an XM-50 membrane.

Sephacryl S-300 chromatography.

Ten ml portions of the concentrated DE-32 cellulose eluate containing approx. 100 mg of protein were applied to a column (2.5 cm x 80 cm) of Sephacryl S-300 equilibrated with 50 mM potassium phosphate buffer, pH 7.5 containing 30% (v/v) glycerol and 0.1 mM DTT. The column was eluted with this buffer and fractions containing pyruvate kinase specific activities of 300 units/mg of protein or greater were pooled and stored in this buffer at $4^{o}C$.

4. SDS-Polyacrylamide Gel Electrophoresis

SDS-Polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970). For analytical procedures, gels were prepared in glass tubes (I.D.=0.6 cm) while for molecular weight determinations, slab gels were prepared in glass cassettes (14.0 cm x 18.0 cm x 0.7 mm). In both cases a 10 cm long running gel was prepared and overlayed with a 2-3 cm long stacking gel. The stacking gel consisted of 7.5% (w/v) acrylamide, 0.2% (w/v) bis-acrylamide, 0.1% (w/v) SDS, 0.125 M Tris-HCl buffer, pH 6.8, 0.05% (v/v) TEMED and 0.07% (w/v) ammonium persulphate. The running gel consisted of 15.0% (w/v) acrylamide, 0.4% (w/v) bis-acrylamide, 0.1% (w/v) SDS, 0.375 M Tris-HCl buffer, pH 8.8, 0.05% (v/v) TEMED and 0.07% (w/v) ammonium persulphate. The electrode buffer contained 0.025 M Trizma base, 0.192 M glycine and 0.1% (w/v) SDS. Protein samples were dissol-

ved in 62.5 mM Tris-HCl buffer, pH 6.8 containing 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0.001% (w/v) bromophenol blue and 2.0% (w/v) SDS. Gels were stained with a solution consisting of 0.25% (w/v) Coomassie brillant blue, 45% (v/v) methanol and 9.2% (v/v) acetic acid and destained with a solution consisting of 7.5% (v/v) acetic acid and 25% (v/v) methanol. For subunit molecular weight determinations bovine serum albumin (M.W. 66,200; Brown, 1975), rabbit muscle pyruvate kinase (M.W. 59,000; Cottam et al., 1969), rabbit muscle creatine kinase (M.W. 40,500; Yue et al., 1967), rabbit muscle lactic dehydrogenase (M.W. 35,500; Holbrook et al., 1975), soybean trypsin inhibitor (M.W. 21,500; Wu & Scheraga, 1962) and lysozyme (M.W. 14,400; Jolles, 1969) were employed as molecular weight marker proteins.

5. Carboxymethylation of Thiol Groups.

Proteins were carboxymethylated according to the method of Anderson & Randall (1975) with the following modifications. Lyophilized protein was dissolved to a concentration of 10 mg/ml in freshly prepared 100 mM Tris-HCl buffer, pH 8.0 containing 8 M urea and 2.0 mM DTT. Iodoacetic acid was added to a final concentration of 10 mM and the reaction was sealed and placed in the dark for 1.5 hours. Reactions were stopped by the addition of 2-mercaptoethanol to a concentration of 25 mM and the protein was freed of excess reagent by dialysis against water and lyophilized. For samples of a small volume, reactions were stopped by the addition of solid trichloroacetic acid to a concentration of 10% (w/v) and the precipitated protein was collected by centrifugation in an Eppendorf microfuge tube. Residual TCA was removed by washing the pellet with ice-cold acetone and the final

precipitate was lyophilized. When radioactive iodoacetic acid was employed it was diluted to a specific radioactivity of 0.5 mC_i/millimole with carrier iodoacetic acid before use.

6. 8 M Urea-Poylacrylamide Gel Electrophoresis

8 M Urea-polyacrylamide gel electrphoresis was carried out under cationic conditions according to the method of Ornstein (1964). The running gel consisted of 7.0% (w/v) acrylamide, 0.187% (w/v) bis-acrylamide, 8 M urea, 0.06 M KOH, 2.66% (v/v) acetic acid and 0.05% (v/v) TEMED. Polymerization was initiated by the addition of ammonium persulphate to a final concentration of 0.07% (w/v) and the gel solution was poured into glass tubes (I.D.=0.6 cm) to a height of 8.0 cm. When polymerization was complete, a stacking gel was poured on top of the running gel in the glass tubes to a height of 1.5 cm. The stacking gel consisted of 3.0% (w/v) acrylamide, 0.08% (w/v) bis-acrylamide, 8 M urea, 0.06 M KOH, 0.375% (v/v) acetic acid, 0.05% (v/v) TEMED and 0.07% (w/v) ammonium persulphate. Carboxymethylated protein samples were dissolved in a solution consisting of 0.24 M KOH, 1.5% (v/v) acetic acid, 8 M urea and 0.02% (w/v) Pyronin Y. The electrode buffer consisted of 0.0323 M β-alanine and 0.024% (v/v) acetic acid. Gels were stained with a solution consisting of 1.0% (w/v) Amido black and 7% (v/v) acetic acid and destained with a solution consisting of 7.5% (v/v) acetic acid and 25% (v/v) methanol.

8 M Urea-polyacrylamide gel electrophoresis was carried out under anionic conditions according to the method of Davis (1964) with the exception that the stacking gel was omitted. The procedure was the same as the method for running 8 M urea

gels under cationic conditions with the following changes. The running gel consisted of 5.0% (w/v) acrylamide, 0.125% (w/v) bis-acrylamide, 8 M urea, 0.234 M Tris-HCl buffer, pH 9.0 and 0.02% (v/v) TEMED. Polymerization was initiated by the addition of ammonium persulphate to a concentration of 0.04% (w/v). Carboxymethylated protein samples were dissolved in a solution containing 8.0 M urea and 0.001% (w/v) bromophenol blue. The electrode buffer consisted of 0.025 M Trizma base and 0.192 M glycine. Urea gels run under anionic conditions were stained with Amido black as described previously.

7. Molecular Weight Determination

The molecular weight of purified eel pyruvate kinase was estimated by gel filtration chromatography on a Sephacryl S-300 column (1.5 cm x 80 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7.5 containing 30% (v/v) glycerol and 0.1 mM DTT. Bovine serum albumin (M.W. 66,200; Brown, 1975), rabbit muscle pyruvate kinase (M.W. 230,000; Cottam et al., 1969) and bovine thyroglobulin (M.W. 669,000; Pharmacia) were employed as molecular weight marker proteins. Fractions of a 1.0 ml volume were collected and assayed for pyruvate kinase activity and their absorbance at 280 nm was recorded.

8. Iscelectric Focusing

Isoelectric focusing was conducted on 6.0% (w/v) polyacrylamide gels according to the method of Wrigley (1971). Gels contained 6.0% (w/v) acrylamide, 0.175% (w/v) bis-acrylamide, 2.0% (w/v) carrier ampholytes (pH range 3-10 or 5-7) and 0.143% (v/v) TEMED. The gel solution was degassed and polymerization was initiated by the addition of ammonium persulphate to a con-

centration of 0.057% (w/v). The gel solution was poured into glass tubes (I.D.=0.6 cm) and overlayed with water. The electrolyte solutions consisted of 0.02 M NaOH for the cathodic chamber and 0.01 M H_3PO_4 for the anodic chamber. The gels were subjected to pre-electrophoresis as follows: 200 volts for 15 minutes, 300 volts for 30 minutes and 400 volts for 30 minutes at 4°C. The cathodic electrolyte solution was removed and protein samples which had been dialyzed against 30% (v/v) aqueous glycerol for 24 hours were loaded on the gels and overlayed with fresh 0.02 M NaOH. Samples were subjected to isoelectric focusing for 18 hours at 300 volts at 4°C. Gels were removed from the glass tubes and fixed with 10% (w/v) TCA overnight and then washed with water to remove carrier ampholytes and acid. Gels were subsequently stained with Amido black as described previously. In order to determine the pH gradient within the gels, duplicate gels were sliced into 0.5 cm portions and soaked in 1.0 ml of distilled water for 18 hours at 4°C and the pH of these fractions was determined. Protein samples were also dialyzed against 30% (v/v) aqueous glycerol containing 0.2 mM FDP for 24 hours prior to being subjected to isoelectric focusing on 6.0% (w/v) polyacrylamide gels containing 0.2 mM FDP.

9. Hydrolysis and Amino Acid Analysis

Protein and peptide samples were hydrolyzed in 6 M HCl in evacuated tubes for 24 hours at 105°C. The HCl was made 1 mM in phenol and 1 mM in 2-mercaptoethanol to prevent the loss of tyrosine and sulphur-containing amino acids. Amino acid analysis was carried out on a Technicon TSM Amino Acid Analyzer by using a single column procedure. An Infotronics CRS-210 Automatic

Digital Integrator was used to quantify the amino acids. For the determination of amino acid compositions, proteins were hydrolyzed for periods of 24, 48 and 72 hours. This enabled corrections to be made for the destruction of serine and threonine and the slow release of valine and isoleucine. Tryptophan content was determined spectrophotometrically by the method of Edelhoch (1967). Molar extinction coefficients were determined as follows: lyophilized pyruvate kinase was dissolved to a concentration of 2.0 mg/ml in 20 mM potassium phosphate buffer, pH 7.0 containing 6.0 M guanidine-HCl and 2.0 mM DTT. The solution was freed of reducing reagent by passage through a column (0.5 cm x 14 cm) of Sephadex G-25 equilibrated with 20 mM potassium phosphate buffer, pH 7.0 containing 6.0 M guanidine-HCl at room temperature. The pyruvate kinase protein, which appeared in the void volume, was collected and the absorbance of this solution at 280 and 288 nm was recorded. In order to determine the protein concentration of this solution, an aliquot of known volume was removed, hydrolyzed with a known amount of a norleucine standard and amino acid analysis was conducted. The protein content of the solution was determined on the basis of the total number of moles of glycine, alanine and valine in the sample as described previously. Molar extinction coefficients of 1.27×10^5 liter/mole·cm and 1.63×10^5 liter/mole·cm were calculated for rabbit and eel muscle pyruvate kinases respectively at 280 nm. The corresponding values at 288 nm were 8.07×10^4 liter/mole·cm for the rabbit enzyme

and 1.02×10^5 liter/mole·cm for the eel enzyme.

10. Carbodiimde Modification of Proteins

The total numbers of aspartic acid and glutamic acid residues in eel and rabbit muscle pyruvate kinases were determined by determined by the method of Carraway & Koshland (1972). Performic acid-oxidized protein (1.5 mg) was dissolved to a concentration of 1.5 mg/ml in 5.0 M guanidine-hydrochloride containing 1.0 M glycine ethyl ester hydrochloride. The pH of the solution was brought to 4.75 by the addition of 1.0 M HCl and the reaction was initiated by the addition of 0.1 ml of 1.0 M ethyldimethyl-aminopropyl carbodiimide. The pH of the reaction solution was maintained at 4.75 at room temperature by the addition of 1.0 M HCl. After 1 hour, the reaction was stopped by the addition of excess 1.0 M sodium acetate buffer, pH 4.75 and the sample was dialyzed exhaustively against water to remove excess reagents and lyophilized. Modified and unmodified protein samples were hydrolyzed and their glycine contents were determined by amino acid analysis.

11. Performic Acid Oxidation

Oxidation of protein samples with performic acid was carried out according to the method of Hirs (1956). Protein was dissolved to a concentration of 10 mg/ml in 99% (v/v) formic acid. An aliquot of 30% (v/v) H_2O_2 was added to the reaction sample to bring the final concentration of H_2O_2 to 0.075% (v/v). The reaction was allowed to proceed for 30 minutes at room temperature and was stopped by the addition of 30 volumes of H_2O and the sample was lyophilized. In order to evaluate the efficiency of the performic acid oxidation, reaction aliquots were removed at timed intervals, lyophilized, hydrolyzed and subjected to amino acid analysis. The reaction conditions employed were sufficient to bring about the complete conversion of cysteine to cysteic acid and methionine to methionine sulfone.

12. N-Terminal Determination

N-terminal analysis was conducted according to the method of Gray (1972). One mg of performic acid-oxidized protein was dissolved in 50 µl of 1.0% (w/v) SDS and heated at 100°C for 5 minutes to ensure thorough unfolding of the polypeptide chains. An equal volume of N-ethyl morpholine was added and the reaction was initiated by the addition of 75 µl of a dansyl chloride solution (25 mg of dansyl chloride dissolved in 1.0 ml of dimethyl formamide). The reaction was allowed to proceed for 2 hours at room temperature and after this time the protein was precipitated by the addition of 2.0 ml of acetone. Protein was collected by centrifugation and washed with acetone 3 times to remove excess reagents. Residual acetone was removed by lyophilization.

The modified protein was subjected to hydrolysis in the usual manner. Hydrochloric acid was evaporated under a stream of nitrogen and dansyl amino acids were extracted by washing the residue 3 times with water-saturated ethyl acetate. The ethyl acetate extracts were pooled and dried under a stream of nitrogen. The dry residue was dissolved in 50% (v/v) aqueous pyridine and portions were spotted about 2 cm from each edge in one corner of a polyamide thin layer sheet (20 cm x 20 cm). The sheet was subjected to ascending chromatography using water-90% (v/v) formic acid (200:3, v/v) as the first solvent. The sheet was dried, turned through 90° and developed with the second solvent which was benzene-acetic acid (9:1, v/v). Thin layer sheets were allowed to air-dry and dansyl amino acids were located by irradiation with U.V. light. The dansyl amino acids were identified on the basis of their mobilities with respect to authentic dansyl amino acid standards.

13. Cyanogen Bromide Peptide Generation

Carboxymethylated pyruvate kinase was dissolved to a concentration of 10 mg/ml in 70% (v/v) formic acid. Solid cyanogen bromide was added to a final concentration of 10 mg/ml and reactions were allowed to proceed at room temperature for 24 hours. The CNBr peptides were dried in a stream of nitrogen, suspended in a small volume of water and lyophilized. Amino acid analysis indicated that under these conditions at least 95% of the methionine residues of protein samples were converted to either homoserine or homoserine lactone.

14. Mapping of CNBr Peptides on SDS Gels

Lyophilized CNBr peptides were subjected to electrophoresis

on 20% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS according to the method of Swank & Munkres (1971). The gel solution consisted of 0.285 M Trizma base, 0.1% (w/v) SDS, 0.200 M H_3PO_4, 20% (w/v) acrylamide, 0.533% (w/v) bis-acrylamide, 0.075% (v/v) TEMED and 4.0 M urea. Polymerization was initiated by the addition of ammonium persulphate to a final concentration of 0.07% (w/v) and the gel solution was poured into glass tubes (I.D.=0.6 cm) to a height of 11.0 cm. The electrode buffer consisted of 0.1 M H_3PO_4, 0.14 M Trizma base and 0.1% (w/v) SDS adjusted to pH 6.8. Peptides were dissolved in 0.01 M H_3PO_4 buffer, adjusted to pH 6.8 with Trizma base, containing 1.0% (w/v) SDS, 4.0 M urea and 0.002% (w/v) bromophenol blue. Gels were stained with Coomassie blue as described previously.

15. Mapping of CNBr Peptides on Triton-Urea Gels

Cyanogen bromide peptides were subjected to electrophoresis on 12% (w/v) polyacrylamide gels containing 7.5 M urea and 6 mM Triton X-100 (Borun et al., 1977). The gel solution consisted of 12% (w/v) acrylamide, 0.32% (w/v) bis-acrylamide, 7.5 M urea, 5.0% (v/v) acetic acid, 6.0 mM Triton X-100 and 0.5% (v/v) TEMED. Polymerization was initiated by the addition of ammonium persulphate to a concentration of 0.06% (w/v) and the gel solution was poured into glass tubes (I.D.=0.6 cm) to a height of 11.0 cm. Following gel polymerization, the gels were overlayed with 100 µl of sample buffer consisting of 5.0% (v/v) acetic acid, 7.5 M urea and 6 mM Triton X-100. Gels were pre-run for 24 hours at 80 volts with 5.0% (v/v) acetic acid as the electrode buffer. Peptide samples were dissolved in the above sample buffer, loaded on gels and subjected to electro-

phoresis with fresh 5.0% (v/v) acetic acid as the electrode buffer. Gels were stained with Amido black as described previously.

16. Fractionation of CNBr Peptides

Cyanogen bromide peptides were solubilized by modification with excess citraconic anhydride (Anderson & Randall, 1975). Peptides (approx. 80 mg) were suspended in 5.0 ml of H_2O and 250 µl of N-ethyl morpholine was added. The reaction was initiated by the addition of 100 µl of citraconic anhydride and the pH was maintained at 8.0 by the addition of 1.0 N NaOH. When the solution was clear and no particulate material was evident a further 50 µl of citraconic anhydride was added to ensure complete solubilization. The reaction was stopped by the addition of excess ammonium bicarbonate and the modified peptides were separated on a column (2.5 cm x 80 cm) of Sephadex G-75 equilibrated with 0.5% (w/v) ammonium bicarbonate. The column was developed with this buffer and the eluate was monitored by measuring the absorbance at 225 nm and by scintillation counting of 0.1 ml aliquots of the 3.5 ml fractions. Separated fractions were concentrated by lyophilization and the citraconyl groups were removed by treatment of the peptides with 5.0% (v/v) acetic acid.

17. Radioactivity Determinations

The radioactivity of peptide and protein samples was determined by adding 5-100 µl aliquots of samples to 10 ml of Aquasol in glass scintillation vials and counting in a Nuclear Chicago Isocap/300 liquid scintillation counter. For samples containing both ^{3}H and ^{14}C, corrections for spill-over were made by counting each radioisotope in Aquasol.

18. Tryptic Digestion of CNBr Peptides

Cyanogen bromide peptides were suspended in 0.5% (w/v) ammonium bicarbonate to a concentration of 10 mg/ml, trypsin was added to a final concentration of 0.08 mg/ml and digestion was allowed to proceed for 12 hours at 37°C with constant stirring. After this time, the reaction was stopped by lyophilization. Residual ammonium bicarbonate was removed by the addition of a few drops of 5.0% (v/v) acetic acid and lyophilization again.

19. Peptide Mapping

For peptide mapping studies, pyruvate kinase (1-3 mg) was carboxymethylated with iodo[2-^{14}C]acetic acid in the presence of 8 M urea and 2.0 mM DTT and subsequently treated with CNBr and trypsin as described previously. All operations were carried out in a 1.5 ml Eppendorf microfuge tube for convenience. Peptides were dissolved in 100-200 µl of pH 6.5 buffer, consisting of acetic acid-pyridine-water (3:100:900, v/v/v), and spotted on a 56 cm-long sheet of Whatman 3MM paper. Electrophoresis was carried out at 3000 volts for 60 minutes at pH 6.5. Following electrophoresis, the air-dried paper was autoradiographed by exposure to Fuji X-ray film for 1 to 2 days. The strip of radioactive peptides was sewn onto a sheet (45 cm x 56 cm) of Whatman 1MM paper and descending chromatography was carried out overnight at 90° to the electrophoretic separation using a buffer (BAWP) consisting of butanol-acetic acid-water-pyridine (15:3:12:10, v/v/v/v). The strip of neutral peptides was located on these maps by autoradiography, excised and subjected to electrophoresis on a sheet (45 cm x 56 cm) of Whatman 1MM paper.

Electrophoresis was carried out at 3000 volts for 30-40 minutes using a pH 2.1 buffer consisting of acetic acid-formic acid-water (4:1:45, v/v/v). Peptide maps were stained with a solution consisting of 0.085% (w/v) ninhydrin, 3.75 mM cadmium acetate, 5% (v/v) acetic acid and 85% (v/v) acetone. Radioactive peptides were located by autoradiography.

20. Purification of Peptides

For preparative procedures, the peptides (approx. 80 mg) derived from the CNBr cleavage and tryptic digestion of ^{14}C-carboxymethylated pyruvate kinase were applied to a sheet (45 cm x 56 cm) of Whatman 3MM paper as a 35 cm-wide band. Electrophoresis was carried out at pH 6.5 as described previously. Radioactive peptides were located on paper by autoradiography and the bands were excised and subjected to descending chromatography and electrophoresis at pH 2.1. As a final purification step, peptides were subjected to electrophoresis at pH 3.5 using a buffer consisting of acetic acid-pyridine-water (10:1:190, v/v/v). Purified peptides were eluted from paper with pH 2.1 buffer. Small CNBr peptides were purified in a similar manner except that the final electrophoretic step at pH 3.5 was omitted as it was not required.

21. Measurements with DTNB

For the determination of the number of cysteine residues in pyruvate kinase, lyophilized proteins were dissolved to a concentration of 1.0-2.0 mg/ml in 100 mM Tris-HCl buffer, pH 8.0 containing 1.0% (w/v) SDS and 2.0 mM DTT. Samples were freed of the reducing reagent by passage through a column (0.5 cm x 14 cm) of Sephadex G-25 equilibrated with 100 mM Tris-HCl

buffer, pH 8.0 containing 1.0% (w/v) SDS. The protein, which appeared in the void volume, was diluted to a concentration of 0.4 mg/ml with the column buffer. Titration reactions were initiated immediately by the addition of 100 µl of a 10 mM solution of DTNB, prepared in 100 mM Tris-HCl buffer, pH 8.0, to 2.5 ml of the 0.4 mg/ml solution of pyruvate kinase. Reactions were conducted in a 1 cm-path-length-cuvette and the absorbance at 412 nm was determined in a Perkin-Elmer Coleman 124 spectrophotometer. The sulfhydryl concentration was calculated assuming a molar extinction coefficient of 13,600 liters/mole·cm for the 5-thio-2-nitrobenzoate anion (Habeeb, 1972).

For the determination of the number of reactive cysteine residues in the native enzyme, concentrated solutions of pyruvate kinase were desalted by passage through a column (2.5 cm x 40 cm) of Sephadex G-25 equilibrated with 100 mM Tris-HCl buffer, pH 8.0 containing 30% (v/v) glycerol at 4°C. Protein samples were not incubated with 2.0 mM DTT prior to application to the Sephadex column. Pyruvate kinase, which appeared in the void volume, was diluted to a concentration of 0.4 mg/ml with the column buffer. Reactions were initiated by the addition of 100 µl of the 10 mM DTNB solution to 2.5 ml of the 0.4 mg/ml pyruvate kinase solution containing 30% (v/v) glycerol. The sulfhydryl concentration was determined spectrophotometrically as before.

22. Chemical Modification of Pyruvate Kinases

Prior to the commencement of chemical modification studies, concentrated solutions of pyruvate kinase were passed through a column (2.5 cm x 40 cm) of Sephadex G-25 at 4°C equilibrated

with 100 mM Tris-HCl buffer, pH 8.0 containing 30% (v/v) glycerol. This step was taken in order to remove the reducing agents and salts which were added to stabilize the enzymes during storage at 4°C. Pyruvate kinase, which eluted in the void volume, was diluted to a concentration of 1.0 mg/ml with the column buffer. These preparations of eel and rabbit muscle pyruvate kinases routinely exhibited specific activities of at least 320 and 350 units/mg of protein and were stable at 4°C for the duration of the day's experiment.

One hundred µl of the 1.0 mg/ml solution of pyruvate kinase was incubated with 40 µl of 100 mM Tris-HCl buffer, pH 8.0 for 5 minutes at 30°C. Chemical modification reactions were initiated by the addition of 10 µl of a sulfhydryl reagent solution prepared in 100 mM Tris-HCl buffer, pH 8.0 and samples were allowed to react in the dark at 30°C. At specific time points, reaction aliquots were removed and assayed for residual activity. For chemical modification studies involving eel pyruvate kinase, reaction aliquots were diluted in 50 mM imidazole-HCl buffer, pH 6.3 containing 30% (v/v) glycerol and assayed immediately for residual activity at pH 6.3, 30°C using the standard assay mixture. For chemical modification studies involving rabbit pyruvate kinase, reaction aliquots were diluted in 50 mM Tris-HCl buffer, pH 7.5 containing 30% (v/v) glycerol and assayed immediately for residual activity at pH 7.5, 30°C using the standard assay mixture. In both cases, the dilution of reaction aliquots was sufficient to stop inactivation and enzyme reaction rates were linear for at least 3 minutes. Residual activities are expressed as a percentage of the initial enzyme activity observed prior to the addition of the sulfhydryl reagent.

In order to determine the protective effects of various compounds against enzyme inactivation due to chemical modification, 100 µl of a 1.0 mg/ml solution of pyruvate kinase, prepared in 100 mM Tris-HCl buffer, pH 8.0 containing 30% (v/v) glycerol, was incubated with 40 µl of 100 mM Tris-HCl buffer, pH 8.0 containing various combinations of cations and substrates at 30°C for 5 minutes. Reactions were initiated by the addition of 10 µl of a sulfhydryl reagent solution prepared in 100 mM Tris-HCl buffer, pH 8.0. For chemical modification studies involving iodoacetamide, the reagent was added to a final concentration of 1.867 mM and 8.000 mM in studies involving eel and rabbit muscle pyruvate kinases respectively. Reactions were conducted in the dark at 30°C and at specific time points aliquots were removed, diluted and assayed for residual activity as described before.

23. Determination of the Number of Reactive Cysteine Residues

In order to determine the number of functional groups reacting with iodoacetamide, the previous chemical modification reactions were conducted using iodo[1-^{14}C]acetamide. The volume of reaction samples was increased 10 fold so the equivalent of 1.0 mg of pyruvate kinase was contained in a final volume of 1.5 ml. For the eel enzyme, chemical modification reactions were initiated by the addition of 100 µl of a 14 mM solution of iodo[1-^{14}C]acetamide, prepared in 100 mM Tris-HCl buffer, pH 8.0. For the rabbit enzyme, the corresponding concentration of the iodo[1-^{14}C]acetamide solution was 60 mM. Radioactively labelled iodoacetamide was diluted to a specific radioactivity of 0.5 mC_i/millimole before use. The chemical modification reactions

were allowed to proceed in the dark at 30°C for 45 minutes in the case of the eel enzyme and 75 minutes in the case of the rabbit enzyme, after which aliquots were assayed for residual activity. The remainder of the pyruvate kinase protein was precipitated by the addition of solid TCA to a concentration of 10% (w/v). Pyruvate kinase was recovered by centrifugation in an Eppendorf microfuge tube and freed of excess radioactive reagent by washing with ice-cold acetone. Residual acetone was removed by lyophilization. The radioactively labelled pyruvate kinase was dissolved in 1.0 ml of 70% (v/v) formic acid and aliquots were removed for scintillation counting.

In order to determine the protein concentration of the formic acid solution, an aliquot was removed, hydrolyzed with a known amount of a norleucine standard and amino acid analysis was conducted. The number of moles of glycine, alanine and valine in the sample were determined. The protein content could then be determined based on the fact that eel pyruvate kinase contains a total of 141 moles of glycine, alanine and valine per mole of enzyme subunit while rabbit pyruvate kinase contains a total of 126 moles of glycine, alanine and valine per mole of subunit.

In parallel experiments, radioactively labelled pyruvate kinase was freed of excess reagent and dissolved in 100 mM Tris-HCl buffer, pH 8.0 containing 1.0% (w/v) SDS and 2.0 mM DTT. The sample was freed of reducing reagent by passage through a column (0.5 cm x 14 cm) of Sephadex G-25 equilibrated with 100 mM Tris-HCl buffer, pH 8.0 containing 1.0% (w/v) SDS. The sulfhydryl content of the radioactively labelled pyruvate kinase was then determined by titration with DTNB as described pre-

viously.

24. Identification of Reactive Cysteine Residues

In order to identify the reactive cysteine residues of eel pyruvate kinase, the native enzyme was chemically modified by iodoacetamide and subsequently carboxymethylated with iodo-[2-^{14}C]acetic acid under denaturing conditions. A 0.667 mg/ml solution of eel pyruvate kinase, prepared in 100 mM Tris-HCl buffer, pH 8.0 containing 20% (v/v) glycerol, was incubated with iodoacetamide at an inhibitor to enzyme subunit mole ratio of 82.5:1 in a sample volume of 1.5 ml. Chemical modification reactions were conducted in the presence and absence of 12.0 mM PEP and 8.0 mM $MgCl_2$. . A control sample consisted of the reaction sample without the sulfhydryl reagent added. Reaction samples were incubated at 30°C in the dark for 45 minutes. Reactions were stopped by the addition of solid TCA to a concentration of 10% (w/v) and pyruvate kinase protein was recovered by centrifugation in an Eppendorf microfuge tube. Excess reagent and acid was removed by washing with ice-cold acetone as described previously. Protein was dissolved in 100 mM Tris-HCl buffer, pH 8.0 containing 8.0 M urea and 2.0 mM DTT and carboxymethylated with iodo[2-^{14}C]acetic acid. Iodo[2-^{14}C]acetic acid was diluted to a specific radioactivity of 2 mC_i/millimole prior to use. ^{14}C-Carboxymethylcysteine-containing peptides were generated by treatment with CNBr followed by trypsin and the radioactive peptides were separated on paper by high voltage electrophoresis and chromatography. The resulting peptide maps were subjected to autoradiography.

In order to quantitate the reactivities of the cysteine

residues of pyruvate kinase, the procedure used to identify the reactive cysteine residues of eel pyruvate kinase was modified. Eel pyruvate kinase was chemically modified by iodoacetamide in the presence and absence of 12.0 mM PEP and 8.0 mM $MgCl_2$ as described previously. A similar procedure was employed for the chemical modification of rabbit muscle pyruvate kinase except iodoacetamide was present at an inhibitor to enzyme subunit mole ratio of 354:1. Reactions were allowed to proceed at 30°C in the dark and at various time points they were stopped by the addition of solid TCA. Pyruvate kinase protein was recovered and carboxymethylated under denaturing conditions as described previously with the exception that iodo-[2-^{3}H]acetic acid was used instead of iodo[2-^{14}C]acetic acid. Tritiated iodoacetic acid was diluted to a specific radioactivity of 10 mC_i/millimole prior to use. Prior to the generation of carboxymethylcysteine-containing peptides, a 0.5 mg portion of ^{14}C-carboxymethylated pyruvate kinase was added to the tritiated, iodoacetamide-treated reaction samples to serve as an internal standard. The ^{14}C-carboxymethylated pyruvate kinase standard was prepared by the alkylation of the enzyme under denaturing conditions with iodo[2-^{14}C]acetic acid which had been diluted to a specific radioactivity of 0.5 mC_i/millimole before use. A 0.5 mg portion of the ^{14}C-carboxymethylated pyruvate kinase was also added to a 0.5 mg portion of ^{3}H-carboxymethylated pyruvate kinase to serve as a control. The ^{3}H-carboxymethylated pyruvate kinase standard was prepared by the alkylation of the enzyme under denaturing conditions with iodo[2-^{3}H]acetic acid which had been diluted to a specific

radioactivity of 10 mC_i/millimole.

Carboxymethylcysteine-containing peptides were generated from these mixtures of ^{14}C-carboxymethylated and ^{3}H-carboxymethylated pyruvate kinases. Radioactive peptides were separated on paper by high voltage electrophoresis and chromatography and located by autoradiography as described previously. Radioactive peptides were eluted from paper with pH 6.5 buffer and a portion was removed for scintillation counting. The $^{3}H/^{14}C$ ratio determined for a peptide derived from a mixture of a tritiated, iodoacetamide-treated reaction sample and the ^{14}C-carboxymethylated pyruvate kinase standard was compared to the $^{3}H/^{14}C$ ratio determined for the corresponding peptide derived from a mixture of ^{3}H-carboxymethylated and ^{14}C-carboxymethylated pyruvate kinase standards. In this manner the percentage of a cysteine residue available for carboxymethylation by ^{3}H-iodoacetic acid following treatment of the native tetrameric enzyme with iodoacetamide could be determined according to the formula:

$$\frac{\left[\frac{^{3}H}{^{14}C}\right]^{R} \times \frac{(\text{Wt. } ^{14}\text{C-CM-PK})^{R}}{(\text{Wt. IAN-PK})^{R}}}{\left[\frac{^{3}H}{^{14}C}\right]^{ST} \times \frac{(\text{Wt. } ^{14}\text{C-CM-PK})^{ST}}{(\text{Wt. } ^{3}\text{H-CM-PK})^{ST}}} \times 100\%$$

where:

$\left[\frac{^3H}{^{14}C}\right]^R_a$ -represents the ratio of the ^3H to ^{14}C counts determined for peptide a derived from a mixture of known amounts of tritiated, iodoacetamide-treated pyruvate kinase and ^{14}C-carboxymethylated pyruvate kinase

$\left[\frac{^3H}{^{14}C}\right]^{ST}_a$ -represents the ratio of the ^3H to ^{14}C counts determined for peptide a derived from a mixture of known amounts of ^3H-carboxymethylated and ^{14}C-carboxymethyated pyruvate kinases

$(\text{Wt. } ^{14}\text{C-CM-PK})^R$ -represents the weight of the ^{14}C-carboxymethylated pyruvate kinase added to the tritiated, iodoacetamide-treated pyruvate kinase

$(\text{Wt. IAN-PK})^R$ -represents the weight of the tritiated, iodoacetamide-treated pyruvate kinase

$(\text{Wt. } ^{14}\text{C-CM-PK})^{ST}$ -represents the weight of the ^{14}C-carboxymethylated pyruvate kinase added to the ^3H-carboxymethylated pyruvate kinase in the control sample

$(\text{Wt. } ^{3}\text{H-CM-PK})^{ST}$ - represents the weight of the ^3H-carboxymethylated pyruvate kinase in the control sample

Since the same amount of ^{14}C-carboxymethylated pyruvate kinase (0.5 mg) was added to both the tritiated, iodoacetamide-treated pyruvate kinase samples and to the ^3H-carboxymethylated pyruvate kinase in the control sample and the weight of ^3H-carboxymethylated pyruvate kinase in the control sample (0.5 mg) is known,

the formula can be simplified to:

$$\frac{\left[\frac{^3H}{^{14}C}\right]^R_a \times 0.5 \text{ mg}}{\left[\frac{^3H}{^{14}C}\right]^{ST}_a \times (\text{Wt. IAN-PK})^R} \times 100\% = \begin{array}{l}\text{Percentage of Unreacted Cysteine} \\ \text{Residue Contained in Peptide } a \\ \text{Derived from Iodoacetamide-} \\ \text{Treated Pyruvate Kinase}\end{array}$$

An aliquot of the tritiated, iodoacetamide-treated pyruvate kinase, dissolved in 70% (v/v) formic acid, was removed prior to the addition of the ^{14}C-carboxymethylated pyruvate kinase standard and the commencement of CNBr cleavage. This sample aliquot was hydrolyzed and subjected to amino acid analysis. Pyruvate kinase content was determined on the basis of the total number of moles of glycine, alanine and valine in the sample as described previously.

25. Amino Acid Sequence Determinations

The amino acid sequence of purified peptides was determined by using a subtractive Edman procedure. Peptides (approx. 100 nmoles) were dissolved in 100 µl of 70% (v/v) redistilled pyridine. Phenyl isothiocyanate was added to a final concentration of 5% (v/v) and the sample was flushed with nitrogen gas, sealed and heated at 53°C for 1 hr. The solution was then extracted twice with 200 µl of benzene to remove excess PITC and then lyophilized. The residue was dried overnight in a dessicator over NaOH pellets in vacuo. To the crystalline residue was added 100 µl of trifluoroacetic acid and the sample was flushed with nitrogen gas, sealed and heated at 53°C for 30 minutes. The TFA was removed under a stream of nitrogen and the sample was dried in a dessicator over NaOH pellets

in vacuo for 15 minutes. To the residue was added 200 µl of distilled, de-ionized water and the aqueous solution was extracted 3 times with 400 µl of water-saturated ethyl acetate. The aqueous solution and the combined ethyl acetate extracts were dried under a stream of nitrogen. The dried aqueous extract was dissolved in 100 µl of 70% (v/v) redistilled pyridine and a 5 µl aliquot was taken for hydrolysis and amino acid analysis. Further rounds of Edman degradations were carried out by repeating the described procedure. The dried ethyl acetate extracts were used to confirm, when necessary, the residues removed by Edman degradations by regeneration of the amino acids from the phenylthiohydantoin derivatives (Lu et al., 1981).

- CHAPTER 3 -

PURIFICATION AND CHARACTERIZATION OF EEL WHITE MUSCLE PYRUVATE KINASE

RESULTS

A typical preparation of pyruvate kinase from eel white muscle is summarized in Table 1. An overall purification of 20 fold was routinely obtained. The major step contributing to this purification was chromatography on DE-32 cellulose carried out as illustrated in Fig. 1. In this step and during the subsequent chromatography on Sephacryl S-300 (Fig. 2), it was necessary to include 30% (v/v) glycerol in the buffers to prevent loss of activity. Attempts to crystallize the purified enzyme by the slow addition of ammonium sulphate were unsuccessful. Under these conditions a fibrous precipitate was obtained.

The purified enzyme gave one band accounting for at least 95% of the protein when electrophoresis was carried out under reducing and non-reducing conditions in SDS polyacrylamide gels (Fig. 3). Electrophoresis in polyacrylamide gels under cationic and anionic conditions in the presence of 8 M urea also indicated that one polypeptide component accounted for at least 95% of the protein (Fig. 4A, B). Under cationic conditions, carboxymethylated eel and rabbit muscle pyruvate kinases exhibited similar electrophoretic mobilities and when the two enzymes were run on the same gel, they were not resolved from each other (Fig. 4A). However, under anionic conditions, the mobility of the carboxymethylated rabbit enzyme was only one-half that of the carboxymethylated eel muscle enzyme (Fig. 4B).

TABLE 1

Summary of the Purification of Pyruvate Kinase from 500g of Eel White Muscle

Step	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Yield (%)
1. Supernatant of pH 5.4 treatment	16,200	280,000	17.3	100
2. 40-75% Saturation $(NH_4)_2SO_4$ Fraction	9,050	239,000	26.4	85
3. CM-Cellulose Supernatant	5,900	203,000	34.4	73
4. DEAE-Cellulose Eluate	320	90,000	281	32
5. Sephacryl S-300 Eluate	180	63,000	350	23

FIGURE 1

DEAE-Cellulose Chromatography of Eel
White Muscle Pyruvate Kinase

Following batch-wise treatment with CM-cellulose, the protein solution was applied onto a column (2.5 cm x 40 cm) of DE-32 cellulose equilibrated with 5.0 mM potassium phosphate buffer, pH 7.8 containing 30% (v/v) glycerol and 0.1 mM DTT. The column was developed as described in the Methods. Fractions of 16.5 ml volume were collected and their absorbance at 280 nm was measured (O——O). Aliquots of the fractions were taken for the determination of pyruvate kinase activity (●——●). The vertical arrow preceeding the pyruvate kinase peak indicates the point at which column elution by the linear KCl graient was initiated. The vertical arrow following the pyruvate kinase peak indicates the point at which column elution by the KCl gradient was completed. The horizontal arrows indicate the fractions which were pooled and concentrated by ultrafiltration.

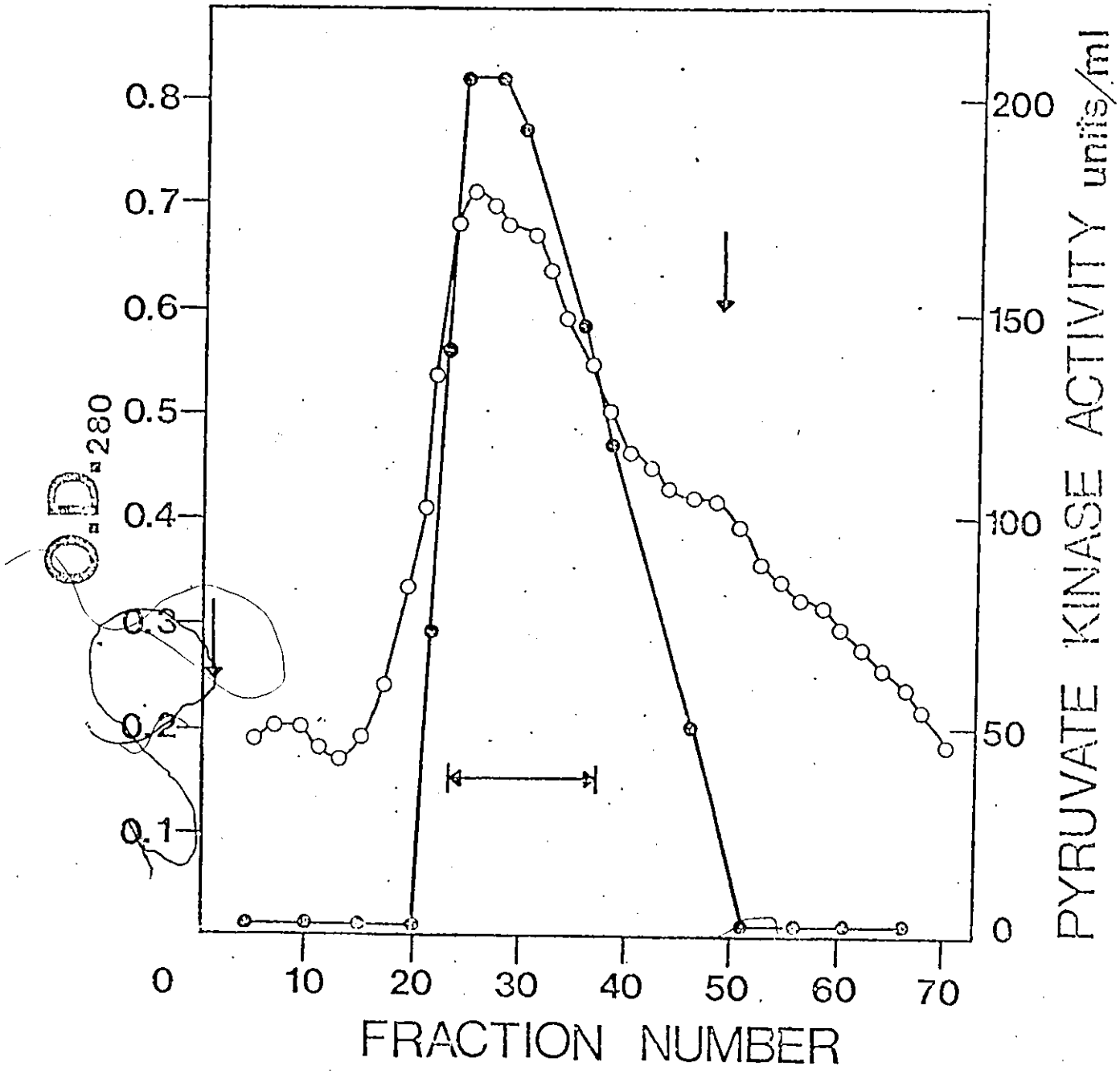
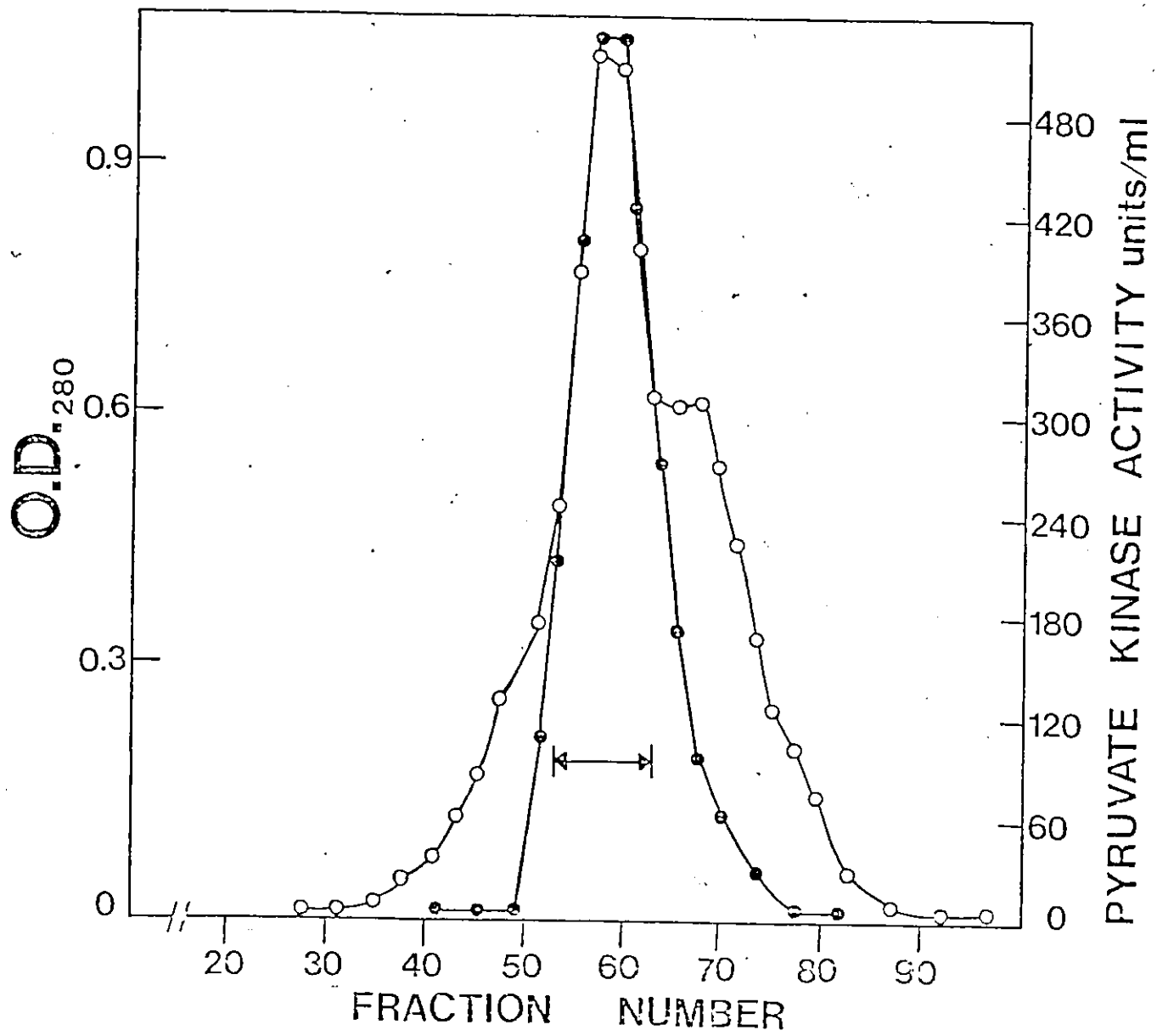

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
O.D.280
200
150
100
50
0
PYRUVATE KINASE ACTIVITY units/ml
0
10
20
30
40
50
60
70
FRACTION NUMBER

FIGURE 2

Sephacryl S-300 Chromatography
of Eel Muscle Pyruvate Kinase

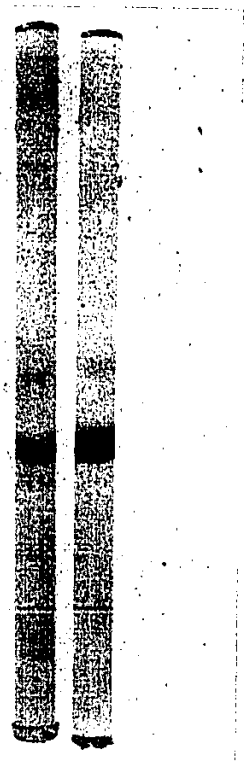


Following concentration by ultrafiltration, the DEAE-cellulose eluate was applied onto a column (2.5 cm x 80 cm) of Sephacryl S-300 equilibrated with 50 mM potassium phosphate buffer, pH 7.5 containing 30% (v/v) glycerol and 0.1 mM DTT. The column was developed with this buffer and 4.67 ml volume fractions were collected and assayed for pyruvate kinase activity (●——●). The absorbance of each fraction at 280 nm was also determined (○——○). Fractions exhibiting the highest pyruvate kinase specific activities (indicated by the horizontal arrows) were pooled.

FIGURE 3

SDS-Polyacrylamide Gel Electrophoresis
of Eel White Muscle Pyruvate Kinase

The purified enzyme was subjected to electrophoresis on SDS-polyacrylamide gels under both non-reducing (gel on the left side) and reducing (gel on the right side) conditions as described in the Methods.



Polyacrylamide gel electrophoresis performed in the presence of 8 M urea has been shown to be an extremely sensitive technique to resolve carboxymethylated protein subunits which differ by as little as one charge change (Perham & Anderson, 1970). The fact that only one protein band was observed for each of the carboxymethylated pyruvate kinases on urea-containing polyacrylamide gels would suggest that the cysteine residues of eel and rabbit muscle pyruvate kinases were completely alkylated under the carboxymethylation reaction conditions employed here. This observation is supported by the fact that carboxymethylated pyruvate kinases did not contain any cysteine residues which were free to react with DTNB. In addition, carboxymethylated pyruvate kinases which were subsequently oxidized by performic acid did not contain any cysteic acid as determined by amino acid analysis.

In order to compare the kinetic properties of eel white muscle pyruvate kinase to those of other pyruvate kinase isozymes, the enzyme was assayed at 30°C. Figure 5 indicates the dependence of the maximal velocity on pH at 30°C. When the enzyme was assayed in the presence of either imidazole or Mes buffer, it exhibited a pH optimum of 6.3. The maximal velocities at the pH optima in these two buffers were comparable. When the enzyme was assayed in potassium phosphate buffer the pH optimum was 6.4 and the maximal velocity obtained at this pH was 1.3 times greater than the maximal velocities obtained for either imidazole or Mes buffers.

Since the eel is a poikilothermic organism, the pH dependency of eel muscle pyruvate kinase was also determined at 18°C (Fig. 5) as this temperature is more in keeping with the range of habitat conditions that this fish is likely to encounter. At 18°C, the pH

FIGURE 4A

8 M Urea-Polyacrylamide Gel Electrophoresis
of Eel and Rabbit Muscle Pyruvate Kinases

Carboxymethylated eel and rabbit muscle pyruvate kinases were subjected to electrophoresis on polyacrylamide gels containing 8 M urea under cationic conditions as described in the Methods. From left to right: gel 1, 50 μg of carboxymethylated eel pyruvate kinase; gel 2, 50 μg of carboxymethylated eel pyruvate kinase plus 50 μg of carboxymethylated rabbit pyruvate kinase; gel 3, 30 μg of carboxymethylated rabbit pyruvate kinase.

FIGURE 4B

8 M Urea-Polyacrylamide Gel Electrophoresis
of Eel and Rabbit Muscle Pyruvate Kinases

Carboxymethylated eel and rabbit muscle pyruvate kinases were subjected to electrophoresis on polyacrylamide gels containing 8 M urea under anionic conditions as described in the Methods. From left to right: gel 1, 50 μg of carboxymethylated eel pyruvate kinase; gel 2, 20 μg of carboxymethylated eel pyruvate kinase plus 50 μg of carboxymethylated rabbit pyruvate kinase; gel 3, 25 μg of carboxymethylated rabbit pyruvate kinase.

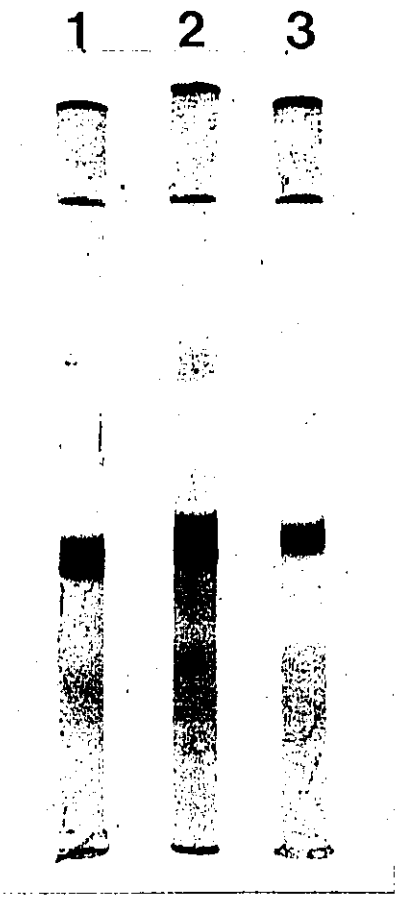


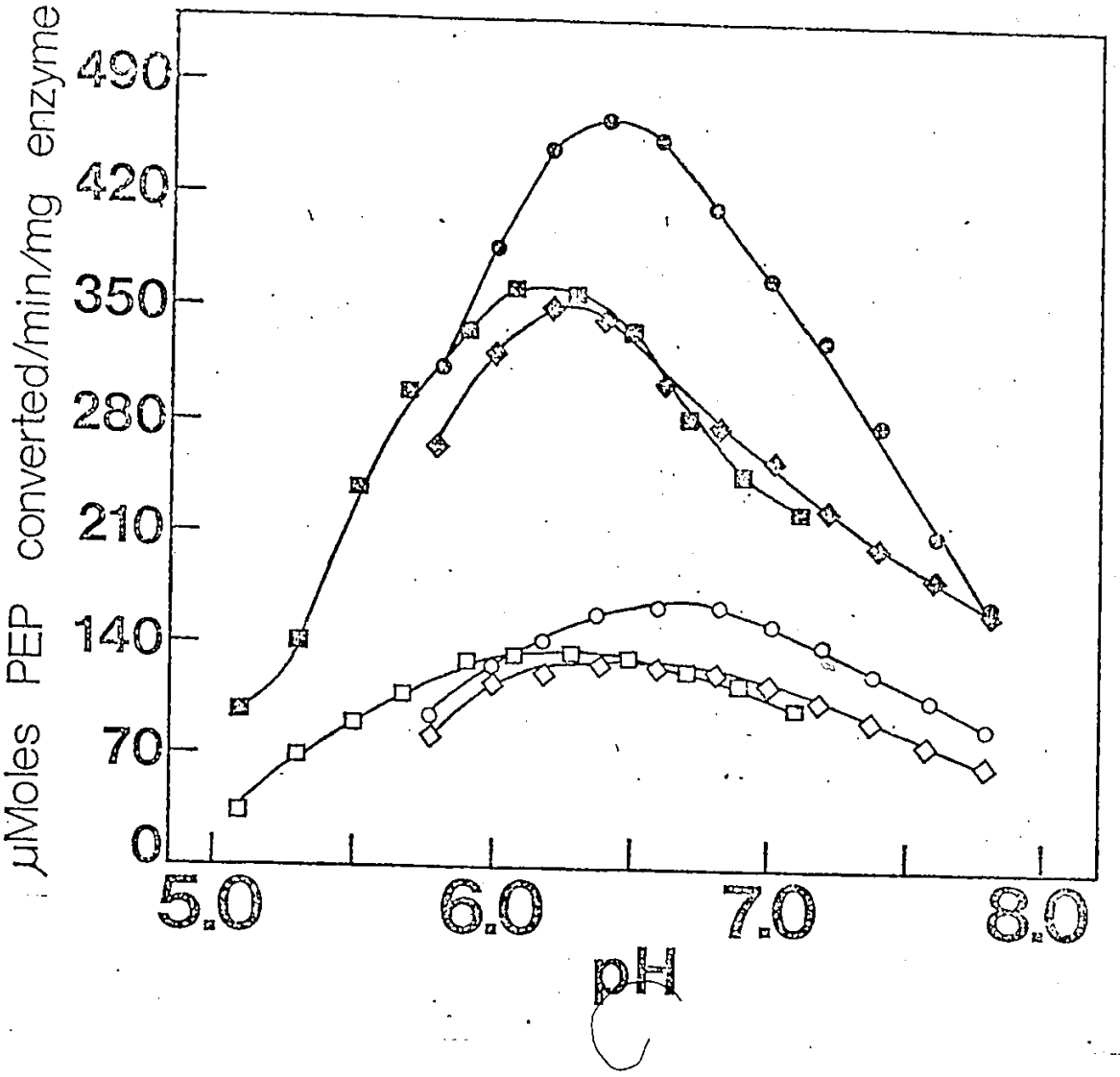
FIG. 4A

FIG. 4B

FIGURE 5

Effect of pH on Eel White Muscle
Pyruvate Kinase Activity

Eel pyruvate kinase activity was assayed in the presence of potassium phosphate (O——O), Mes (□——□) and imidazole (◇——◇) buffers. The enzyme was assayed in the presence of 50 mM buffer containing 2.5 mM PEP, 2.0 mM ADP, 8.0 mM $MgCl_2$, 45 mM KCl, 0.15 mM β-NADH and 3 units of dialyzed lactic dehydrogenase at 30°C (closed symbols) and 18°C (open symbols).


µMoles PEP converted/min/mg enzyme
490
420
350
280
210
140
70
0
5.0
6.0
7.0
8.0
pH

optimum in Mes buffer was unchanged but the pH optimum in imidazole buffer shifted to 6.5. In the presence of potassium phosphate buffer the pH optimum was 6.7 and the maximal velocity was 1.3 times greater than the maximal velocities obtained for either Mes or imidazole buffers at 18°C.

The sensitivity of eel pyruvate kinase to inhibition by imidazole buffer was investigated. At pH 6.3 and 30°C, the maximum velocity exhibited by eel pyruvate kinase was constant for a range of imidazole-HCl concentrations between 0.025 and 0.250 M. For the subsequent kinetic experiments, 0.05 M imidazole-HCl was employed as the assay buffer since it did not appear to inhibit the eel enzyme or activate it like potassium phosphate buffer.

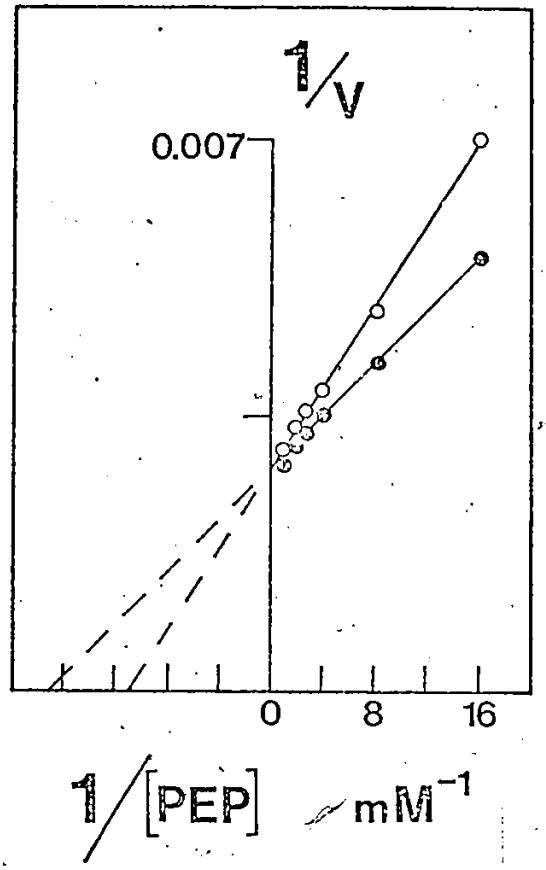
Figures 6, 7 and 8 indicate the dependence of the initial reaction velocity on the concentration of the substrate PEP in the presence and absence of 0.5 mM FDP at 30°C. Table 2 summarizes the kinetic parameters obtained from these plots. At pH 6.3, a hyperbolic substrate saturation plot was obtained with an apparent K_m value of 0.09 mM in the absence of FDP and a Hill coefficient of 1.05 was determined (Fig. 6). There was a 1.5 fold decrease in the apparent K_m when the enzyme was assayed in the presence of FDP but the $V_{max.}$ value and the Hill coefficient remained unchanged. When the pH was increased to 7.2 at 30°C, there was a 3.1 fold increase in the apparent K_m and a 1.6 fold decrease in the $V_{max.}$ in the absence of FDP (Fig. 7). FDP could increase the enzyme's affinity for the substrate 2.8 fold and the maximal velocity in the presence of FDP was comparable to the maximal velocity at pH 6.3. A complex

FIGURE 6

Affinity of Eel Pyruvate Kinase
for PEP at pH 6.3, 30°C

Eel pyruvate kinase was assayed in the presence of 50 mM imidazole-HCl buffer, pH 6.3 containing 2.0 mM ADP, 8.0 mM $MgCl_2$, 45 mM KCl, 0.15 mM β-NADH and 3 units of dialyzed lactic dehydrogenase at 30°C. Enzyme was assayed in the absence (O——O) and presence (●——●) of 0.5 mM FDP. Lineweaver-Burk plots of the data obtained appear in the upper panel while the corresponding Hill plots appear in the lower panel.

Enzyme activities (v and $V_{max.}$) are expressed in terms of μmoles of PEP converted to pyruvate per minute per mg of enzyme.


1/v
0.007
0
8
16
1/[PEP] mM⁻¹

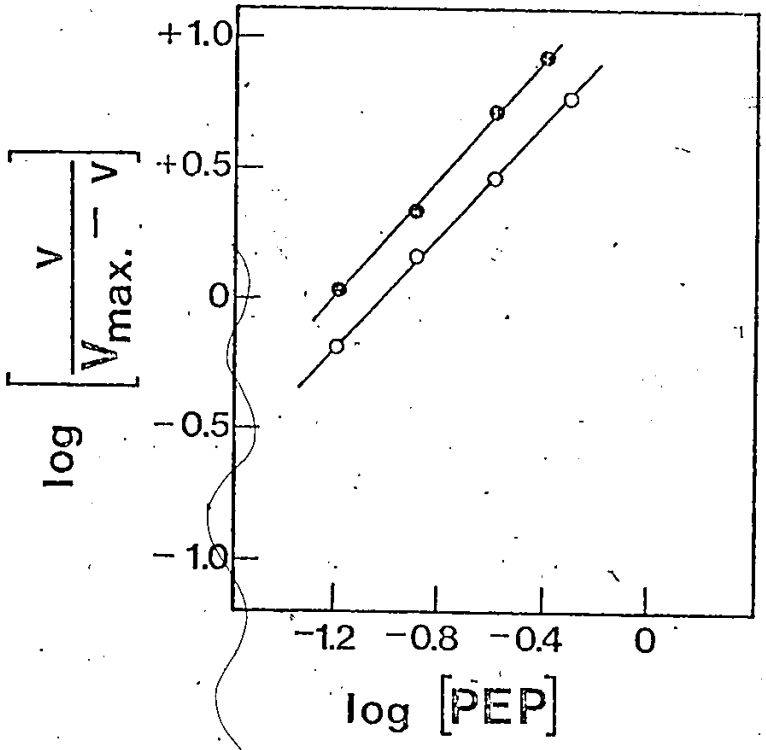
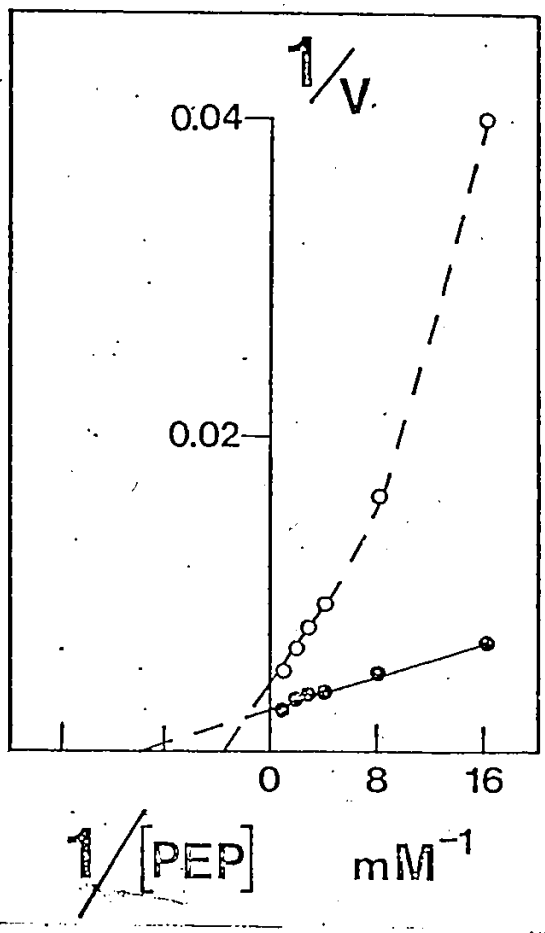

+1.0
+0.5
0
−0.5
−1.0
log [v/(Vmax. − v)]
−1.2
−0.8
−0.4
0
log [PEP]

FIGURE 7

Affinity of Eel Pyruvate Kinase
for PEP at pH 7.2, 30°C

Eel pyruvate kinase was assayed in the presence of 50 mM imidazole-HCl buffer, pH 7.2 in the absence (O——O) and presence (●——●) of 0.5 mM FDP at 30°C. The enzyme was assayed as described in Figure 6.


1/V
0.04
0.02
0
8
16
1/[PEP] mM⁻¹

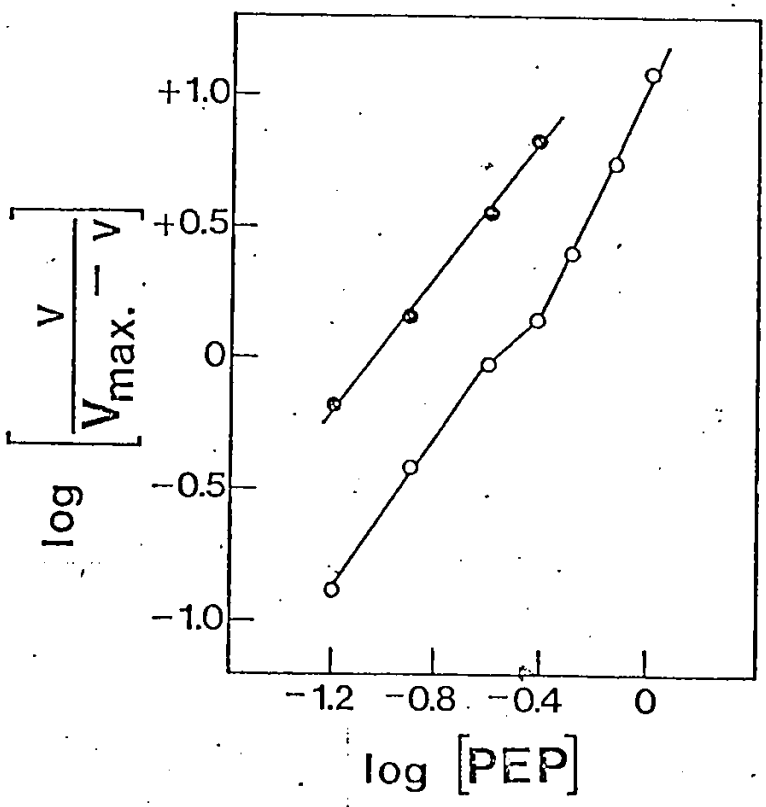
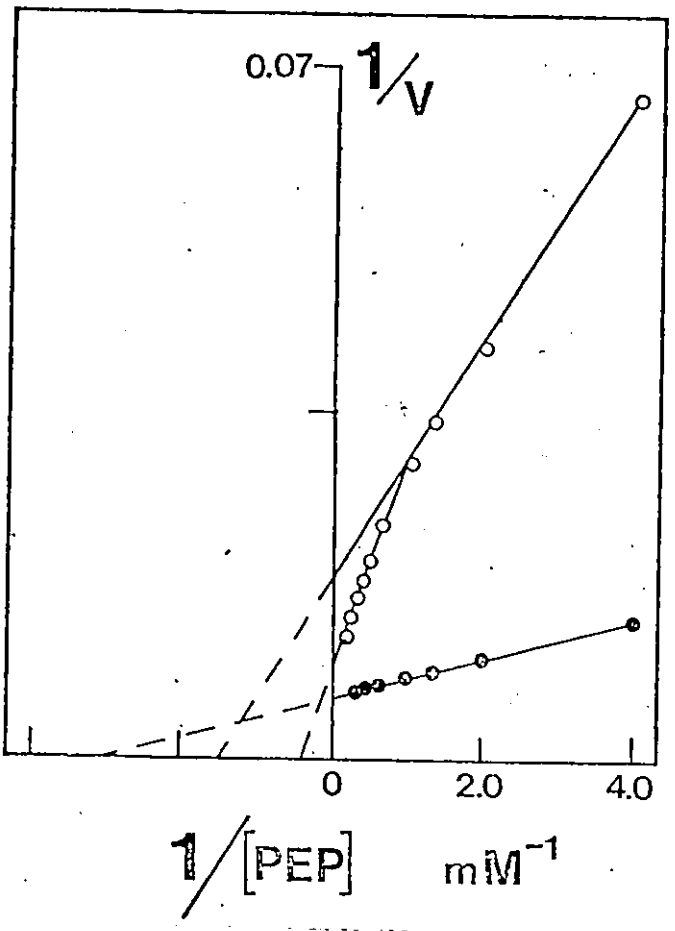
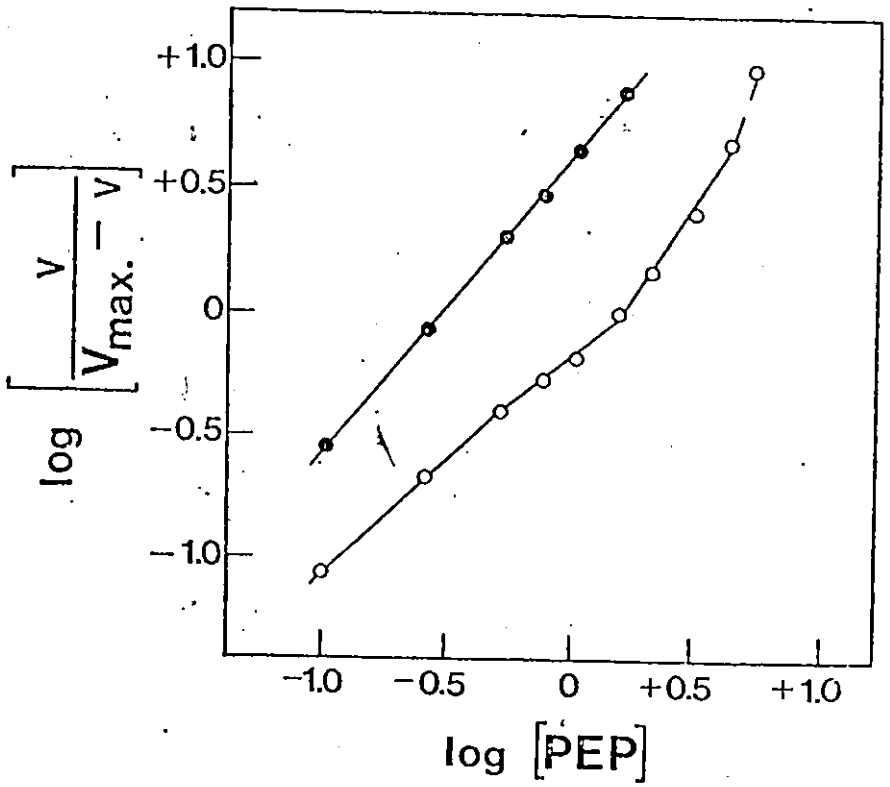

+1.0
+0.5
0
−0.5
−1.0
log [V / (Vmax. − V)]
−1.2
−0.8
−0.4
0
log [PEP]

FIGURE 8

Affinity of Eel Pyruvate Kinase
for PEP at pH 8.0, 30°C

Eel pyruvate kinase was assayed in the presence of 50 mM Tris-HCl buffer, pH 8.0 in the absence (O——O) and presence (●——●) of 0.5 mM FDP at 30°C. The enzyme was assayed as descibed in Figure 6.


0.07
1/v
0
2.0
4.0
1/[PEP] mM⁻¹


+1.0
+0.5
0
−0.5
−1.0
log [v / (Vmax. − v)]
−1.0
−0.5
0
+0.5
+1.0
log [PEP]

Hill plot was generated from the data obtained in the absence of FDP at pH 7.2 (Fig. 7). A Hill coefficient of 1.47 was obtained for PEP concentrations less than 0.25 mM while a Hill coefficient of 2.10 was obtained for PEP concentrations greater than 0.375 mM. In the presence of FDP, a single straight line was obtained on a Hill plot with a coefficient of 1.25.

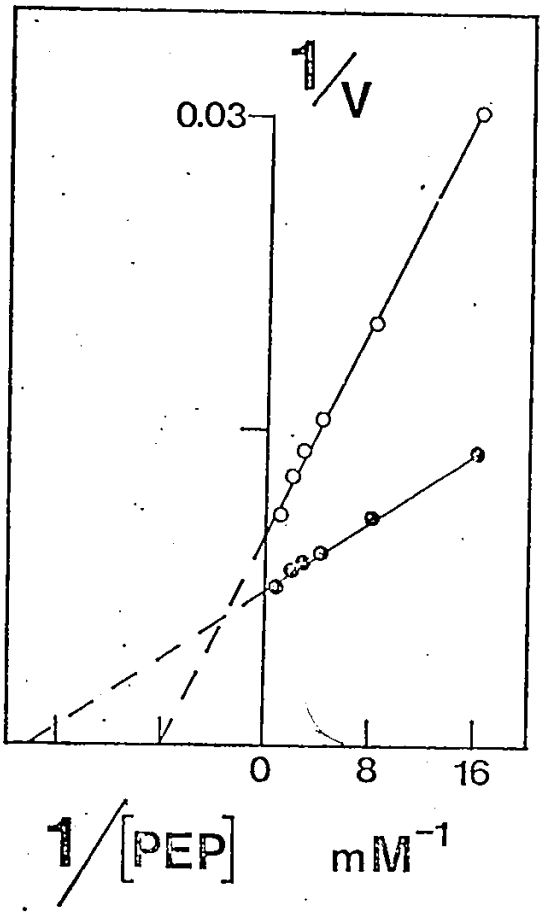
As shown in Figure 8, the enzyme's dependency on FDP for activation was even more pronounced at pH 8.0 using 0.05 M Tris-HCl as the assay buffer. In this case, the maximal velocity increased 1.9 fold in the presence of FDP. Lineweaver-Burk plots of the data obtained in the absence of FDP were biphasic and apparent K_m values of 0.66 mM for low concentrations of PEP and 2.40 mM for high concentrations of the substrate were estimated. In the presence of FDP, a single straight line was obtained on a Lineweaver-Burk plot and an apparent K_m of 0.33 mM was calculated. A complex Hill plot was generated from the data obtained in the absence of FDP at pH 8.0. A Hill coefficient of 1.00 was obtained for PEP concentrations less than 0.5 mM while a Hill coefficient of 1.48 mM was obtained for PEP concentrations greater than 1.5 mM (Fig. 8). In the presence of FDP, a single straight line was obtained on a Hill plot with a coefficient of 1.17.

The enzyme's affinity for the substrate PEP was also examined under conditions which are more in keeping with the physiological conditions that this fish is likely to encounter. When the assay temperature was reduced from 30°C to 18°C at pH 7.2, there was a 2.3 fold decrease in the apparent K_m and 2.2 fold decrease in the $V_{max.}$ in the absence of FDP. FDP could decrease the apparent K_m by

FIGURE 9

Affinity of Eel Pyruvate Kinase
for PEP at pH 7.2, 18°C

Eel pyruvate kinase was assayed in the presence of 50 mM imidazole-HCl buffer, pH 7.2 in the absence (O——O) and presence (●——●) of 0.5 mM FDP at 18°C. The enzyme was assayed as described in Figure 6.


1/V
0.03
0
8
16
1/[PEP] mM⁻¹

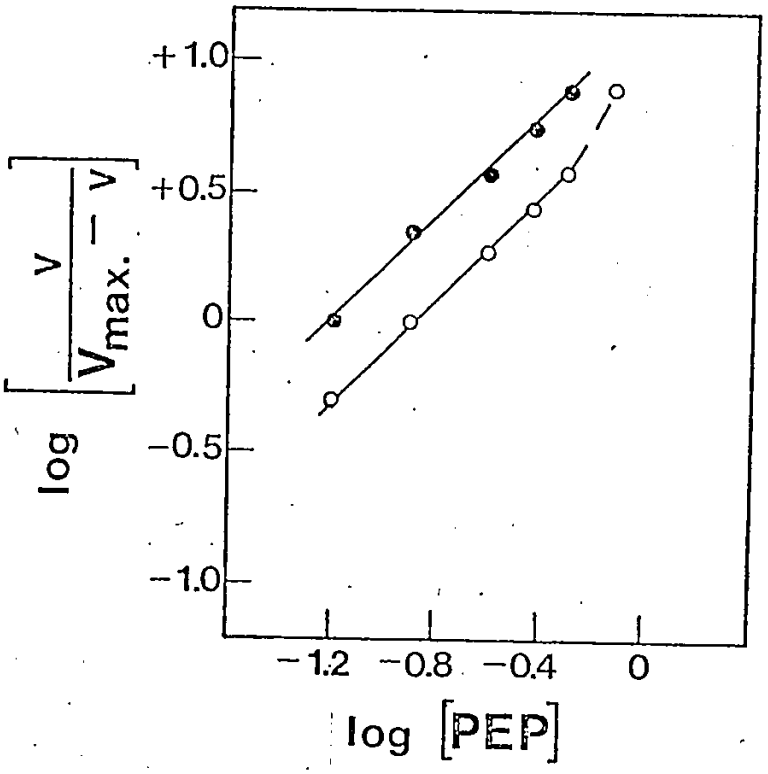

+1.0
+0.5
0
−0.5
−1.0
−1.2
−0.8
−0.4
0
log [V/(Vmax. − V)]
log [PEP]

TABLE 2

Effect of pH and FDP on the Affinity of Eel Pyruvate Kinase for PEP

pH	Temperature (°C)	FDP	Apparent K_m (PEP) (mM)	$V_{max.}$	n_H
6.3	30	-	0.09	350	1.05
		+	0.06	350	1.15
7.2	30	-	0.28	220	1.47 (< 0.25mM)
					2.10 (> 0.37mM)
		+	0.10	355	1.25
8.0	30	-	0.66, 2.40	85	1.00 (< 0.50mM)
					1.48 (> 1.50mM)
		+	0.33	163	1.17
7.2	18	-	0.12	98	1.00
		+	0.06	133	1.03

Eel pyruvate kinase was assayed at either 18°C or 30°C in the presence of 50 mM imidazole-HCl buffer, pH 7.2 and at 30°C in the presence of 50 mM imidazole-HCl buffer, pH 6.3 or 50 mM Tris-HCl buffer, pH 8.0. Enzyme was assayed in buffer containing 2.0 mM ADP, 8.0 mM $MgCl_2$, 45 mM KCl, 0.15 mM β-NADH and 3 units of dialyzed lactic dehydrogenase in the absence (-) or presence (+) of 0.5 mM FDP. Apparent K_m (PEP) and $V_{max.}$ values were obtained from the double reciprocal plots shown in Figures 6, 7, 8 and 9. Enzyme activity ($V_{max.}$) is expressed in terms of μmoles of PEP converted to pyruvate per minute per mg of enzyme. Hill coefficient values (n_H) were obtained from Hill plots of the data and are applicable to the specific PEP concentration ranges indicated in brackets.

TABLE 3

Effect of Temperature on the Affinity of Eel Pyruvate Kinase for PEP

pH	Temperature (^{o}C)	Apparent K_m (PEP) (mM)
6.3	13	0.08
	21	0.09
	30	0.09
7.2	13	0.10
	21	0.14
	30	0.28

Enzyme was assayed at either pH 6.3 or pH 7.2 in the presence of 50 mM imidazole buffer containing 2.0 mM ADP, 45 mM KCl, 8.0 mM $MgCl_2$, 0.15 mM β-NADH and 3 units of dialyzed lactic dehydrogenase.

a factor of 2.0 and increase the $V_{max.}$ by a factor of 1.4 at pH 7.2 and 18°C (Fig. 9). A reduction in the assay temperature from 30°C to 18°C could also abolish the complex co-operative behaviour exhibited by the enzyme in the absence of FDP at pH 7.2. A Hill coefficient of 1.0 was obtained for the enzyme assayed in the presence or absence of FDP at pH 7.2 and 18°C (Fig. 9).

The effects of activators, inhibitors, pH and temperature on substrate affinities and maximum activities exhibited by purified eel white muscle pyruvate kinase are complex. The dependence of the initial reaction velocity on the PEP concentration was investigated at 3 different temperatures. At pH 6.3, virtually no change in the enzyme's affinity for PEP could be detected as the assay temperature was decreased from 30 to 13°C (Table 3). However at pH 7.2, the enzyme's affinity for PEP increased over 2 fold as the assay temperature was decreased from 30 to 13°C (Table 3).

Figure 10 indicates the dependence of the reaction velocity on the concentration of 4 different monovalent cations at the pH optimum. At pH 6.3, potassium ion was the most effective activator of eel pyruvate kinase followed by ammonium ion, sodium ion and lastly lithium ion. At saturating levels of PEP, ADP and magnesium ion, the apparent K_a values for K^+, NH_4^+, Na^+ and Li^+ were determined to be 4.5, 4.9, 28.6 and 11.4 mM respectively at pH 6.3 and 30°C. Although the affinity of the enzyme for both K^+ and NH_4^+ was similar, the $V_{max.}$ of the enzyme was 1.4 times as great in the presence of saturating concentrations of K^+. The enzyme exhibited a much lower affinity for Na^+ and Li^+ and these cations only activate the enzyme to a small degree. In the absence of any monovalent

cation, no enzymatic activity could be detected. Ammonium ion concentrations in excess of 50 mM were found to be inhibitory but this was not the case for any of the other monovalent cations tested.

The cyclohexylammonium salts of PEP, ADP and β-NADH were employed during the course of this investigation of the monovalent cation dependency of eel pyruvate kinase. Since the enzyme did not exhibit any activity in the absence of any additional monovalent cation, it was concluded that the obligatory cyclohexylammonium cation could not activate eel pyruvate kinase. In order to investigate whether the cyclohexylammonium cation was inhibitory, the potassium ion dependency of eel pyruvate kinase was investigated using the potassium salts of PEP, ADP and β-NADH. When allowances were made for the presence of additional K^+ due to the use of the potassium salts of the substrates, the affinity of eel pyruvate kinase for K^+ and the $V_{max.}$ were unchanged.

The eel enzyme also exhibited an absolute requirement for a divalent cation. At saturating levels of PEP, ADP and potassium ion, the apparent K_a for Mg^{+2} was determined to be 0.84 mM at the pH optimum and 30°C (data not shown).

PEP substrate saturation plots were determined for saturating concentrations of 3 different monovalent cations. At pH 6.3 and 30°C, the enzyme exhibited a similar affinity for PEP in the presence of K^+ and NH_4^+ but the maximal velocity was 1.4 times greater

FIGURE 10

Effect of the Concentration of Various Monovalent Cations on the Activity of Eel Pyruvate Kinase

Eel pyruvate kinase was assayed in the presence of 50 mM imidazole-HCl buffer, pH 6.3 containing 2.5 mM PEP, 2.0 mM ADP, 8.0 mM $MgCl_2$, 0.15 mM β-NADH and 3 units of dialyzed lactic dehydrogenase at 30°C. Enzyme was assayed in the presence of varying concentrations of KCl (■——■), NH_4Cl (□——□), NaCl (●——●) and LiCl (○——○).

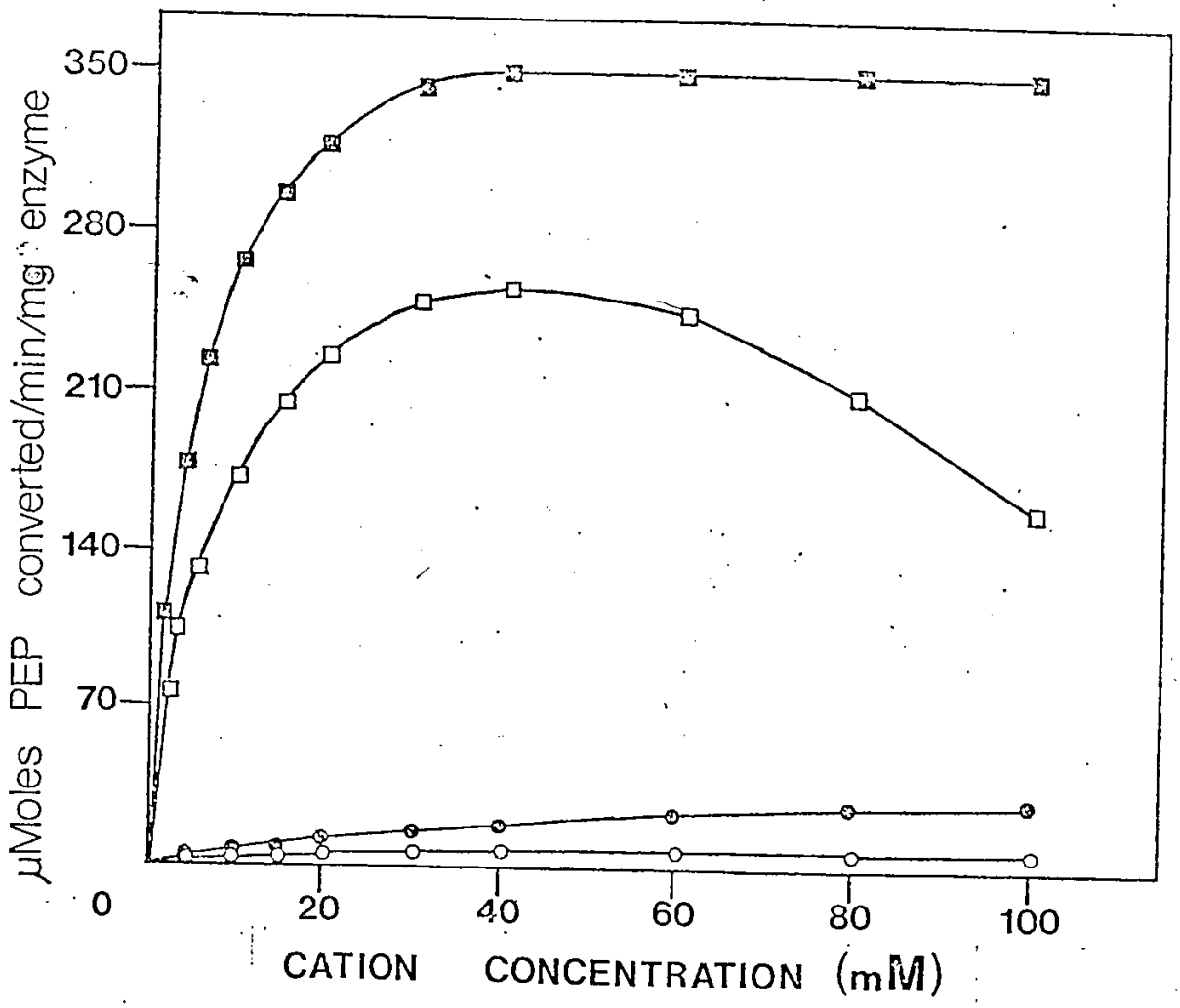

350
280
210
140
70
0
µMoles PEP converted/min/mg enzyme
20
40
60
80
100
CATION CONCENTRATION (mM)

TABLE 4

Effect of the Type of Monovalent Cation on the Affinity of Eel Pyruvate Kinase for PEP

pH	Temperature (^{o}C)	Type of Cation	Apparent K_m(PEP) mM	$V_{max.}$	n_H
6.3	30	K^+	0.09	350	1.05
		NH_4^+	0.12	250	1.17
		Na^+	0.32	29	1.11

Enzyme was assayed at 30^{o}C in 50 mM imidazole buffer, pH 6.3 containing 2.0 mM ADP, 8.0 mM $MgCl_2$, 0.15 mM β-NADH and 3 units of dialyzed lactic dehydrogenase in the presence of either 45 mM KCl, 40 mM NH_4Cl or 100 mM NaCl.

in the presence of K^+ than in the presence of NH_4^+ (Table 4). Sodium ion was a poor activator of the enzyme and the apparent K_m (PEP) was significantly greater in the presence of this cation. The Hill coefficients determined for the enzyme assayed in the presence of saturating levels of K^+, NH_4^+ or Na^+ ions were comparable.

The effect of PEP concentration on the enzyme's affinity for ADP was investigated (Fig. 11). At pH 6.3 and 30°C, the apparent K_m for ADP was found to be 0.35 mM and this was not changed by varying the PEP concentration from 0.125 mM to 2.5 mM. Similarly, the apparent K_m for PEP was constant at 0.09 mM for a range of ADP concentrations between 0.1 and 2.0 mM at pH 6.3 and 30°C (Fig. 12).

The effects of the known inhibitors of the mammalian pyruvate kinase isozymes on the activity of eel muscle pyruvate kinase were examined. The sensitivity of the eel enzyme to inhibition by ATP at pH 7.2 and 30°C was tested at a PEP concentration of 0.25 mM in order to compare these results with those obtained for other pyruvate kinases. A pronounced sigmoidal inhibition curve was obtained for increasing concentrations of ATP (Fig. 13). Since the inhibitory effect of ATP could possibly be attributed to the fact that it can chelate with the divalent cation required for catalytic activity, the inhibitory effect of an equimolar amount of Mg^{+2} and ATP was examined and found to be comparable to that of ATP alone for concentrations less than 8 mM. However, Mg^{+2}-ATP was significantly less inhibitory for concentrations in excess of 8 mM (Fig. 13). When the concentration of PEP in the assay buffer was increased from 0.25 to 1.0 mM, concentrations of ATP as high as 10 mM had a negligible inhibitory effect on the eel enzyme.

FIGURE 11

Effect of PEP Concentration on the Affinity of Eel Pyruvate Kinase for ADP

Enzyme was assayed in the presence of 50 mM imidazole-HCl buffer, pH 6.3 containing either 0.125 mM (■——■), 0.250 mM (□——□), 0.500 mM (●——●) or 2.500 mM (○——○) PEP and 8.0 mM $MgCl_2$, 45 mM KCl, 0.15 mM β-NADH and 3 units of dialyzed lactic dehydrogenase at 30°C.

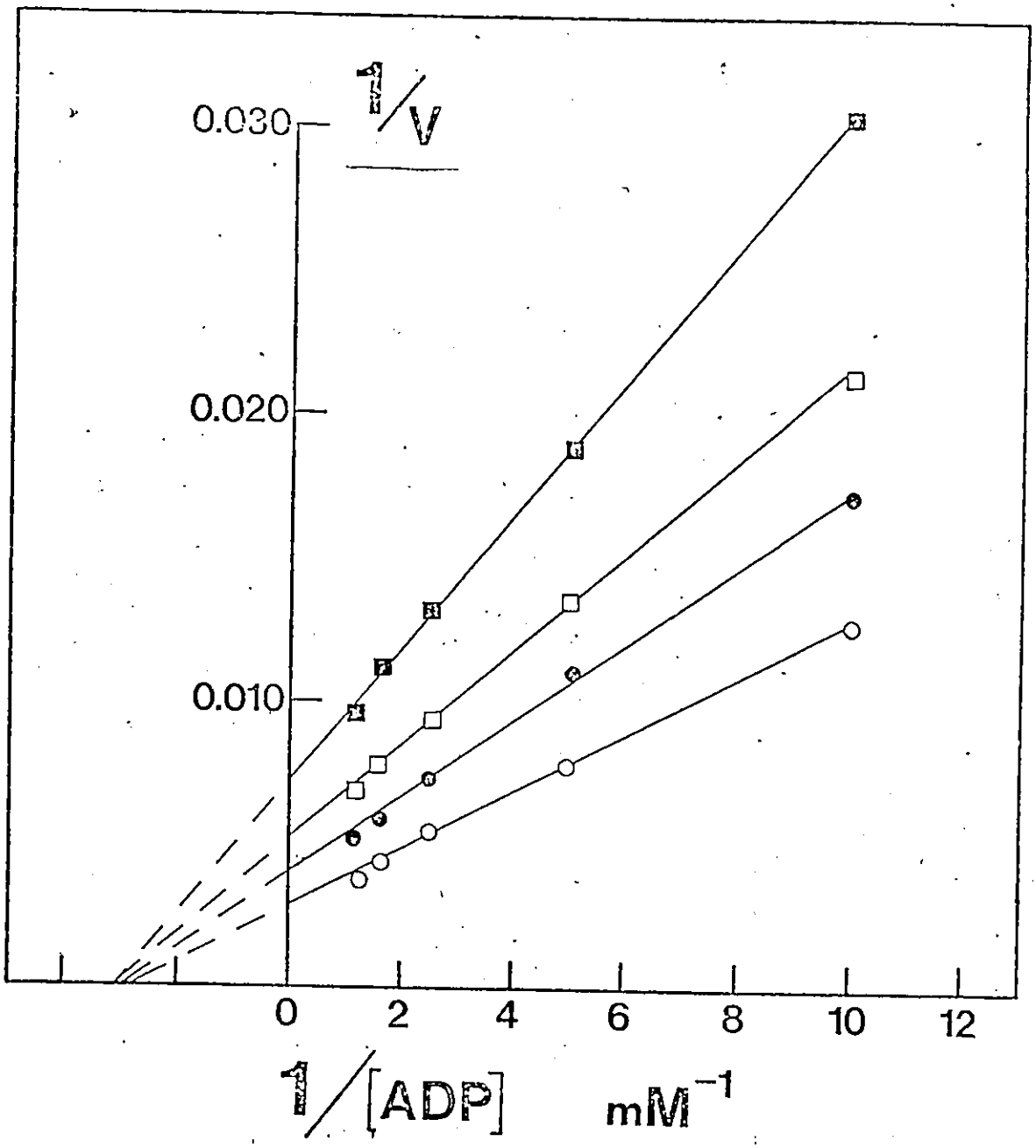

0.030
0.020
0.010
1/V
0
2
4
6
8
10
12
1/[ADP] mM⁻¹

TABLE 5

Effect of PEP Concentration on the Affinity of Eel Pyruvate Kinase for ADP

Concentration of PEP (mM)	Apparent K_m (ADP) (mM)	Maximum Velocity (%)
0.125	0.33	40.0
0.250	0.33	54.2
0.500	0.34	74.8
2.500	0.35	100.0

Enzyme was assayed at pH 6.3, 30°C in imidazole buffer as described in Figure 11.

FIGURE 12

Effect of ADP Concentration on the Affinity of Eel Pyruvate Kinase for PEP

Enzyme was assayed in the presence of 50 mM imidazole buffer, pH 6.3 containing either 0.1 mM (▲——▲), 0.2 mM (■——■), 0.4 mM (□——□), 1.0 mM (●——●) or 2.0 mM (○——○) ADP and 8.0 mM $MgCl_2$, 45 mM KCl, 0.15 mM β-NADH and 3 units of dialyzed lactic dehydrogenase at 30°C.

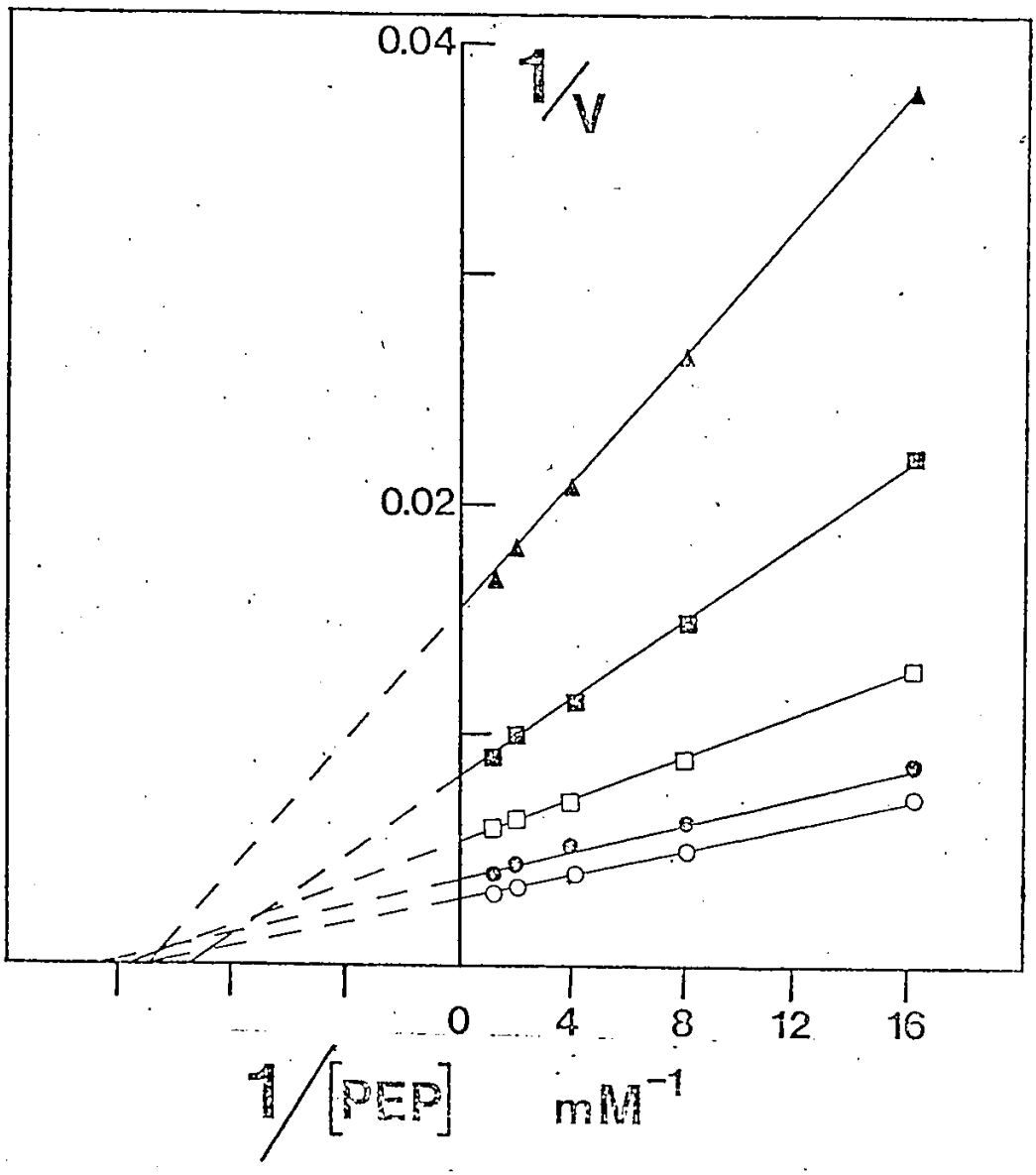

0.04
1/v
0.02
0
4
8
12
16
1/[PEP] mM⁻¹

TABLE 6

Effect of ADP Concentration on the Affinity of Eel Pyruvate Kinase for PEP

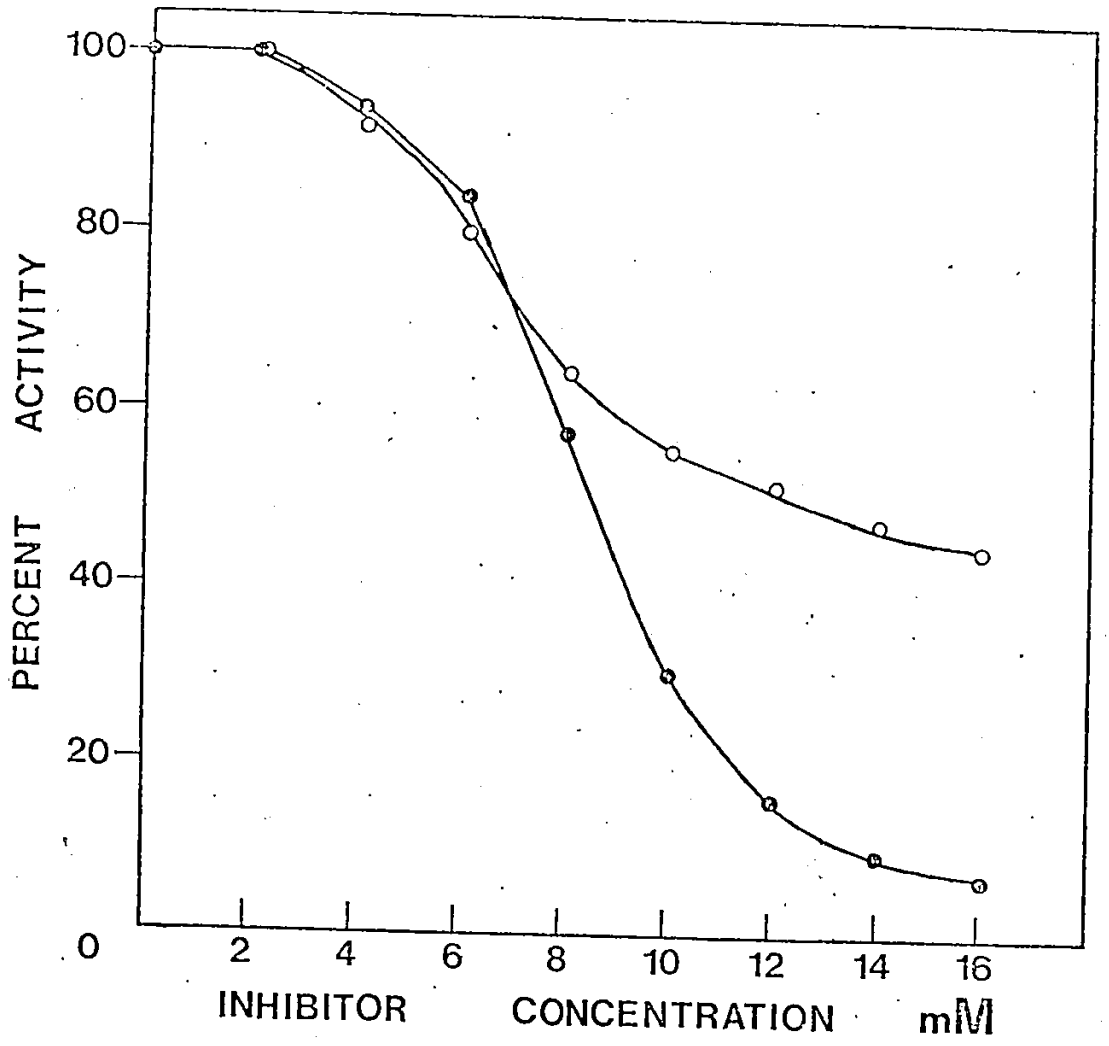
Concentration of ADP (mM)	Apparent K_m (PEP) (mM)	Maximum Velocity (%)
0.10	0.09	18.2
0.20	0.11	35.3
0.40	0.09	52.9
1.00	0.08	80.0
2.00	0.09	100.0

Enzyme was assayed at pH 6.3, 30°C in imidazole buffer as described in Figure 12.

FIGURE 13

Inhibition of Eel Pyruvate Kinase
by ATP and Mg^{+2}·ATP

Eel pyruvate kinase was assayed at 30°C in the presence of 50 mM imidazole-HCl buffer, pH 7.2 containing 0.25 mM PEP, 2.0 mM ADP, 45 mM KCl, 8.0 mM $MgCl_2$, 0.15 mM β-NADH and 3 units of dialyzed lactic dehydrogenase with varying concentrations of ATP (●——●) or an equimolar amount of ATP and $MgCl_2 \cdot 6H_2O$ (○——○). Enzyme activity is expressed as a percentage of the activity observed in the absence of inhibitor.


100
80
60
40
20
0
PERCENT ACTIVITY
2
4
6
8
10
12
14
16
INHIBITOR CONCENTRATION mM

Both the amino acids alanine and phenylalanine could substantially inhibit eel muscle pyruvate kinase. Phenylalanine was the more potent of the two amino acids and its inhibitory effect could be reduced by increasing the concentration of PEP in the assay buffer (Fig. 14). Figure 15 shows the effect of phenylalanine on the activity of the eel enzyme in the presence and absence of FDP as a function of PEP concentration. It can be seen that the presence of the amino acid decreased the affinity of the enzyme for PEP. In the absence of FDP, the $S_{0.5}$ value for PEP increased from 0.28 to 0.67 mM when 2.0 mM phenylalanine was added but there was no effect on the $V_{max.}$. When FDP was present, the $S_{0.5}$ value increased from 0.10 to 0.23 mM when phenylalanine was added to a concentration of 2.0 mM and the $V_{max.}$ decreased by 25%.

FIGURE 14

Inhibition of Eel Pyruvate Kinase
by Alanine and Phenylalanine

Enzyme was assayed at 30°C in imidazole buffer, pH 7.2 containing 2.0 mM ADP, 45 mM KCl, 8.0 mM $MgCl_2$, 0.15 mM β-NADH and 3 units of dialyzed lactic dehydrogenase in the presence of either 0.25 mM PEP (closed symbols) or 1.0 mM PEP (open symbols). The effects of varying concentrations of phenylalanine (○——○) and alanine (□——□) on enzyme activity were examined.

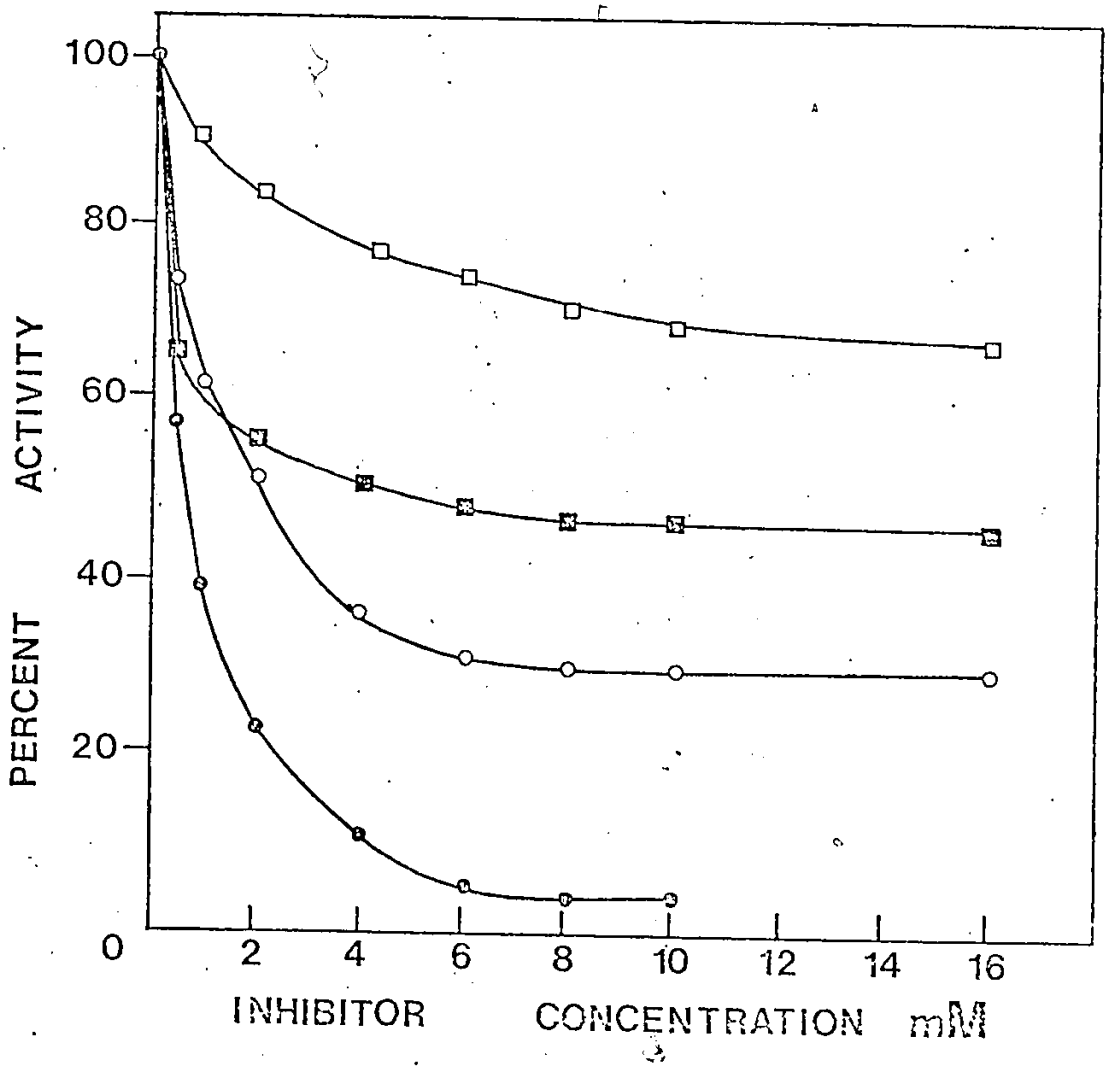
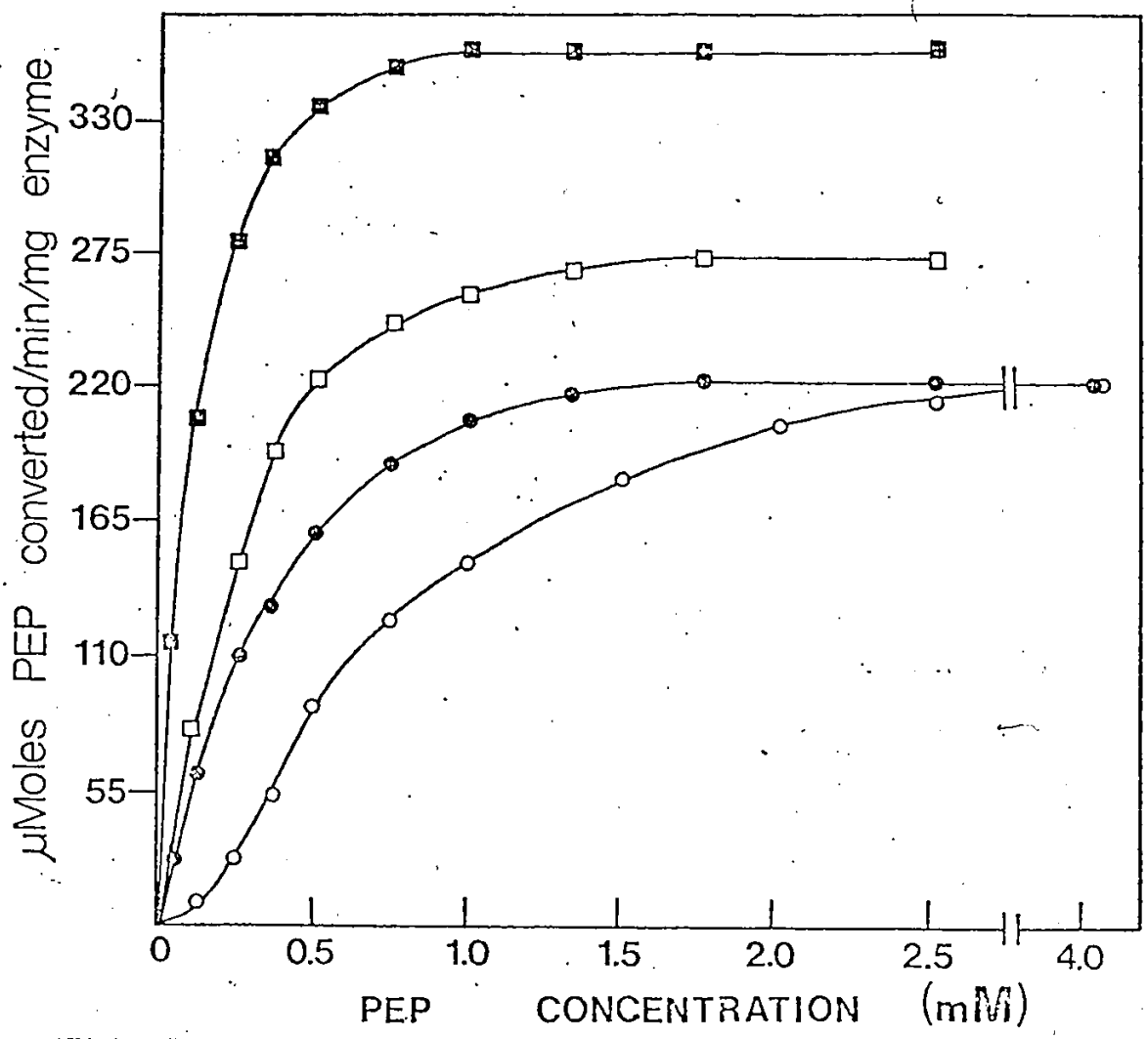

100
80
60
40
20
0
PERCENT ACTIVITY
2
4
6
8
10
12
14
16
INHIBITOR CONCENTRATION mM

FIGURE 15

Effect of Phenylalanine on the Activity of Eel Pyruvate Kinase at pH 7.2, 30°C

Eel pyruvate kinase activity was assayed at 30°C in the presence of 50 mM imidazole-HCl buffer, pH 7.2 containing 2.0 mM ADP, 8.0 mM $MgCl_2$, 45 mM KCl, 0.15 mM β-NADH and 3 units of dialyzed lactic dehydrogenase. The enzyme was assayed in the presence (○——○) and absence (●——●) of 2.0 mM phenylalanine without FDP and in the presence (□——□) and absence (■——■) of 2.0 mM phenylalanine with 0.5 mM FDP present.


µMoles PEP converted/min/mg enzyme
330
275
220
165
110
55
0
0.5
1.0
1.5
2.0
2.5
4.0
PEP CONCENTRATION (mM)

DISCUSSION

The purification procedure described allows the preparation of pyruvate kinase from the white muscle of the American eel in pure form, as judged by polyacrylamide gel electrophoresis, and in amounts sufficient for structural studies. The protein was purified by a modification of the method used to purify sturgeon muscle pyruvate kinase (Randall & Anderson, 1975). The enzyme was precipitated by concentrations of ammonium sulphate which are similar to the conditions used to precipitate mammalian M_1 and M_2 type pyruvate kinases (Harada et al., 1978). Like the sturgeon enzyme, eel pyruvate kinase was adsorbed on DEAE-cellulose under conditions that allow the adsorption of the mammalian liver isozyme but not the mammalian muscle isozyme (Tanaka et al., 1967). The specific activity of the final preparation was comparable to that of highly purified preparations of other muscle pyruvate kinases (Hall & Cottam, 1978). Unlike the sturgeon enzyme, eel pyruvate kinase was found to be susceptible to denaturation and it was necessary to add glycerol to the purification buffers. This precaution has been taken by others during the purification of invertebrate (Giles et al., 1976c), yeast (Hunsley & Seulter, 1969), mammalian liver (Riou et al., 1978) and M_2 type pyruvate kinases (Berglund et al., 1977). When glycerol was present in the buffers, the purified eel enzyme was stable at $4^{\circ}C$ for at least 6 months.

The pyruvate kinase protein concentration in eel white muscle was estimated to be 1.6 mg per gm fresh weight of tissue based on the total amount of assayable activity in the pH 5.4 extract. A preliminary study was performed in order to quantitate the amount of pyruvate kinase protein in eel white muscle. The

method employed was based on that of Anderson (1976) and involves the use of double radioisotope labelling and peptide quantification to determine the amount of individual proteins in mixtures. Based on this method, the pyruvate kinase protein concentration in eel white muscle was determined to be 2.8 mg per gm fresh weight of tissue. By way of comparison, Johnson & Veneziale (1980) reported that the pyruvate kinase concentration in rabbit skeletal muscle was 2.5 mg per gm fresh weight of tissue by radioimmunassay. Based on the total amount of assayable activity in an extract of rabbit skeletal muscle, the authors reported a value of 1.8 mg/gm fresh weight for the concentration of pyruvate kinase. It is apparent from these series of studies that the pyruvate kinase concentrations in eel white muscle and rabbit skeletal muscle are comparable and the assayable activity in a muscle extract does not reflect enzyme concentration.

The enzyme appears to consist of only one type of subunit as only one band was obtained on SDS gels (Fig. 3) and also on 8 M urea-containing gels run under both cationic and anionic conditions (Fig. 4A, B). Eel pyruvate kinase, like the sturgeon enzyme (Anderson & Randall, 1975), exhibited an electrophoretic mobility which was twice that of the rabbit enzyme on 8 M urea gels run under anionic conditions (Fig. 4B). No evidence could be obtained for the presence of disulphide bonds linking subunits together because eel pyruvate kinase exhibited the same electrophoretic mobility on SDS gels run under both reducing and non-reducing conditions (Fig. 3).

The enzyme activity was found to be maximal between pH 6.0 and pH 6.5 in three different buffers at 30°C (Fig. 5). A pH optimum in the range 6.0 to 6.5 is considerably lower than that observed for the enzyme isolated from the muscle of a variety of mammalian species (Baranowska & Baranowski, 1975; Berglund & Humble, 1979; Cardenas et al., 1973) and the river sturgeon (Randall & Anderson, 1975). However, it is similar to the values obtained for the enzyme isolated from the muscle of several fish species (Fields et al., 1978; Guppy & Hochachka, 1979; Johnston, 1975) and for some mammalian liver (Hall & Cottam, 1978) and M_2 type pyruvate kinases (Schering et al., 1982). It, therefore, seems clear that the pH optimum of pyruvate kinase isozymes is not constant for a given tissue or organ, but varies with the requirements of individual species.

The type of assay buffer had little effect on the value of the pH optimum or the pH sensitivity of the enzyme at 30°C but the maximal velocity obtained in the presence of potassium phosphate buffer was significantly higher than the maximal velocities in Mes or imidazole buffers (Fig. 5). As reported previously (Moon & Hulbert, 1980a), potassium phosphate buffer can activate eel pyruvate kinase and this can not be attributed to the presence of additional K^+ as the enzyme is already saturated under the assay conditions employed. Phosphate buffer has been found to activate human muscle (Baranowska & Baranowski, 1975) and pig liver pyruvate kinase (Ljungström et al., 1976) as well as some invertebrate isozymes (Giles et al., 1976c; Kapoor, 1975). The activation of pyruvate kinase isozymes by phosphate buffer may be a general feature of this enzyme.

A decrease in temperature has been found to decrease the pH sensitivity of tuna muscle pyruvate kinase (Guppy & Hochachka, 1979). No appreciable change in the pH sensitivity of the eel enzyme could be detected when the assay temperature was decreased from 30°C to 18°C. The decrease in temperature had no effect on the value of the pH optimum in Mes buffer but there was a slight shift in the pH optima observed in imidazole and potassium phosphate buffers (Fig. 5).

A previous study of a partially purified pyruvate kinase from eel white muscle indicated a pH optimum close to 6.0 at 18°C and a considerably narrower activity as a function of pH profile (Moon & Hulbert, 1980a). This partially purified enzyme was prepared by homogenization and extraction of eel white muscle with 100 mM Tris-Maleate buffer, pH 7.4 containing 2 mM EDTA followed by ammonium sulphate fractionation. A specific activity value of 4 units/mg of protein was reported for this enzyme preparation at pH 6.0 and 20°C (Moon & Hulbert, 1980a). Following ammonium sulphate fractionation, a specific activity value of 26 units/mg of protein was obtained for partially purified pyruvate kinase in the present study at pH 6.3 and 30°C. When corrections are made for the different assay temperatures employed, the specific activity value obtained in the present study is still 3.5 times greater than the value reported for a partially purified pyruvate kinase previously (Moon & Hulbert, 1980a). In the previous study, the enzyme preparation was dialyzed against the tissue extraction buffer for 12 hours in the absence of anti-oxidants and stabilizing agents prior to the commencement of kinetic experiments. It is more than likely that the enzyme was denatured as a result of this treatment and this would have altered its kinetic properties.

Eel pyruvate kinase is activated by fructose-1,6-bisphosphate in a complex and pH-dependent manner. At all pH values examined, the affinity of the enzyme for the substrate PEP was increased in the presence of FDP, but an increase in the $V_{max.}$ due to the presence of FDP was only observed at pH values greater than 6.3 (Table 2). This was particularly evident at pH 8.0 where the enzyme exhibited two apparent K_m values for PEP of 0.66 and 2.40 mM in the absence of FDP but in its presence only one K_m value of 0.33 mM was observed and there was a 1.9 fold increase in the maximum velocity. An identical finding has been reported for the M_2 isozyme but not the M_1 isozyme of the pig (Berglund & Humble, 1979).

In some cases, the biphasic nature of a Lineweaver-Burk plot can be attributed to the phenomenon of negative co-operativity (Dixon & Webb, 1979). However in this instance, Hill plots of the data obtained in the absence of FDP at pH 8.0 indicated that the enzyme exhibited no co-operativity, either negative or positive, in the binding of the substrate PEP for concentrations less than 0.5 mM while slightly positive co-operative binding kinetics prevailed for PEP concentrations greater than 1.5 mM. Similar complex Hill plots exhibiting inflection points between two linear sections have been described for the M_2 and L type pyruvate kinases of rat liver (Van Berkel et al., 1973; Van Berkel, 1974; Van Berkel et al., 1977). Such kinetic behaviour can be attributed to the presence of 2 kinetically different forms of the same enzyme which exhibit different affinities for the substrate PEP.

In mammals, pyruvate kinase isozymes can be distinguished on the basis of their affinities for the substrate PEP and their res-

ponse to FDP. The enzyme from skeletal muscle shows hyperbolic binding kinetics and this is not influenced by the presence of FDP (Hall & Cottam, 1978). The enzyme from liver shows pronounced co-operative effects in the binding of PEP and FDP greatly increases the affinity of the enzyme for this substrate (Hall & Cottam, 1978). The binding of PEP to M_2-type isozymes is much less co-operative than is the binding of this substrate to the mammalian liver isozyme, however it is facilitated by the presence of FDP and the effect of FDP increases with increasing pH (Berglund & Humble, 1979; Ibsen et al., 1981; Van Berkel et al., 1973). Therefore, with respect to its affinity for PEP and its response to FDP, pyruvate kinase from eel muscle most resembles the mammalian M_2-type isozyme. Comparison with data obtained for other species indicates that, with the exception of birds and mammals, most muscle pyruvate kinases exhibit increased affinities for the substrate PEP in the presence of FDP (Zammit et al., 1978). Although there is considerable variation in the details of this process between species, this observation suggests that the more primitive forms of the enzyme are FDP-sensitive and the development of FDP-insensitivity represents a more recently evolved property of the muscle enzyme.

In common with what has been reported for other poikilothermic animals (Somero & Hochachka, 1968; Randall & Anderson, 1975; Guppy & Hochachka, 1979), the apparent K_m for PEP of eel pyruvate kinase was found to decrease with decreasing temperature, although the effect was pH-dependent. At pH 6.3, the apparent K_m for PEP increased slightly from 0.08 to 0.09 mM over the temperature range

13 to 30°C. However at pH 7.2, the apparent K_m increased from 0.10 mM at 13°C to 0.28 mM at 30°C (Table 3). This is in contrast to a previous report using a partially purified eel white muscle pyruvate kinase in which the apparent K_m for PEP was observed to increase with decreasing temperature (Moon & Hulbert, 1980a). The low specific activity of this preparation or its contamination by impurities may account for the discrepancy.

Eel pyruvate kinase exhibited a decreased sensitivity to activation by FDP at lower assay temperatures (Table 2). This observation coupled with the temperature dependency of the enzyme's affinity for PEP would suggest that eel pyruvate kinase is subject to temperature dependent interconversion between kinetically different forms. A similar observation has been reported for the muscle pyruvate kinases of the Alaskan king crab (Somero, 1969), trout (Somero & Hochachka, 1968) and sturgeon (Randall & Anderson, 1975).

Eel pyruvate kinase, like all other vertebrate pyruvate kinases described to date, exhibited an absolute requirement for a monovalent and a divalent cation for catalytic activity. The enzyme's affinity for the divalent cation Mg^{+2} is comparable to that of mammalian muscle pyruvate kinases but the affinity for the monovalent cation is 2 times greater. Some mammalian M_2 (Pogson, 1968) and L type pyruvate kinases (Ekman et al., 1976) also exhibit affinities for K^+ which are at least twice as high as those of the mammalian M_1 type pyruvate kinase. Of the four monovalent cations tested, K^+ is the best activator of the enzyme followed by NH_4^+ then Na^+ and lastly Li^+. This is similar to the manner in which rabbit muscle pyruvate kinase responds to mono-

valent cation activators (Kayne, 1971).

Previously it was reported that a partially purified eel pyruvate kinase exhibited hyperbolic binding kinetics with respect to PEP in the presence of K^+ but sigmoidal binding kinetics in the presence of Na^+ (Moon & Hulbert, 1980a). Although purified white muscle pyruvate kinase did exhibit a reduced affinity for PEP when assayed in the presence of Na^+ at the pH optimum (Table 4) the degree of co-operativity displayed was much less dependent on the type of monovalent cation present.

Eel pyruvate kinase behaves in a manner more similar to mammalian isozymes than to some other fish pyruvate kinases with respect to its affinity for one substrate in the presence of varying concentrations of the second substrate. The kinetic data obtained in the present study is consistent with random substrate binding for ADP and PEP as has been reported for mammalian M_1 (Reynard et al., 1961; Ainsworth & Macfarlane, 1973) and M_2 type pyruvate kinases (Jimenez de Asua et al., 1971; Walker & Potter, 1973). In contrast, it has been reported that a decrease in the concentration of one substrate results in an increase in the affinity of the muscle enzyme of several types of fish for the second substrate (Fields et al., 1978; Guderley & Cardenas, 1980b)

The enzyme from eel white muscle is much less sensitive to ATP inhibition than pyruvate kinases from other sources. ATP inhibits all isozymes from mammals (Hall & Cottam, 1978), although the L-type isozyme is by far the most sensitive form. Some fish isozymes from liver and kidney (Guderley et al., 1978) and skeletal muscle (Guderley & Cardenas,1980b; Johnston, 1975; Somero & Hochachka, 1968) have also been shown to be sensitive to inhibition by ATP.

However, the pyruvate kinases of eel liver and tuna skeletal muscle have been reported to be similar to the eel white muscle isozyme with respect to their sensitivities to inhibition by ATP (Moon & Hulbert, 1980b; Guppy & Hochachka, 1979). The significance of the relative insensitivity of eel white muscle pyruvate kinase to ATP inhibition is therefore uncertain.

Alanine and phenylalanine both inhibited pyruvate kinase from eel white muscle and the effect is only partially reversed by the addition of FDP. The effects of amino acids on pyruvate kinase isozymes from other species are variable. For example, alanine inhibits L-type pyruvate kinase from rats (Imamura et al., 1972) but has no effect on the bovine liver isozyme (Cardenas et al., 1975b). Pyruvate kinase from frog skeletal muscle is not inhibited by alanine or phenylalanine but the enzyme from frog heart is inhibited by both amino acids (Flanders et al., 1971). Only the mammalian M_2-type isozyme has consistently been shown to be sensitive to inhibition by both alanine and phenylalanine and this inhibition is only partially reversed by the presence of FDP (Schering et al., 1982). This suggests that structural similarities accounting for amino acid binding exist between the eel muscle and mammalian M_2-type pyruvate kinases.

The kinetic properties of eel pyruvate kinase are similar to those of the muscle enzymes of the carp (Johnston, 1975), the tuna (Guppy & Hochachka, 1979), the turtle (Storey & Hochachka, 1974) and some osteoglossid fish (Fields et al., 1978). However eel pyruvate kinase is different kinetically from the muscle enzymes of the salmon (Guderley & Cardenas, 1980b), the trout (Somero &

Hochachka, 1968) and some erythrinid fish (Fields et al., 1978). It is likely that the pyruvate kinases of these different species exhibit varying sensitivities to modulators of enzyme activity because they are adapted to suit the specific physiological conditions of each species.

The pyruvate kinases are a complex family of enzymes. Examination of some of the kinetic properties of eel pyruvate kinase has indicated that it probably has many structural features in common with the mammalian M_2 isozyme. Like the mammalian M_2-type isozyme, eel pyruvate kinase exhibits slight co-operativity in the binding of PEP at physiological pH and is activated by FDP in a pH-dependent manner and is inhibited by both phenylalanine and alanine. Further study of the structural basis of these properties and comparison with features of the mammalian M_2 isozyme and other isozymes may therefore help to define the origin, development and role of pyruvate kinase isozymes.

- CHAPTER 4 -

EXAMINATION OF THE SUBUNIT AND PRIMARY STRUCTURES OF EEL & RABBIT MUSCLE PYRUVATE KINASES

RESULTS

Since the kinetic properties of eel white muscle pyruvate kinase were found to be substantially different from those of the M_1 isozyme from rabbit muscle, the structural features of these 2 different muscle isozymes were determined.

The molecular weight of eel muscle pyruvate kinase was found to be 229,000 ± 16,000 as determined by gel filtration chromatography on a Sephacryl S-300 column (Fig. 1). When eel and rabbit muscle pyruvate kinases were applied onto the Sephacryl column together, they appeared together in the same elution volume. SDS-polyacrylamide gel electrophoresis indicated that both the eel and rabbit enzymes consisted of a single type of subunit of 59,000 ± 700 molecular weight (Fig. 2). The subunits of eel and rabbit muscle pyruvate kinases were found to have the same electrophoretic mobility on SDS gels.

In the absence of FDP, eel pyruvate kinase gave 2 major bands and one minor band when isoelectric focusing was conducted on polyacrylamide gels according to the method of Wrigley (1971). The pI values of the 2 major bands were determined to be 5.92 and 6.35 while the minor band had a pI value of 6.62. In the presence of FDP, eel pyruvate kinase gave only one band on isoelectric focusing gels with a pI value of 5.90. In the presence or absence of FDP, rabbit muscle pyruvate kinase gave only one band on isoelectric focusing gels with a pI value of 8.83 (data not shown).

FIGURE 1

Molecular Weight Determination
of Eel Pyruvate Kinase

The molecular weight of the native eel muscle enzyme was determined by gel filtration chromatography as described in the Methods. Distribution coefficient values (K_d) are equivalent to $(V_e-V_o)/V_s$ where V_e is the elution volume of the protein, V_o is the void volume and V_s is the column bed volume. The intersection of the vertical dotted lines with the horizontal axis indicates the range of K_d values obtained for eel pyruvate kinase. THY, thyroglobulin (M.W. 669,000); RPK, rabbit muscle pyruvate kinase (M.W. 230,000); BSA, bovine serum albumin (M.W. 66,200).

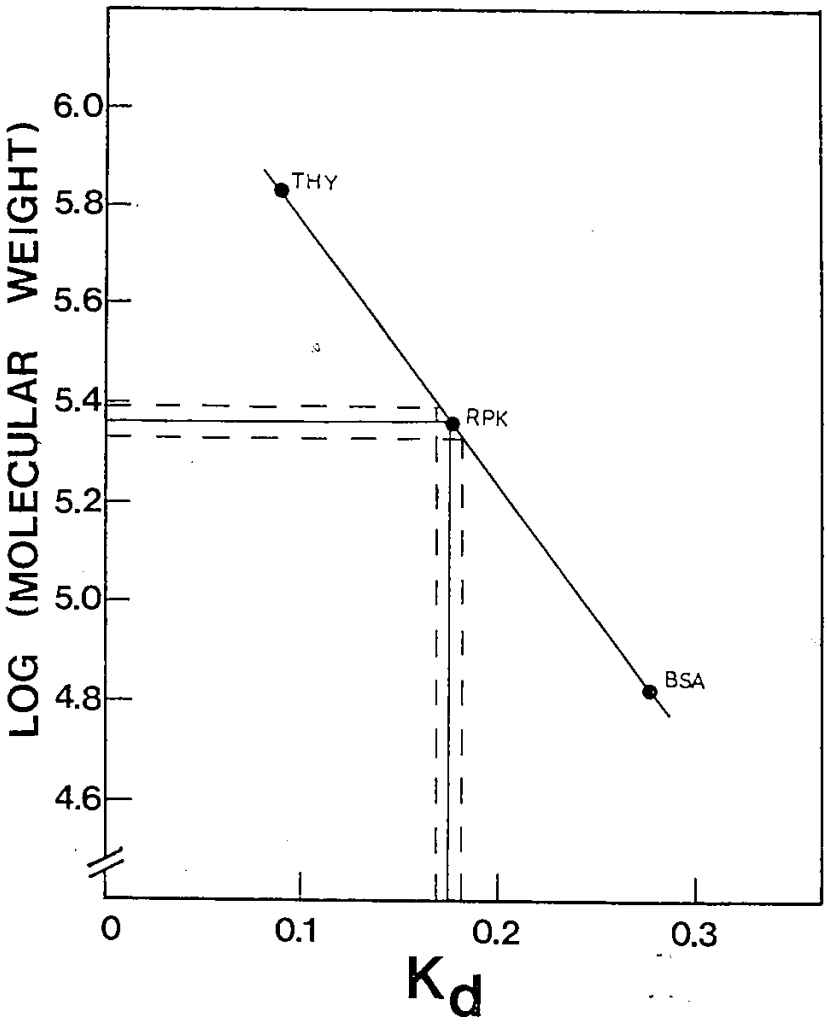
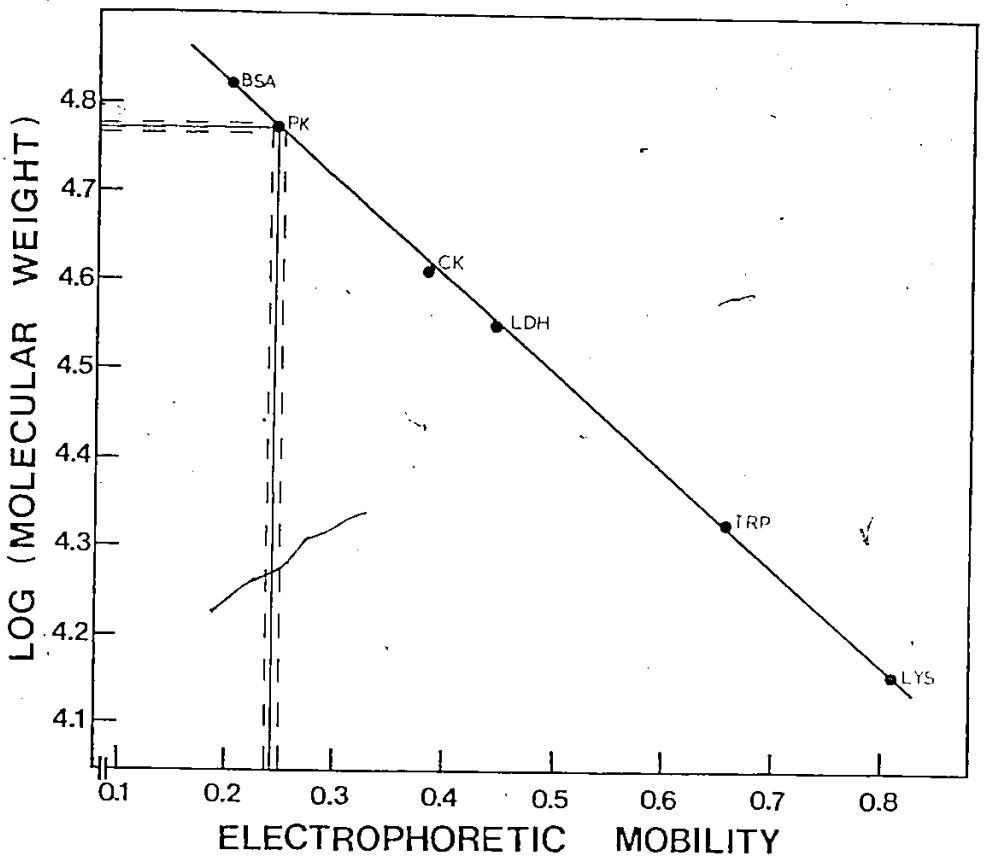

LOG (MOLECULAR WEIGHT)
6.0
5.8
5.6
5.4
5.2
5.0
4.8
4.6
THY
RPK
BSA
0
0.1
0.2
0.3
K_d

FIGURE 2

Subunit Molecular Weight Determination
of Eel Pyruvate Kinase

Subunit molecular weight determinations were conducted on SDS-polyacrylamide gels as described in the Methods. The electrophoretic mobilities of the marker proteins are expressed with respect to the tracking dye, bromophenol blue. The intersection of the vertical dotted lines with the horizontal axis indicates the range of electrophoretic mobility values obtained for eel muscle pyruvate kinase. BSA, bovine serum albumin (M.W. 66,200); PK, rabbit muscle pyruvate kinase (M.W. 59,000); CK, rabbit muscle creatine kinase (M.W. 40,500); LDH, rabbit muscle lactic dehydrogenase (M.W. 35,500); TRP, soybean trypsin inhibitor (M.W. 21,500); LYS, lysozyme (M.W. 14,400).


LOG (MOLECULAR WEIGHT)
4.8
4.7
4.6
4.5
4.4
4.3
4.2
4.1
BSA
PK
CK
LDH
TRP
LYS
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
ELECTROPHORETIC MOBILITY

The amino acid compositions of eel and rabbit pyruvate kinases are given in Table 1. The results are expressed per subunit and assume that the enzymes are composed of identical subunits of 59,000 molecular weight. The compositions of the 2 enzymes are very similar with the greatest differences being in the smaller number of alanine residues and larger number of threonine residues found in the eel enzyme.

The N-terminal amino acid of eel pyruvate kinase was determined to be proline. Authentic dansyl-proline was found to co-migrate on polyamide thin layer sheets with the dansyl amino acid derived from a hydrolysate of dansylated eel pyruvate kinase. Under similar conditions, rabbit muscle pyruvate kinase failed to give a positive N-terminal reaction. (data not shown).

Saheki et al. (1982a) recently characterized the 4 pyruvate kinase isozymes of the rat on the basis of their cyanogen bromide peptide maps on polyacrylamide gels. In order to compare the structural features of eel and rabbit muscle pyruvate kinases to those of the 4 isozymes of the rat, cyanogen bromide peptides derived from carboxymethylated pyruvate kinases were mapped on SDS-polyacrylamide gels run under anionic conditions (Fig. 3A) and on Triton-urea polyacrylamide gels run under cationic conditions (Fig. 3B). Although carboxymethylated eel and rabbit muscle pyruvate kinases appear to share some common cyanogen bromide peptides, the peptide banding patterns are distinctly different. The peptide banding patterns of carboxymethylated rabbit muscle pyruvate kinase on SDS and Triton-urea gels are almost identical to those reported previously for the M_1 isozyme of the rat (Saheki et al., 1982a).

TABLE 1

Amino Acid Compositions of
Rabbit and Eel Muscle Pyruvate Kinases

Amino Acid	Mole/Mole of Enzyme Subunit	
	Rabbit	Eel
Cysteine	8.9	6.1
Aspartic Acid and Asparagine	50.6	53.2
Threonine	27.3	36.4
Serine	28.8	34.5
Glutamic Acid and Glutamine	52.5	49.2
Proline	19.2	22.0
Glycine	42.8	44.4
Alanine	57.5	46.2
Valine	40.4	35.3
Methionine	16.6	16.8
Isoleucine	34.6	34.0
Leucine	39.0	38.2
Tyrosine	9.9	13.6
Phenylalanine	16.3	16.6
Histidine	15.9	16.5
Tryptophan	3.4	4.1
Lysine	38.5	37.4
Arginine	33.7	31.5
Aspartic Acid and Glutamic Acid	66.8	68.5

Amino acid compositions were determined as described in the Methods. Cysteine and tryptophan were determined spectrophotometrically by the methods of Habeeb (1972) and Edelhoch (1967) respectively. The total content of aspartic acid and glutamic acid was determined by carbodiimide modification according to the method of Carraway & Koshland (1972).

FIGURE 3A

Cyanogen Bromide Peptide Mapping
on SDS-Polyacrylamide Gels

The cyanogen bromide peptides derived from carboxymethylated eel and rabbit muscle pyruvate kinases and carboxymethylated rat liver pyruvate kinase were subjected to electrophoresis on polyacrylamide gels containing SDS as described in the Methods. From left to right: gel 1, 75 µg of carboxymethylated rat liver PK CNBr peptides; gel 2, 75 µg of carboxymethylated rabbit PK CNBr peptides; gel 3, 75 µg of carboxymethylated eel PK CNBr peptides.

FIGURE 3B

Cyanogen Bromide Peptide Mapping
on Triton-Urea Polyacrylamide Gels

The cyanogen bromide peptides derived from carboxymethylated eel and rabbit muscle pyruvate kinases were subjected to electrophoresis on polyacrylamide gels containing 7.5 M urea and 6 mM Triton X-100 as described in the Methods. From left to right: gel 1, 75 µg of carboxymethylated rabbit PK CNBr peptides; gel 2, 75 µg of carboxymethylated eel PK CNBr peptides.

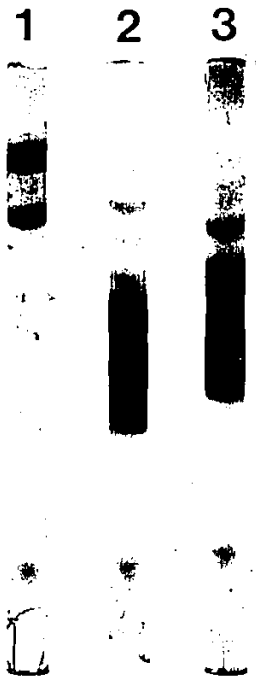


FIG. 3A

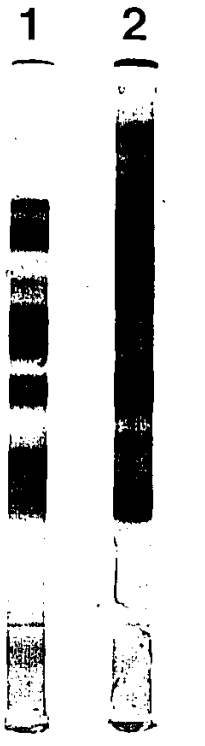


FIG. 3B

However, the peptide banding patterns of carboxymethylated eel muscle pyruvate kinase on SDS and Triton-urea gels are very similar to those reported previously for the M_2 isozyme of the rat. By way of comparison, the peptide banding pattern of carboxymethylated rat liver pyruvate kinase on SDS gels (Fig. 3A) reported here is very similar to the pattern described previously (Saheki et al., 1982a).

The cyanogen bromide peptides of ^{14}C-carboxymethylated eel and rabbit pyruvate kinases were separated on a Sephadex G-75 column as illustrated in Figures 4A and 4B. Peptides eluted in the final radioactive peak might be expected to be of relatively low molecular weight and therefore amenable to purification by high voltage paper electrophoresis. One major radioactive peptide was purified in this manner from each of the 2 muscle pyruvate kinases and the amino acid compositions of these peptides were found to be very similar (Table 2). The asparagine and glutamine contents of these peptides were not determined directly. Instead this information was inferred from the fact that both eel CNBr peptide EX1 and rabbit CNBr peptide RX1 migrated slightly further toward the cathode than ε-DNP-lysine at pH 6.5. The N-terminal amino acids of the eel and rabbit cyanogen bromide peptides were determined to be threonine and isoleucine respectively.

In order to further investigate the structural similarities of the eel and rabbit muscle isozymes, peptides were generated from ^{14}C-carboxymethylated pyruvate kinases by cyanogen bromide cleavage followed by tryptic digestion and these peptides were mapped on paper. Figure 5A represents a map of the acidic and basic peptides

FIGURE 4A

Sephadex G-75 Chromatography of the Cyanogen Bromide Peptides Derived from ^{14}C-Carboxymethylated Rabbit Pyruvate Kinase

Rabbit pyruvate kinase was treated with iodo[2-^{14}C]acetic acid, CNBr and citraconic anhydride as described in the Methods prior to application to a column of Sephadex G-75 equilibrated with 0.5 % (w/v) ammonium bicarbonate. The column was developed with this buffer and the eluate was monitored by measuring the absorbance at 225 nm (●·····●) and by scintillation counting of 0.1 ml aliquots of 3.5 ml fractions (●——●).

FIGURE 4B

Sephadex G-75 Chromatography of the Cyanogen Bromide Peptides Derived from ^{14}C-Carboxymethylated Eel Pyruvate Kinase

The cyanogen bromide peptides derived from ^{14}C-carboxymethylated eel pyruvate kinase were fractionated on a column of Sephadex G-75 as described above. The absorbance at 225 nm (●·····●) and the radioactivity in 0.1 ml aliquots of fractions (●——●) was monitored.

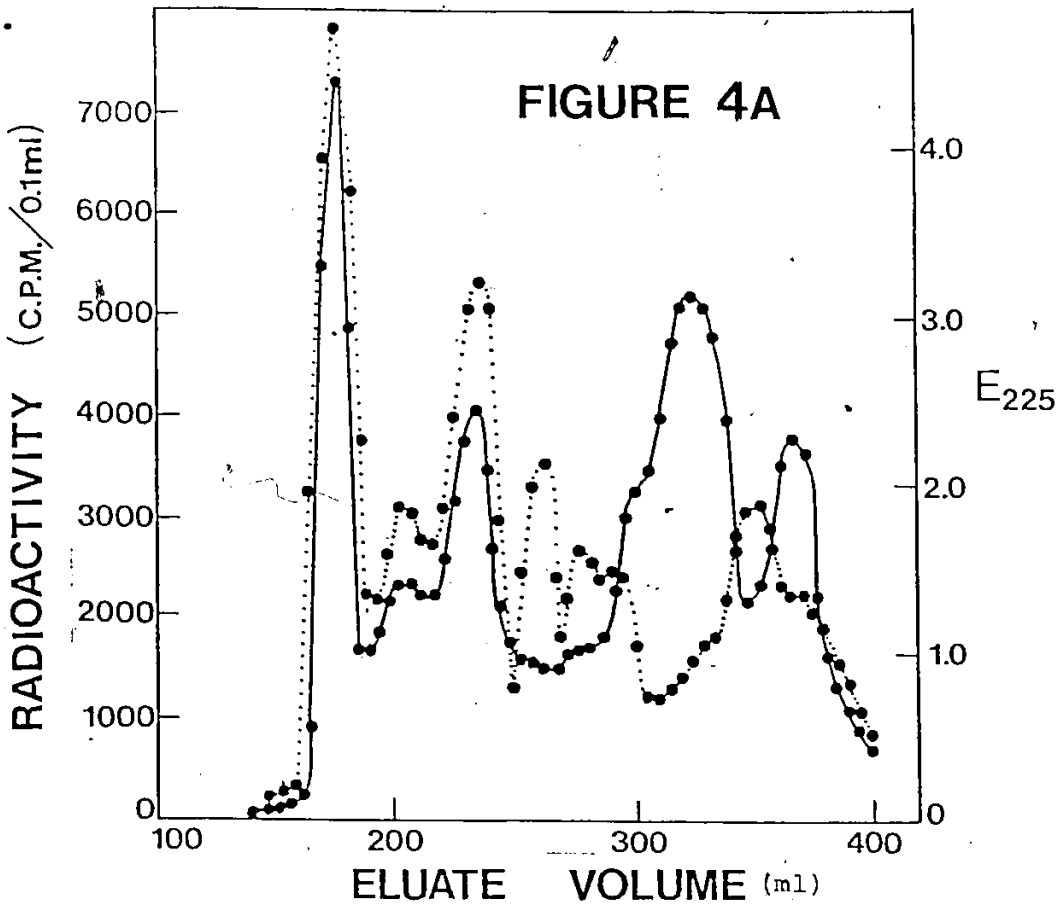


FIGURE 4A

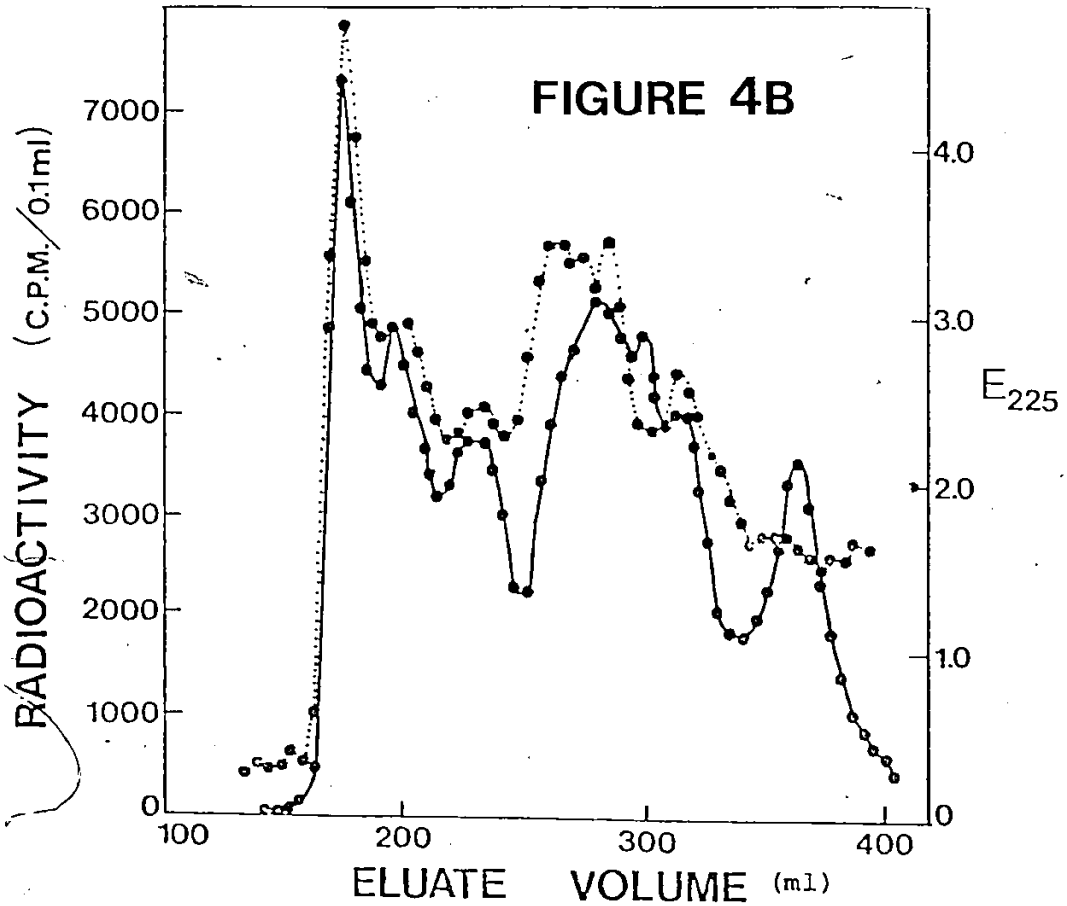


FIGURE 4B

TABLE 2

Amino Acid Compositions of the Radioactive, Low Molecular Weight Cyanogen Bromide Peptides of ^{14}C-Carboxymethylated Eel and Rabbit Pyruvate Kinases

Amino Acid	Mole/Mole of Peptide (Nearest Integer)	
	EX1	RX1
Carboxymethylcysteine	2	2
Asparagine	1	1
Threonine	3	1
Glutamine	1	1
Proline	1	1
Glycine	2	2
Alanine	1	2
Valine	-	1
Isoleucine	2	3
Lysine	1	1
Arginine	2	2
Homoserine/Homoserine Lactone	1	1
Total Number of Residues	17	18

at pH 6.5 derived from rabbit muscle pyruvate kinase. The strip of neutral peptides was not stained on this map and instead these peptides were mapped separately. Figure 5B represents the corresponding peptide map for the eel enzyme. A comparison of these maps indicated that although there are many differences between the 2 muscle isozymes, they appear to share several common peptides with similar electrophoretic and chromatographic mobilities. Twelve of the 26 acidic and basic peptides derived from the rabbit enzyme (Fig. 5A) also appear to be present in the eel enzyme (Fig. 5B).

Similarly Figure 6A represents a map of the neutral peptides derived from rabbit muscle pyruvate kinase while Figure 6B represents the corresponding peptide map for the eel enzyme. Nine of the 15 neutral peptides derived from the eel enzyme also appear to be present in the rabbit enzyme. A total of 39 eel pyruvate kinase peptides and 41 rabbit pyruvate kinase peptides were resolved and detected on these maps. In addition, a number of faintly stained ninhydrin-positive spots could be detected. Since both eel and rabbit pyruvate kinases contain relatively high contents of lysine and arginine residues, one might expect that as many as 70 peptides might be resolved on these maps. This apparent discrepancy can be attributed to the fact that some insoluble material was present in tryptic digests of cyanogen bromide peptides. This insoluble material was removed by centrifugation prior to the separation of peptides by high voltage paper electrophoresis. The insoluble material accounted for less than 5% of the total radioactivity of the tryptic digest.

FIGURE 5A

Peptide Map of ^{14}C-Carboxymethylated
Rabbit Muscle Pyruvate Kinase

and

FIGURE 5B

Peptide Map of ^{14}C-Carboxymethylated
Eel Muscle Pyruvate Kinase

Pyruvate kinases were treated with iodo[2-^{14}C]acetic acid, CNBr and trypsin as described in the Methods. The resulting peptides were separated on paper by electrophoresis at pH 6.5 followed by descending chromatography (BAWP) at 90^{o} to the electrophoretic separation. Peptides were detected on paper by staining with ninhydrin. Peptides which appear to be present in the maps of both enzymes are circled with a solid line. Peptides which are unique to each muscle isozyme are circled with a broken line. Radioactive peptides which were detected by autoradiography are shaded.

FIGURE 5A

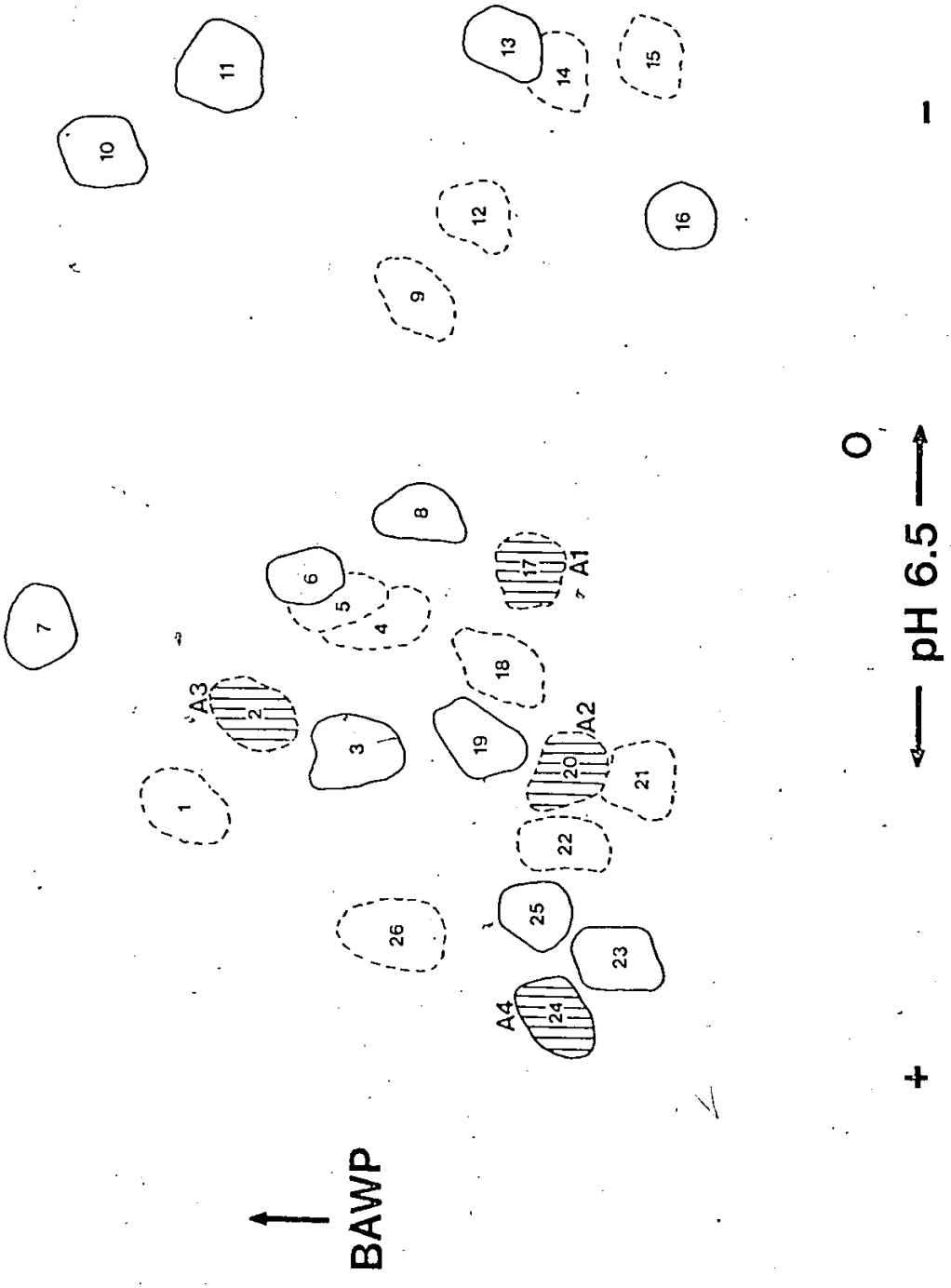

BAWP
pH 6.5
O
+
-
A1
A2
A3
A4
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26

FIGURE 5B

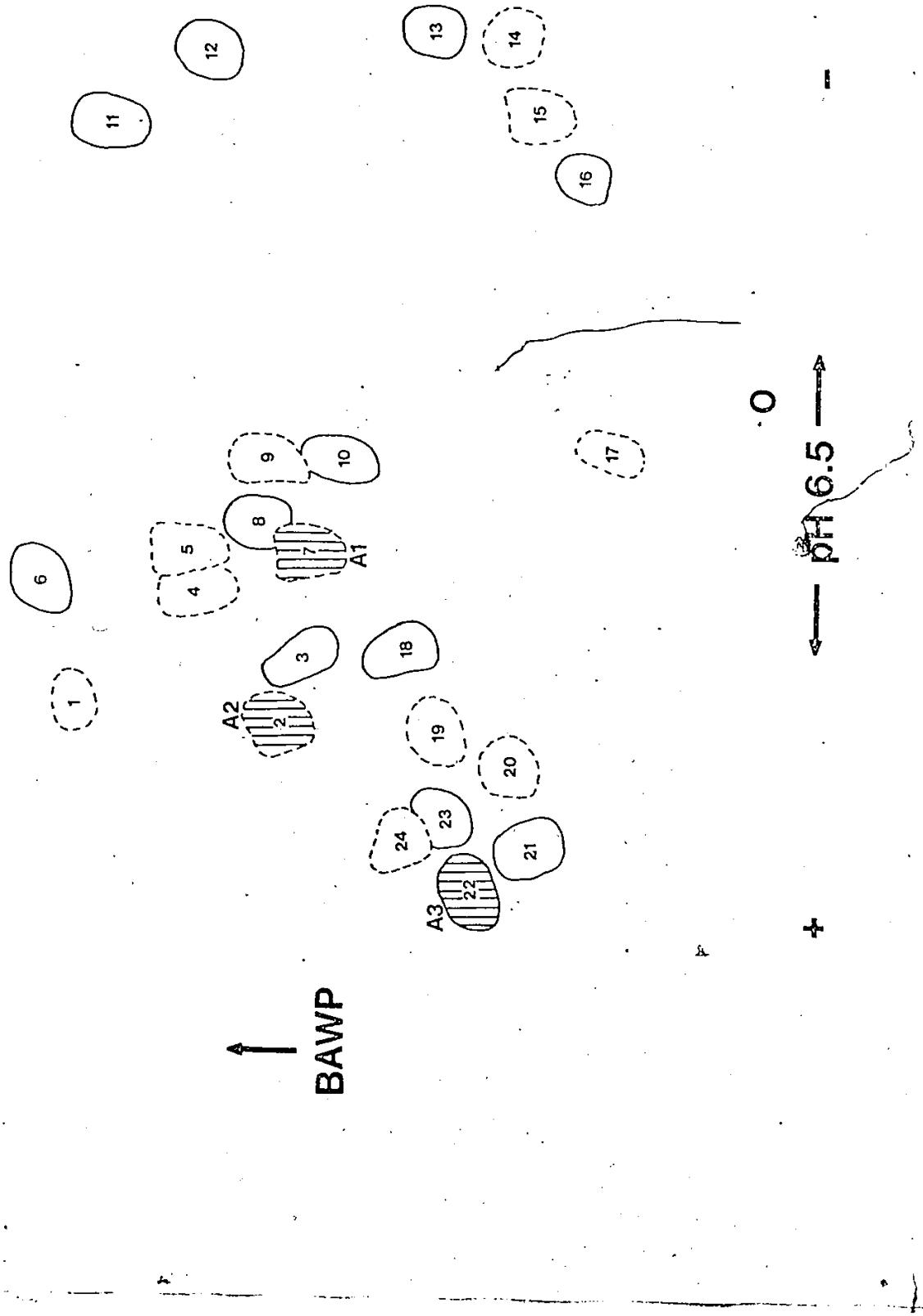

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
A1
A2
A3
BAWP
pH 6.5
0
+
-

FIGURE 6A

Map of the Neutral Peptides of ^{14}C-Carboxymethylated Rabbit Muscle Pyruvate Kinase

and

FIGURE 6B

Map of the Neutral Peptides of ^{14}C-Carboxymethylated Eel Muscle Pyruvate Kinase

The neutral peptides contained in the maps shown in Figures 5A and 5B were mapped separately on paper by electrophoresis at pH 2.1. Peptides were detected on paper by staining with ninhydrin. Peptides which appear to be present in the maps of both enzymes are circled with a solid line. Peptides which are unique to each muscle isozyme are circled with a broken line. Radioactive peptides which were detected by autoradiography are shaded.

FIGURE 6A

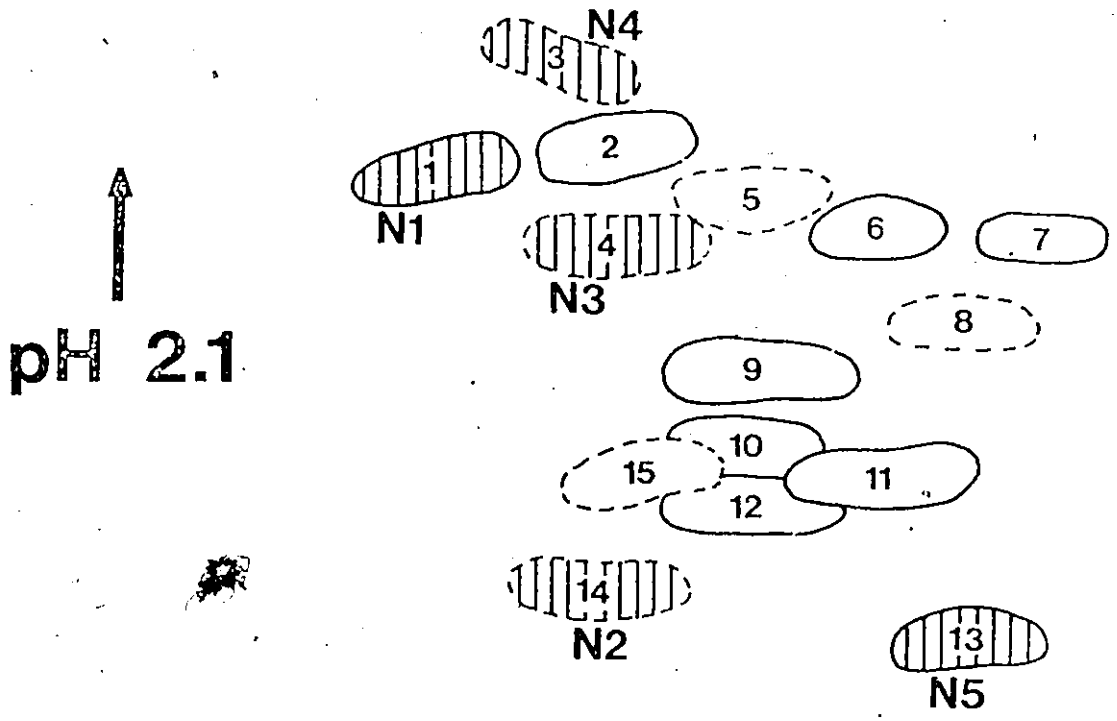
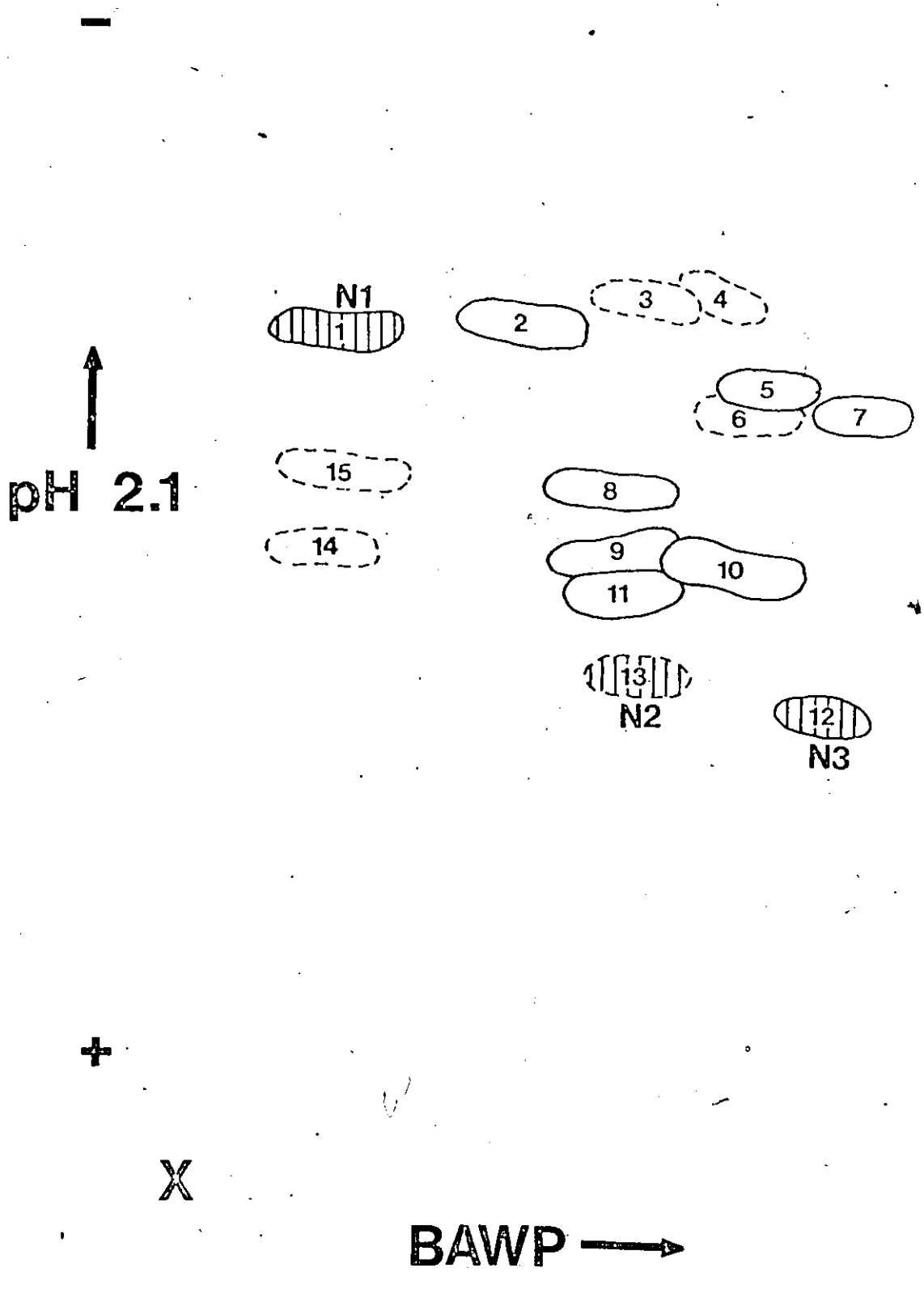


FIGURE 6B



Autoradiography revealed that 4 of the acidic peptides (Fig. 5A) and 5 of the neutral peptides (Fig. 6A) derived from ^{14}C-carboxymethylated rabbit muscle pyruvate kinase were radioactively labelled. Similarly 3 of the acidic peptides (Fig. 5B) and 3 of the neutral peptides (Fig. 6B) derived from ^{14}C-carboxymethylated eel muscle pyruvate kinase were radioactively labelled. These radioactively labelled peptides were purified by high voltage paper electrophoresis and paper chromatography and their amino acid compositions appear in Tables 3 and 4. All of these radioactively labelled peptides were found to contain ^{14}C-carboxymethylcysteine. The asparagine and glutamine contents of the neutral peptides were not determined directly but are inferred from the fact that these peptides exhibited the same electrophoretic mobility as ϵ-DNP-lysine at pH 6.5.

Four of the 6 carboxymethylcysteine-containing peptides derived from eel pyruvate kinase are homologous to 4 of the 9 carboxymethylcysteine-containing peptides derived from rabbit muscle pyruvate kinase. Peptides EN1 and RN1 exhibit the same amino acid compositions and peptide map locations. In addition, carboxymethylcysteine was determined to be the N-terminal residue of both these peptides by Edman degradation. Therefore, the peptides EN1 and RN1 have the sequence Cmc-Asn-Arg since they were obtained by tryptic digestion and arginine is most probably the C-terminal amino acid. Peptides EN3 and RN5 also exhibit the same amino acid compositions and peptide map locations. These peptides contain N-terminal asparagine residues and a threonine residue was released from both of these peptides by a second round of Edman degradations.

TABLE 3

Amino Acid Compositions of the ^{14}C-Carboxymethylcysteine-Containing Peptides of Rabbit Pyruvate Kinase

Amino Acid	Mole/Mole of Peptide (Nearest Integer)								
	~RN1	~RN2	RN3	RN4	~RN5	RA1	RA2	RA3	~RA4
Cmc	1	1	1	1	1	1	1	1	1
Asx						3	2	2	4
Asp	-	-	-	-	-				
Asn	1	-	1	-	1				
Thr	-	1	-	-	2	1	-	-	-
Ser	-	-	-	-	1	1	-	1	1
Glx						1	1	2	1
Glu	-	-	-	-	-				
Gln	-	1	-	-	-				
Pro	-	1	-	-	1	2	-	1	-
Gly	-	1	-	-	2	1	-	1	3
Ala	-	2	-	-	1	3	-	1	4
Val	-	1	-	-	-	1	-	1	2
Ile	-	1	1	-	2	2	1	1	1
Leu	-	-	-	-	-	2	1	1	1
Tyr	-	-	-	-	-	1	1	-	-
Phe	-	-	-	-	-	1	-	1	-
His	-	-	-	-	-	-	-	-	-
Lys	-	1	1	-	-	1	1	1	-
Arg	1	-	-	1	1	1	-	1	-
Hser/Hserl	-	1	-	-	-	-	-	-	1
Total Number of Residues	3	11	4	2	12	22	8	15	19

~ Homologous peptide present in eel muscle pyruvate kinase.

TABLE 4

Amino Acid Compositions of the ^{14}C-Carboxymethylcysteine-Containing Peptides of Eel Pyruvate Kinase

Amino Acid	Mole/Mole of Peptide (Nearest Integer)					
	~EN1	~EN2	~EN3	EA1	EA2	~EA3
Cmc	1	1	1	1	1	1
Asx				2	2	4
Asp	-	-	-			
Asn	1	-	1			
Thr	-	2	2	2	1	-
Ser	-	-	1	2	1	1
Glx				3	1	1
Glu	-	-	-			
Gln	-	1	-			
Pro	-	1	1	1	1	-
Gly	-	1	2	2	-	3
Ala	-	1	1	2	1	4
Val	-	-	-	2	1	2
Ile	-	1	2	2	1	1
Leu	-	-	-	3	2	1
Tyr	-	-	-	1	-	-
Phe	-	-	-	1	-	-
His	-	-	-	-	-	-
Lys	-	1	-	1	-	-
Arg	1	-	1	1	1	-
Hser/Hserl	-	1	-	-	-	1
Total Number of Residues	3	10	12	26	13	19

~ Homologous peptide present in rabbit muscle pyruvate kinase.

From the amino acid compositions of cyanogen bromide peptides EX1 and RX1, it seemed likely that 2 carboxymethylcysteine-containing peptides could be generated from each of these peptides by tryptic digestion. Peptides EN1 and EN2 were obtained from the tryptic digestion of cyanogen bromide peptide EX1 while peptides RN1 and RN2 were obtained from peptide RX1.

Although peptides EN2 and RN2 exhibit similar but not identical amino acid compositions and peptide map locations, they are also homologous. The N-terminal amino acid of peptide EN2 was determined to be threonine while the N-terminal residue of peptide RN2 was alanine. Apart from this difference, the amino acid compositions of these peptides are the same except for the presence of an extra valine residue in the rabbit peptide RN2. A glycine residue was released from both these peptides by a second round of Edman degradations.

Peptides EA3 and RA4 also exhibit the same amino acid compositions and peptide map locations. Further evidence of their identity came from sequencing studies which indicated that alanine and either a glutamine or a glutamic acid residue were the first and second amino acids released respectively from both peptides EA3 and RA4 by two rounds of Edman degradations.

Peptides EA1 and EA2 derived from eel pyruvate kinase did not correspond to any of the carboxymethylcysteine-containing peptides derived from rabbit pyruvate kinase.

DISCUSSION

Eel and rabbit muscle pyruvate kinases both have a molecular weight of 229,000 ± 16,000 and a subunit molecular weight of 59,000 ± 700. Both isozymes appear to consist of only one type of subunit as only one band was obtained for each enzyme on SDS gels and also on 8 M urea gels run under both cationic and anionic conditions (Chapter 3). This would suggest that eel pyruvate kinase, like the M_1 isozyme from rabbit muscle, is a homotetrameric enzyme consisting of 4 identical subunits. The native enzyme and subunit molecular weights determined here for the rabbit enzyme are in agreement with the values reported previously (Cottam et al., 1969). Therefore, with respect to the size and organization of its subunits, eel muscle pyruvate kinase is very similar to the M_1 and M_2 isozymes purified from a variety of mammalian species (Saheki et al., 1982b).

Proline was determined to be the only N-terminal amino acid of eel pyruvate kinase. This finding supports the observation that the eel enzyme is composed of only one type of subunit. Proline is also the N-terminal amino acid of the M_1 and M_2 type pyruvate kinases of the mouse and rat (Saheki et al., 1982b). Under identical conditions, the N-terminal amino acid of the rabbit enzyme could not be determined. This is in agreement with the previous finding that the N-terminus of the rabbit enzyme is blocked (Brummel et al., 1976).

In addition to their similar subunit sizes, eel and rabbit muscle pyruvate kinases share similar subunit amino acid compositions. The amino acid composition reported here for the rabbit enzyme is very similar to the compositions reported previously (Cottam et

al., 1969; Anderson & Randall, 1975; Saheki et al., 1982b). The total number of asparagine and glutamine residues determined here per mole of rabbit pyruvate kinase subunit is quite close to the value reported previously for the number of moles of ammonia released per mole of rabbit pyruvate kinase subunit upon hydrolysis (Cottam et al., 1969). Although the amino acid composition of eel pyruvate kinase is similar to that of the M_1 isozyme of the rabbit, it more closely resembles the composition of the muscle isozyme of the sturgeon (Anderson & Randall, 1975). Both eel and sturgeon muscle pyruvate kinases contain relatively higher threonine and lower alanine contents than the rabbit isozyme, although the differences are not as pronounced in the sturgeon enzyme. Eel muscle pyruvate kinase might be expected to bear more of a resemblance structurally to the muscle isozyme of an amphibian than to that of a mammal. However, the amino acid compositions of eel and frog muscle pyruvate kinases (Saheki et al., 1982b) do not appear to be any more similar than the compositions of eel and rabbit muscle pyruvate kinases.

In spite of their similar amino acid compositions, eel and rabbit pyruvate kinases could be separated electrophoretically on 8M urea gels. At the anionic conditions employed, both denatured eel and rabbit muscle pyruvate kinases would be expected to carry a weak negative charge on the basis of their amino acid compositions. Slight differences in the number of acidic and basic residues in these 2 proteins could contribute to a significant difference in their electrophoretic mobilities. This may account for the fact that the mobility of the eel enzyme was twice that of the rabbit enzyme on 8 M urea gels run under anionic conditions.

Sturgeon muscle pyruvate kinase also exhibits an electrophoretic mobility which is twice that of the rabbit muscle isozyme on 8 M urea gels (Anderson & Randall, 1975). Hence, the amino acid composition similarities of eel and sturgeon muscle pyruvate kinases give rise to similar charge properties of the denatured polypeptide chains.

Eel and rabbit muscle pyruvate kinases also exhibit similar subunit structures. The elution profiles from a Sephadex G-75 column of the cyanogen bromide peptides derived from the two muscle isozymes were very similar (Fig. 4). Similar elution profiles have been reported for the cyanogen bromide peptides derived from the muscle pyruvate kinases of the sturgeon (Anderson & Randall, 1975) and the cat (Harkins & Fothergill, 1977). The smallest cyanogen bromide peptide derived from eel pyruvate kinase is structurally homologous to the corresponding peptide derived from the rabbit enzyme. Tryptic digestion of eel peptide EX1 gave rise to the 2 carboxymethylcysteine-containing peptides EN1 and EN2. The compositions of peptides EN1 and EN2 account for all of the amino acid residues of cyanogen bromide peptide EX1 except for one residue each of threonine, isoleucine, glycine, and arginine. Peptide EN2 corresponds to the C-terminal portion of cyanogen bromide peptide EX1 because it contains a homoserine residue. Since the N-terminal amino acid of peptide EN1 is CM-Cys , the tryptic tetrapeptide Thr-(Ile, Gly)-Arg is likely to correspond to the N-terminal portion of peptide EX1. Similarly, tryptic digestion of rabbit cyanogen bromide RX1 gave rise to the 2 carboxymethylcysteine-containing peptides RN1 and RN2. The arrangement of these tryptic peptides within the structure of peptide RX1 has been

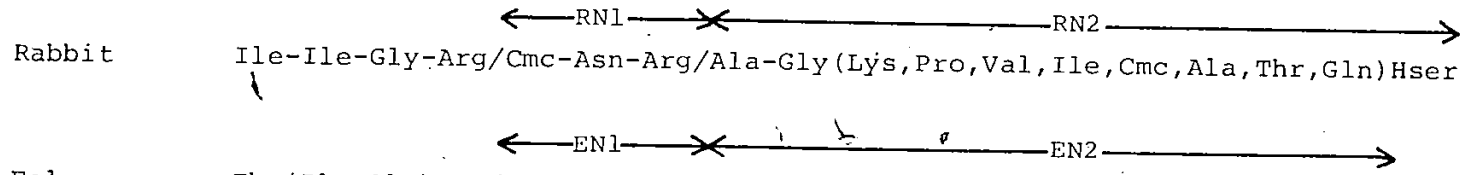
described previously (Anderson & Randall, 1975). Figure 7 summarizes the data obtained in these studies and compares the sequences of cyanogen bromide peptides EX1 and RX1 to the homologous sequences of sturgeon muscle pyruvate kinase (Anderson & Randall, 1975) and yeast pyruvate kinase (Burke et al., 1983). These regions of the polypeptide chains of the four pyruvate kinase isozymes exhibit a high degree of homology although there are some variations in the sequences. However, a comparison of the eel muscle and yeast pyruvate kinase sequences indicates that 4 of the 6 amino acid replacements can be accounted for by a single base change in 4 of the codons of the pyruvate kinase gene. The fact that this particular structural feature has been conserved in these evolutionary diverse species suggests that it has an important function.

Peptide mapping studies indicated that eel and rabbit muscle pyruvate kinases share several polypeptide chain regions that are likely to exhibit a high degree of homology. Of the 41 rabbit pyruvate kinase peptides which were resolved and detected on these maps, 21 also appear to be present in the eel enzyme. Chemical modification studies have indicated that rabbit muscle pyruvate kinase contains at least one catalytically important cysteine residue (Flashner et al., 1972; Chalkley & Bloxham, 1976; Bloxham et al., 1978). A comparative study of the primary structures around the cysteine residues of eel and rabbit muscle pyruvate kinases might be expected therefore to be of value in the correlation of pyruvate kinase structure and function. Four of the 6 carboxymethylcysteine-containing peptides of eel pyruvate kinase were found to be homologous to 4 of the 9 carboxymethylcysteine-containing peptides of rabbit pyruvate kinase. In addition to the homologous carboxymethyl-

FIGURE 7

Comparison of the Sequences of Cyanogen Bromide Peptides EX1 and RX1 Derived from Eel and Rabbit Muscle Pyruvate Kinases Respectively to the Homologous Sequences of Sturgeon Muscle and Yeast Pyruvate Kinases

The sequences of the radioactive, low molecular weight cyanogen bromide peptides EX1 and RX1, derived from ^{14}C-carboxymethylated eel and rabbit muscle pyruvate kinases respectively, are compared to the homologous sequences of sturgeon (Anderson & Ranndall, 1975) and yeast (Burke et al., 1983) pyruvate kinases. The horizontal arrows indicate the positions of the carboxymethylcysteine-containing peptides RN1 and RN2 which were obtained from a tryptic digest of CNBr peptide RX1. Similarly horizontal arrows indicate the positions of peptides EN1 and EN2 within the structure of CNBr peptide EX1. Diagonal lines indicate tryptic cleavage sites. The order of the amino acid residues enclosed in brackets is unknown.


RN1
RN2
Rabbit
Ile-Ile-Gly-Arg/Cmc-Asn-Arg/Ala-Gly(Lys,Pro,Val,Ile,Cmc,Ala,Thr,Gln)Hser
EN1
EN2
Eel
Thr(Ile,Gly)Arg/Cmc-Asn-Arg/Thr-Gly(Lys,Pro,Ile,Cmc,Ala,Thr,Gln)Hser
Sturgeon
Ile-Gly-Arg/Cmc-Asn-Lys/Ala(Gly,Lys,Pro,Val,Ile,Cmc,Ala,Thr,Gln)Hser
Yeast
Leu-Ile-Ala-Lys-Ser-Asn-Leu-Ala-Gly-Lys-Pro-Val-Ile-Cmc-Ala-Thr-Gln-Met

cysteine-containing peptides contained in the cyanogen bromide peptides EX1 and RX1, peptides EN3 and RN3 are homologous and have the sequence Asn-Thr-(Cmc, Thr, Ser, Pro, 2Gly, Ala, 2Ile)-Arg. It is possible that these peptides correspond to a 12 amino acid-long portion of yeast pyruvate kinase located at residues 413 to 424 (Burke et al., 1983). The function of this particular conserved structural feature has yet to be determined.

Peptides EA3 and RA4 have the sequence Ala-Glx-(Gly, Ser, Asx, Val, Ala, Asx, Ala, Val, Leu, Asx, Gly, Ala, Asx, Cmc, Ile, Gly)-Hser. These peptides are homologous to an 18 amino acid-long portion of yeast pyruvate kinase located at residues 313 to 330 (Burke et al., 1983). Peptides EA3 and RA4 are also homologous to the N-terminal portion of a 34 residue tryptic peptide, isolated from bovine muscle pyruvate kinase, which has been reported to contain a catalytically important lysine residue (Johnson et al., 1979). This structural feature may be conserved in these evolutionary diverse organisms in order to maintain the proper orientation of a catalytically important lysine residue at the active site of pyruvate kinase.

In spite of their similar subunit sizes, amino acid compositions and structures, eel pyruvate kinase more closely resembles a mammalian M_2 isozyme than the M_1 isozyme of the rabbit. Cyanogen bromide peptide mapping on SDS and Triton-urea gels indicated that the rabbit muscle enzyme shares the same structural features as the M_1 isozyme of the rat (Saheki et al., 1982a). However, while eel and rabbit muscle pyruvate kinases appear to share some common cyanogen bromide peptides, the peptide banding patterns of eel pyruvate kinase on SDS and Triton-urea gels are very similar to those

reported previously for the M_2 isozyme of the rat (Saheki et al., 1982a).

Eel and rabbit pyruvate kinases exhibit distinctly different isoelectric points. The pI value determined for the rabbit enzyme is in agreement with previous reports (Susor et al., 1969; Ibsen et al., 1976) and is very similar to the value reported for the M_1 isozyme of the ox (Cardenas et al., 1973). In the absence of FDP, the pI values of the 3 isoelectric forms of eel pyruvate kinase were at least 2 pH units lower than the pI value determined for the rabbit enzyme. In the presence of FDP, the most acidic isoelectric form of the eel enzyme predominated and a pI value of 5.90 is in agreement with the value determined previously for a partially purified eel white muscle pyruvate kinase (Moon & Hulbert, 1980a). The fact that rabbit and eel muscle pyruvate kinases exhibit distinctly different isoelectric points in spite of their similar subunit amino acid compositions and structures suggests that these different muscle isozymes exist in substantially different conformations.

Of the mammalian isozymes, the M_1 type exhibits a pI value in the range 7.2-8.8 (Susor et al., 1969; Cardenas et al., 1973; Ibsen et al., 1976; Berglund et al., 1977; Ibsen et al., 1981; Nagao et al., 1982) and this is not altered by the presence of FDP. The L type exists as multiple isoelectric forms in the absence of FDP but in its presence, only one isoelectric form is observed with a pI value in the range 5.1-5.5 (Ibsen et al., 1976; Hall & Cottam, 1978). The M_2 type also exists as multiple isoelectric forms in the absence of FDP but it is the only mammalian isozyme which appears as one isoelectric form in

the presence of FDP with a pI value in the range 5.8-6.2 (Berglund et al., 1977; Ibsen et al., 1981; Schering et al., 1982; Nagao et al., 1982). Therefore with respect to its surface charge properties in the presence and absence of FDP, eel pyruvate kinase most closely resembles the mammalian M_2 type isozyme.

These series of studies have indicated that eel white muscle pyruvate kinase is not only kinetically but also structurally very similar to a mammalian M_2 pyruvate kinase. Part of the difficulty in determining the relationships and roles of the mammalian M_1 and M_2 isozymes is obtaining suitable amounts of the M_2 isozyme. As eel muscle pyruvate kinase can be obtained with relative ease and in good yield, further examination of the structural features of eel and rabbit muscle pyruvate kinases could provide valuable information concerning the structure-function relationships of the mammalian M_1 and M_2 isozymes.

- CHAPTER 5 -

DETERMINATION OF THE NUMBER, LOCATION AND REACTIVITY OF THE SULFHYDRYL GROUPS OF EEL & RABBIT MUSCLE PYRUVATE KINASES

RESULTS

A comparative study of the role of the sulfhydryl groups in eel and rabbit muscle pyruvate kinases might be expected to shed some light on the structural basis for the kinetic differences exhibited by these 2 isozymes. An examination of the number, reactivity and location of the sulfhydryl groups of eel and rabbit muscle pyruvate kinases was therefore undertaken.

Eel muscle pyruvate kinase contains 6.1 cysteine residues per enzyme subunit as determined by reaction with DTNB in the presence of 1.0% (w/v) SDS whereas rabbit pyruvate kinase contains 8.9 cysteine residues per enzyme subunit (Chapter 4). An estimate of the cysteine contents of these muscle isozymes was also made by determining the amount of radioactivity incorporated into pyruvate kinases carboxymethylated with iodo[2-^{14}C]acetic acid in the presence of 8 M urea and 2.0 mM DTT. Eel pyruvate kinase was determined to contain 6.3 cysteine residues per subunit by this method while the rabbit enzyme contained 9.2 cysteine residues per subunit.

In the presence of 30% (v/v) glycerol, 5 of the 6 cysteine residues per subunit of eel pyruvate kinase reacted with DTNB in the native enzyme. In the absence of glycerol, the number of reactive cysteine residues in the native enzyme could not be determined due to the instability of the enzyme. In the presence of 30% (v/v) glycerol, only one of the 9 cysteine residues per subunit of rabbit

TABLE 1

Inactivation of Eel Muscle Pyruvate Kinase due to Chemical Modification by Three Different Sulfhydryl Reagents

Name of Inhibitor	Concentration of Inhibitor (mM)	Inhibitor to Enzyme Subunit Mole Ratio	Inactivation Rate (min^{-1})
Iodoacetic acid	0.915	81:1	-0.0023
Iodoacetamide	0.915	81:1	-0.0390
DTNB	0.0226	2:1	-0.0687

One hundred µl of a 1.0 mg/ml solution of eel pyruvate kinase, prepared in 100 mM Tris-HCl buffer, pH 8.0 containing 30% (v/v) glycerol, was incubated with 40 µl of 100 mM Tris-HCl buffer, pH 8.0 for 5 min at 30°C. Chemical modification reactions were initiated by the addition of 10 µl of a sulfhydryl reagent solution prepared in 100 mM Tris-HCl buffer, pH 8.0 and samples were allowed to react in the dark at 30°C. At specific time points, reaction sample aliquots were removed, diluted and assayed for residual activity at pH 6.3, 30°C as described in the Methods.

muscle pyruvate kinase reacted with DTNB and this was followed by a very slow titration reaction. However, in the absence of glycerol, all of the 9 cysteine residues per subunit of rabbit pyruvate kinase reacted with DTNB. For subsequent studies, glycerol was added to all enzyme solutions to ensure that identical reaction conditions were employed for both isozymes.

The effects of several sulfhydryl reagents on the catalytic activity of eel pyruvate kinase were examined. The inactivation rates determined for three different inhibitors appear in Table 1. Although DTNB was a very powerful inhibitor of eel pyruvate kinase activity, it was not employed in subsequent experiments because the modification of enzyme sulfhydryl groups by this reagent is readily reversible and it is subject to disulfide exchange reactions (Flashner et al., 1972). Iodoacetamide was employed for the remaining experiments because eel pyruvate kinase could be completely inhibited by this reagent in an irreversible manner at comparatively moderate inhibitor to enzyme subunit mole ratios.

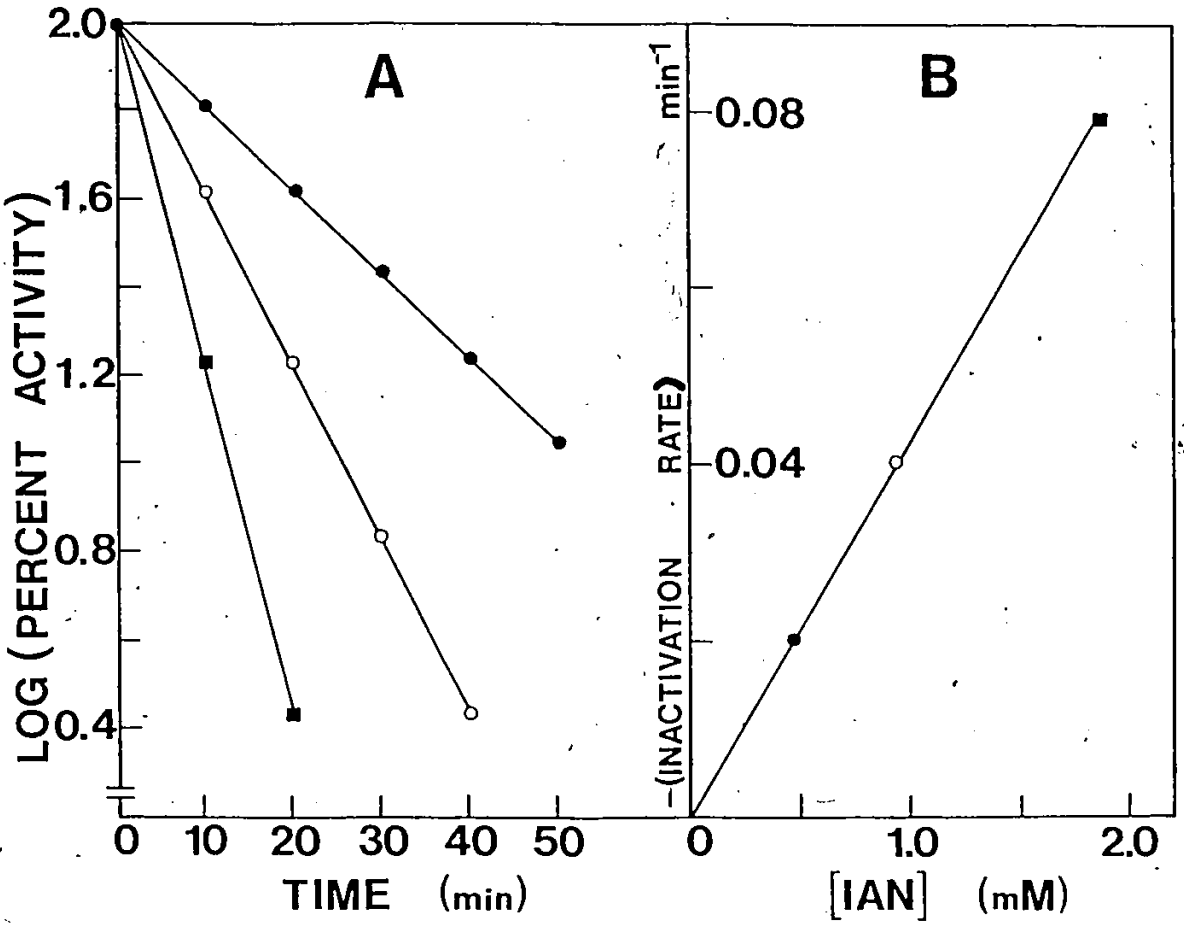
Fig. 1A shows the loss of eel pyruvate kinase activity as a function of time for 3 different concentrations of iodoacetamide. In each case, linear inactivation curves were obtained from which apparent first-order rates of inactivation were calculated. Fig. 1B indicates that the apparent first-order inactivation rate was directly proportional to the concentration of iodoacetamide used. From the slope of Fig. 1B, an apparent first-order inactivation rate constant of $-0.0429\ min^{-1}$ was calculated for the eel enzyme. Under identical conditions, rabbit muscle pyruvate kinase was also inactivated by iodoacetamide however the rabbit enzyme was much less sensitive to this sulfhydryl reagent (Fig. 2A). The

FIGURES 1A and 1B

Effect of Incubation of Eel
Pyruvate Kinase with Iodoacetamide

Panel A: One hundred µl of a 1.0 mg/ml solution of eel pyruvate kinase, prepared in 100 mM Tris-HCl buffer, pH 8.0 containing 30% (v/v) glycerol, was incubated with 40 µl of 100 mM Tris-HCl buffer, pH 8.0 for 5 min at 30°C. Chemical modification reactions were initiated by the addition of iodoacetamide to a final concentration of either 0.466 mM (●——●), 0.933 mM (○——○) or 1.866 mM (■——■). Samples were allowed to react in the dark at 30°C and at specific time points, reaction sample aliquots were removed and assayed for residual activity at pH 6.3, 30°C as described in the Methods.

Panel B: Apparent first-order rates of inactivation were calculated for the linear inactivation curves shown in Panel A and plotted as a function of the final concentration of iodoacetamide in the reaction sample.

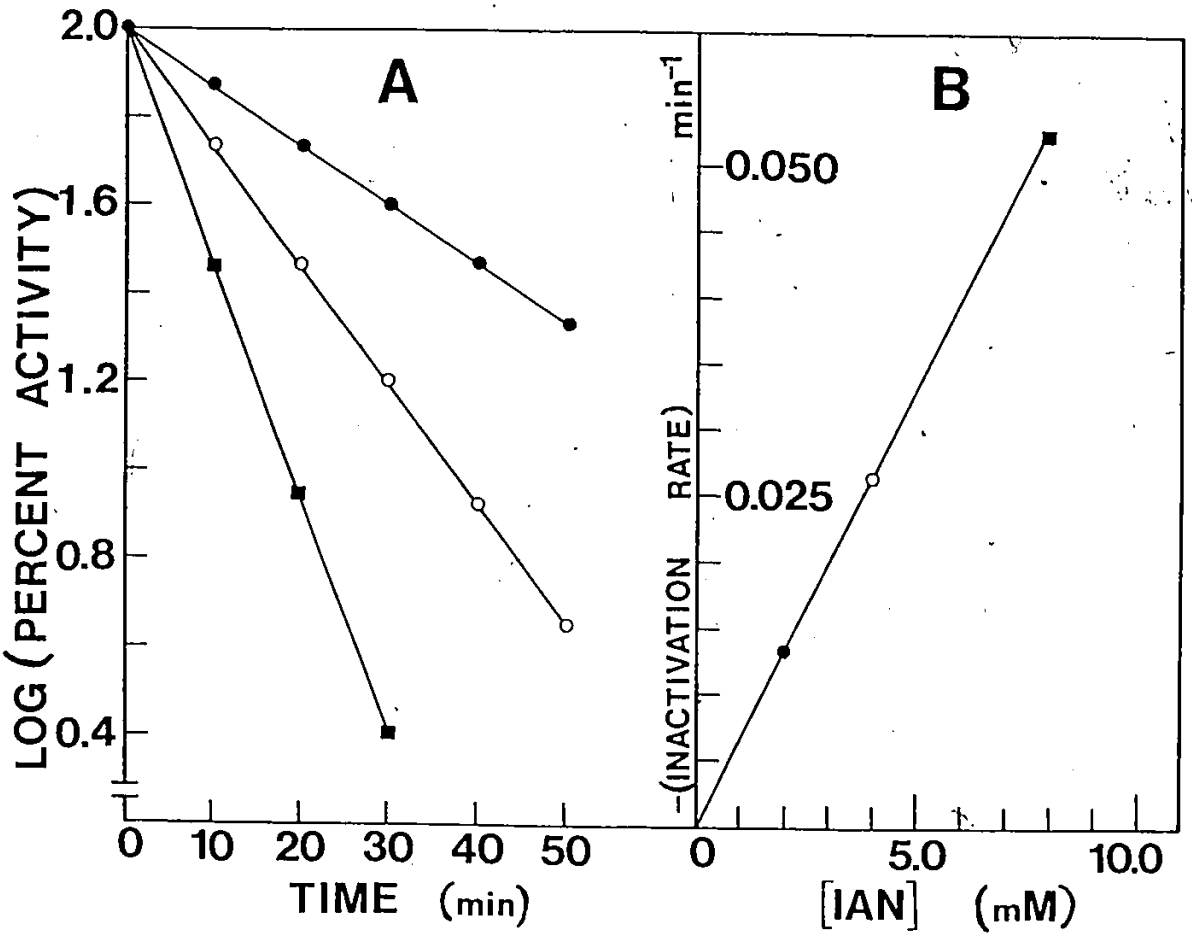

A
B
2.0
1.6
1.2
0.8
0.4
LOG (PERCENT ACTIVITY)
0
10
20
30
40
50
TIME (min)
0.08
0.04
-(INACTIVATION RATE) min-1
0
1.0
2.0
[IAN] (mM)

FIGURES 2A and 2B

Effect of Incubation of Rabbit
Pyruvate Kinase with Iodoacetamide

Panel A: One hundred µl of a 1.0 mg/ml solution of rabbit pyruvate kinase, prepared in 100 mM Tris-HCl buffer, pH 8.0 containing 30% (v/v) glycerol, was incubated with 40 µl of 100 mM Tris-HCl buffer, pH 8.0 for 5 min at 30°C. Chemical modification reactions were initiated by the addition of iodoacetamide to a final concentration of either 2.00 mM (●——●), 4.00 mM (○——○) or 8.00 mM (■——■). Samples were allowed to react in the dark at 30°C and at specific time points, reaction sample aliquots were removed and assayed for residual activity at pH 7.5, 30°C as described in the Methods.

Panel B: Apparent first-order rates of inactivation were calculated for the linear inactivation curves shown in Panel A and plotted as a function of the final concentration of iodoacetamide in the reaction sample.


A
B
LOG (PERCENT ACTIVITY)
2.0
1.6
1.2
0.8
0.4
0
10
20
30
40
50
TIME (min)
-(INACTIVATION RATE) min⁻¹
0.050
0.025
0
5.0
10.0
[IAN] (mM)

apparent first-order rate constant of inactivation for eel pyruvate kinase was 6.4 times greater than the corresponding rate constant of inactivation for the rabbit enzyme (Fig. 2B).

The effects of various substrate and cation combinations on the protection of eel pyruvate kinase against inactivation by iodoacetamide were examined at an inhibitor to enzyme subunit mole ratio of 165:1 (Table 2). Alkylation reactions were initiated by the addition of iodoacetamide and after 20 minutes reaction aliquots were removed, diluted in 50 mM imidazole buffer, pH 6.3 and assayed for residual activity at pH 6.3. Under these conditions, eel pyruvate kinase was almost completely inactivated and the addition of K^+ or Mg^{+2} either alone or in combination provided little protection against inactivation. FDP could partially protect the eel enzyme from inactivation and it could partially re-activate the iodoacetamide-inactivated enzyme. The residual activity exhibited by the iodoacetamide-inactivated enzyme assayed in the presence of 0.5 mM FDP was equivalent to the residual activity of the enzyme partially protected by 0.5 mM FDP against inactivation by iodoacetamide. While ADP provided little protection against inactivation, PEP could partially protect against inactivation. The amount of protection afforded by PEP increased with increasing PEP concentrations. The addition of K^+ to the alkylation reaction sample to a concentration of 44 mM could not improve the protective effect of PEP but in the presence of 8 mM $MgCl_2 \cdot 6H_2O$ and 12 mM PEP, eel pyruvate kinase was completely protected against inactivation.

Similarly rabbit muscle pyruvate kinase was incubated with iodoacetamide for 30 minutes at an inhibitor to enzyme subunit mole ratio of 708:1 and assayed for residual catalytic activity

TABLE 2

Effects of Various Substrate and Cation Combinations on the Protection of Eel Pyruvate Kinase Against Inactivation due to Chemical Modification by Iodoacetamide

Sample	Additions	Percent Residual Activity
Control	None	97.0
1.866 mM IAN	None	2.7
	44 mM KCl	2.0
	8.0 mM $MgCl_2$	6.5
	0.5 mM FDP	30.2
	2.0 mM ADP	4.1
	1.0 mM PEP	45.7
	4.0 mM PEP	64.6
	12.0 mM PEP	70.8
	12.0 mM PEP and 8.0 mM $MgCl_2$	100.0

One hundred µl of a 1.0 mg/ml solution of eel pyruvate kinase, prepared in 100 mM Tris-HCl buffer, pH 8.0 containing 30% (v/v) glycerol, was incubated with 40 µl of 100 mM Tris-HCl buffer, pH 8.0 containing various combinations of cations and substrates at 30°C for 5 min. Reactions were initiated by the addition of iodoacetamide to a final concentration of 1.866 mM. Reactions were conducted in the dark at 30°C and after 20 minutes, reaction aliquots were removed and assayed for residual activity at pH 6.3, 30°C as described in the Methods.

at pH 7.5. Under these conditions, rabbit pyruvate kinase was almost completely inactivated and the cations K^+ and Mg^{+2}, either alone or in combination and the substrate ADP could provide no protection against enzyme inactivation. Although PEP could partially protect the rabbit enzyme from inactivation, only the combination of 12 mM PEP and 8 mM Mg^{+2} could provide complete protection. Rabbit pyruvate kinase, unlike the eel enzyme, was not partially protected by 0.5 mM FDP from inactivation and FDP could not re-activate the iodoacetamide-treated enzyme (data not shown).

The number of reactive enzyme functional groups was determined by measuring the amount of radioactivity incorporated into pyruvate kinases alkylated with iodo[1-^{14}C]acetamide. Eel pyruvate kinase was incubated with radioactive iodoacetamide at an inhibitor to enzyme subunit mole ratio of 82.5:1 for 45 minutes and the amount of radioactivity incorporated into the enzyme was determined. Eel pyruvate kinase was completely inactivated under these conditions and this was accompanied by the incorporation of 0.9 moles of iodo[1-^{14}C]acetamide per mole of eel pyruvate kinase subunit and the loss of the equivalent of 1.1 cysteine residues per enzyme subunit which could react with DTNB under denaturing conditions. Similarly when rabbit muscle pyruvate kinase was incubated with iodo[1-^{14}C]acetamide at an inhibitor to enzyme subunit ratio of 354:1 for 75 minutes, the equivalent of 2.2 moles of iodo[1-^{14}C]acetamide were incorporated per mole of enzyme subunit. This was accompanied by complete enzyme inactivation and the loss of the equivalent of 2.5 cysteine residues per subunit which could react with DTNB under denaturing conditions. Therefore the alkylation of the equivalent of 0.9 cysteine residues per sub-

unit of eel pyruvate kinase and 2.2 cysteine residues per subunit of rabbit pyruvate kinase can completely account for the observed levels of incorporation of the radioactively labelled sulfhydryl reagent.

The number and location of the reactive sulfhydryl groups of eel pyruvate kinase were investigated. The unreacted sulfhydryl groups remaining after exposure of the native enzyme to iodoacetamide in the presence and absence of Mg^{+2}•PEP were alkylated with iodo[2-^{14}C]acetic acid. Alkylated eel pyruvate kinase was subsequently fragmented by treatment with cyanogen bromide followed by digestion with trypsin. The ^{14}C-carboxymethylcysteine-containing peptides were separated on paper by electrophoresis at pH 6.5 followed by descending chromatography and the autoradiograms of these peptide maps appear in Figure 3. In the control sample, all 6 of the cysteine residues per subunit of eel pyruvate kinase reacted with iodo[2-^{14}C]acetic acid (Fig. 3A). Incubation of the native enzyme with iodoacetamide at an inhibitor to enzyme subunit mole ratio of 82.5:1, in the absence of Mg^{+2}-PEP, resulted in complete enzyme inactivation by 45 minutes and this was accompanied by a reduction in the amount of the cysteine residue, contained in peptide EN2, which was available for alkylation by iodo[2-^{14}C]acetic acid (Fig. 3B). The combination of Mg^{+2}•PEP could completely protect against enzyme inactivation and this was accompanied by an increase, relative to the unprotected enzyme, in the amount of the cysteine residue, contained in peptide EN2, which was available for alkylation by iodo[2-^{14}C]acetic acid (Fig. 3C). Therefore, this experiment suggested that the basis for the protection against inactivation

FIGURE 3

Identification of the Reactive
Sulfhydryl Groups of Eel Pyruvate Kinase

The native eel enzyme was incubated in the presence and absence of Mg^{+2}•PEP with iodoacetamide at an inhibitor to enzyme subunit mole ratio of 82.5:1 for 45 minutes at 30°C in the dark. Unreacted sulfhydryl groups remaining after exposure of the native enzyme to iodoacetamide were alkylated by iodo[2-^{14}C] acetic acid in the presence of 8.0 M urea and 2.0 mM DTT as described in the Methods. ^{14}C-Carboxymethylcysteine-containing peptides were generated by treatment with CNBr followed by trypsin and the radioactive peptides were separated on paper by high voltage electrophoresis and chromatography. The resulting peptide maps were subjected to autoradiography.

Fig. 3A: Control sample, eel pyruvate kinase incubated at 30°C for 45 minutes in the absence of iodoacetamide.

Fig. 3B: Eel pyruvate kinase incubated with iodoacetamide in the absence of Mg^{+2}•PEP.

Fig. 3C: Eel pyruvate kinase incubated with iodoacetamide in the presence of 8.0 mM $MgCl_2$ and 12.0 mM PEP.

Note: There is no significance in the fact that peptide A1 is circled in these autoradiograms.

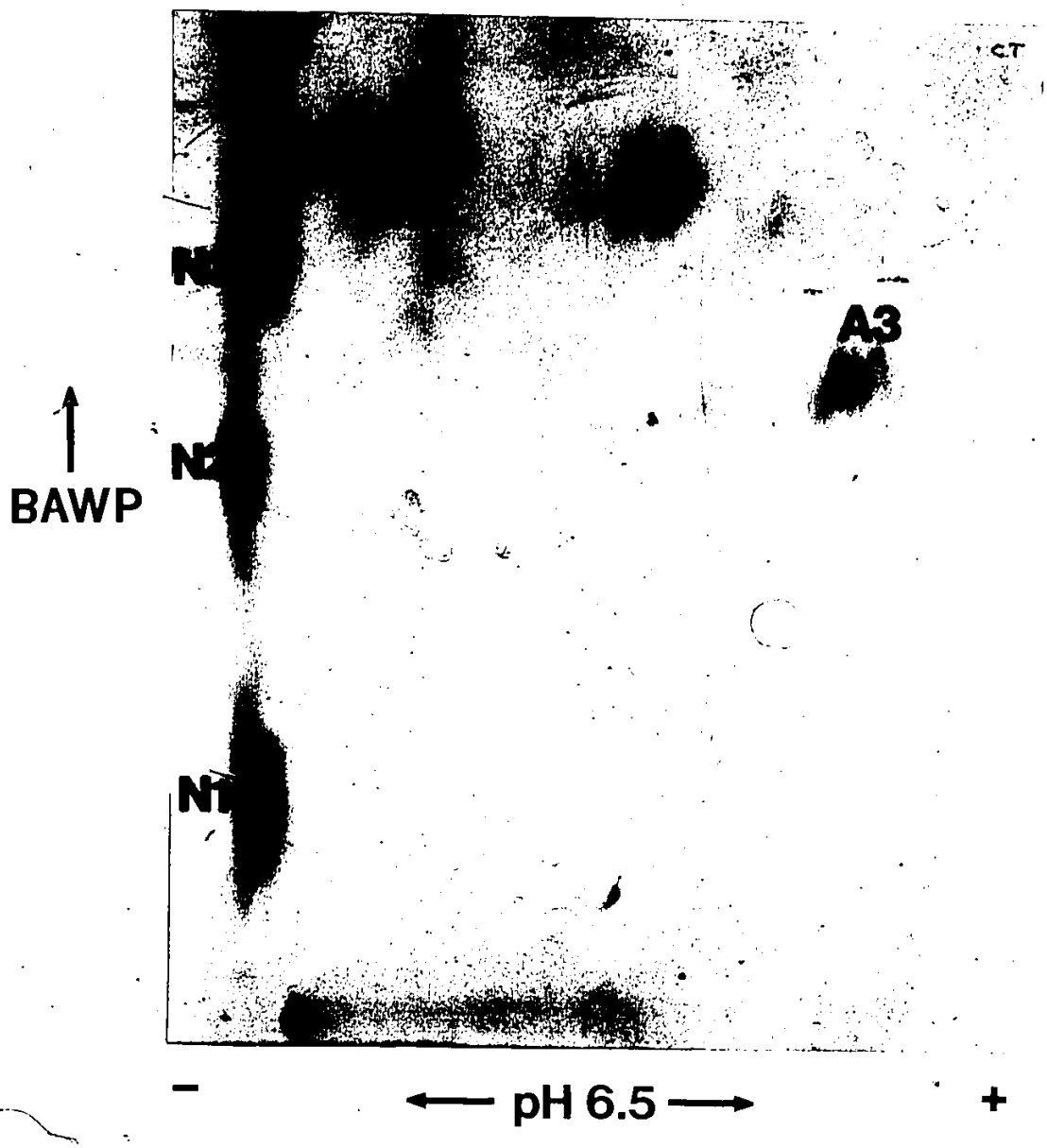


FIGURE 3A

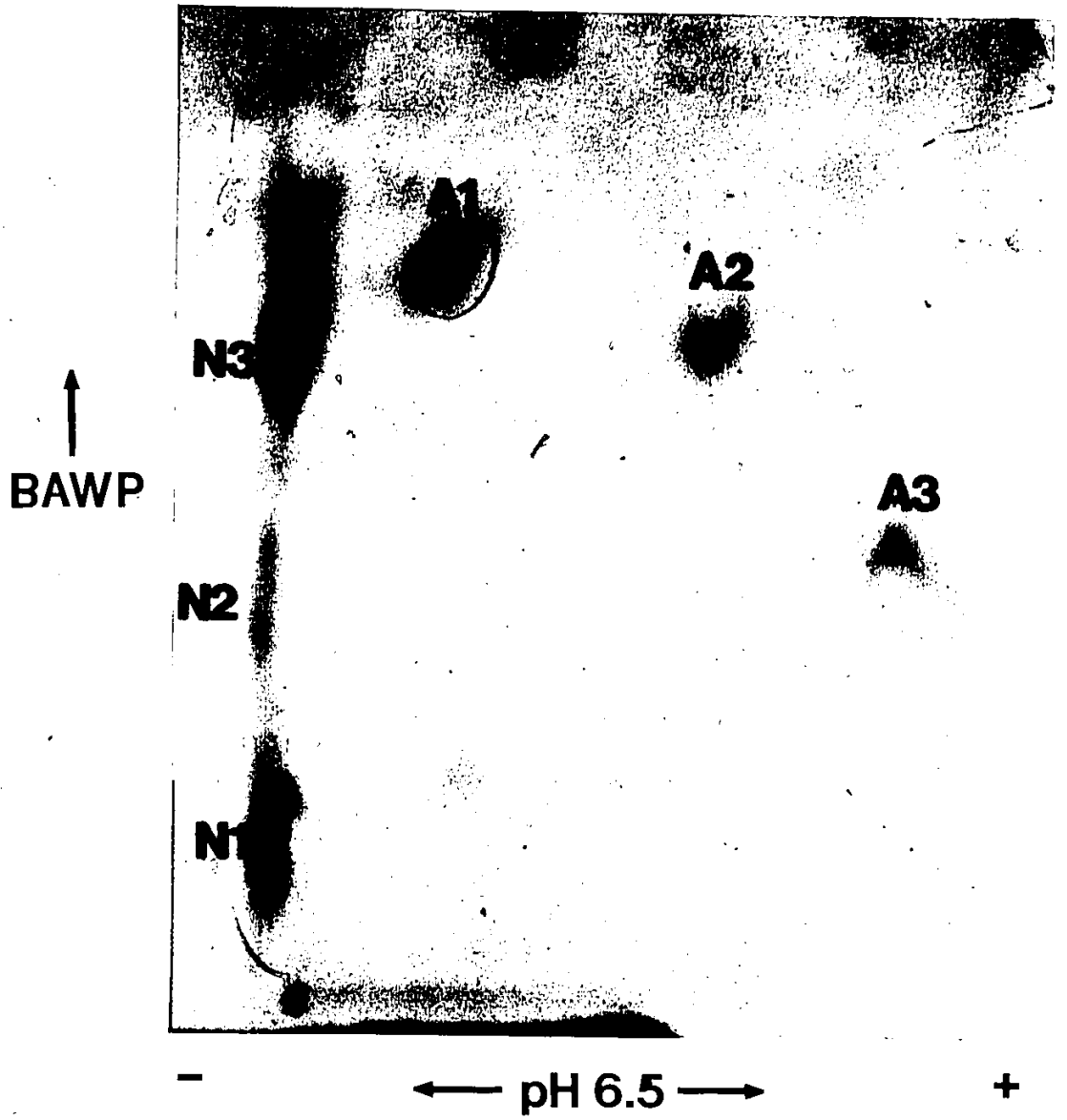


FIGURE 3B

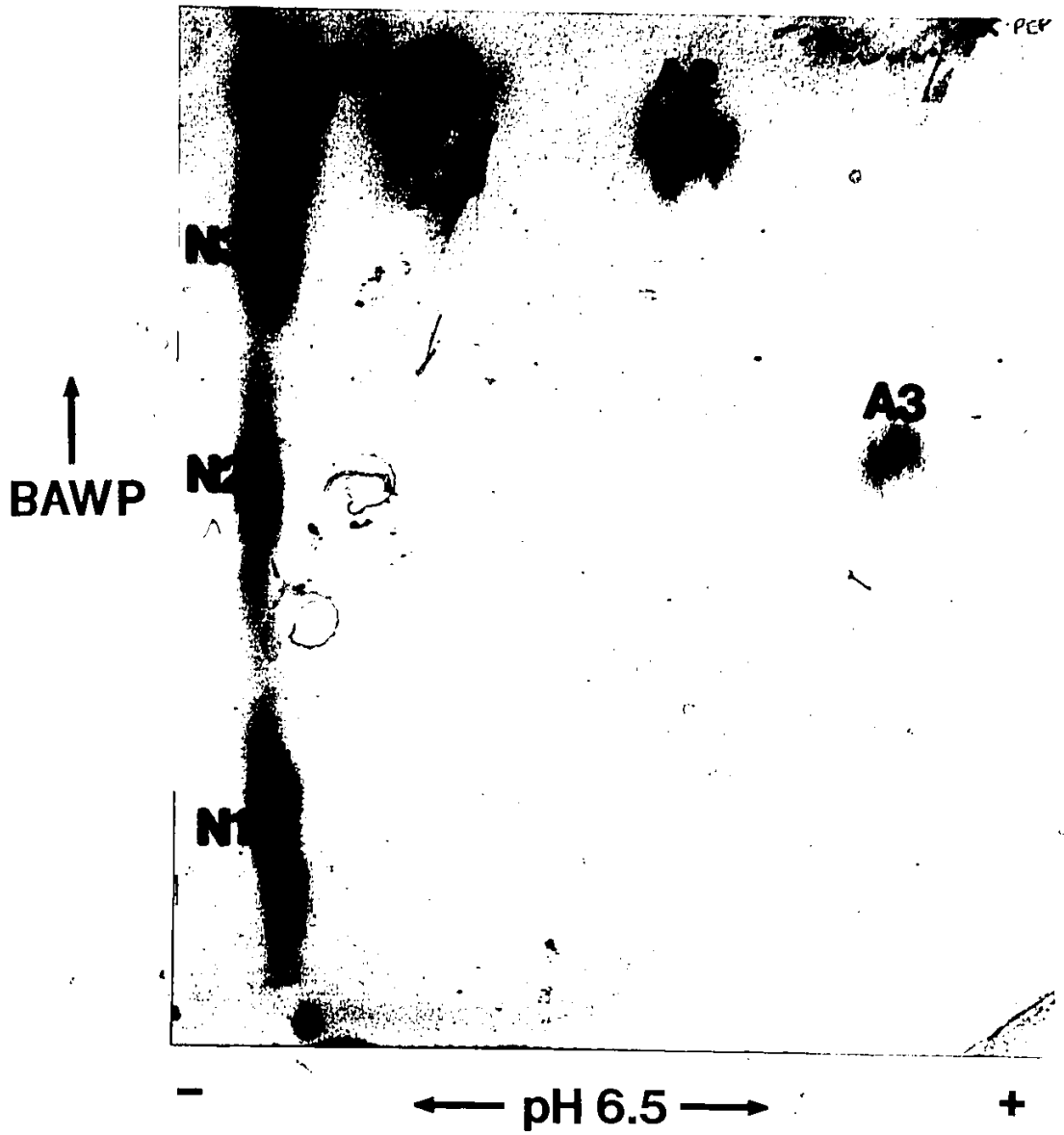


FIGURE 3C

afforded by the combination of Mg^{+2} and PEP was due to the protection of the cysteine residue, contained in peptide EN2, against alkylation by iodoacetamide.

However, it was apparent from the autoradiograms that complete alkylation of this particular cysteine residue of eel pyruvate kinase was not required in order to bring about complete inactivation of the enzyme. Therefore a quantitative measure of the amount of a cysteine residue in the native tetrameric enzyme reacting with iodoacetamide was desired.

The unreacted sulfhydryl groups remaining after exposure of the native enzyme to iodoacetamide in the presence and absence of Mg^{+2}·PEP were alkylated with iodo[2-^{3}H]acetic acid. A known amount of ^{14}C-carboxymethylated pyruvate kinase was added to the tritiated reaction samples to serve as an internal standard. Peptides were generated, mapped on paper and located by autoradiography as described previously. Radioactive peptides were eluted from paper and aliquots were removed for scintillation counting. In this manner the $^{3}H/^{14}C$ ratios of the carboxymethylcysteine-containing peptides of pyruvate kinase could be determined. By comparison of these ratios with those of a standard mixture of ^{3}H-carboxymethylated pyruvate kinase and ^{14}C-carboxymethylated pyruvate kinase, the percentage of a cysteine residue available for carboxymethylation by ^{3}H-iodoacetic acid following treatment of the native enzyme with iodoacetamide could be determined.

Eel pyruvate kinase was incubated with iodoacetamide at an inhibitor to enzyme subunit mole ratio of 82.5:1 and at specific time points, the number of unreacted cysteine residues was determined

TABLE 3

Determination of the Percentage of a Cysteine Residue Available for Carboxymethylation by ^{3}H-IAA Following Treatment of Native Eel Pyruvate Kinase with Iodoacetamide

Sample	Cysteine Residue Location	Percentage Unreacted Cysteine Residue 30 min	60 min	90 min
Unprotected Enzyme	EN1	99.0	100.1	106.2
	EN2	63.5	49.3	42.0
	EN3	114.2	117.9	110.8
	EA1	92.3	83.5	60.1
	EA2	100.0	100.0	100.0
	EA3	117.7	117.3	115.6
Mg^{+2}·PEP Protected Enzyme	EN1	108.6	92.4	99.9
	EN2	111.9	103.0	109.7
	EN3	101.3	100.4	100.3
	EA1	77.1	83.8	76.7
	EA2	100.0	100.0	100.0
	EA3	94.2	98.0	85.5

Eel pyruvate kinase was incubated at pH 8.0, 30°C with iodoacetamide at an inhibitor to enzyme subunit mole ratio of 82.5:1 in the presence and absence of 12 mM PEP and 8 mM $MgCl_2$. At specific time points, reactions were stopped and the percentage of a cysteine residue, contained within a specific peptide, which was available for carboxymethylation by ^{3}H-IAA was determined as described in the Methods. The percentage availability of the cysteine residue, contained in peptide EA3, was determined to be 100±7% at all time points. For the purposes of comparison, the percentage of unreacted cysteine residues in other peptides was expressed relative to that of the cysteine residue contained in peptide EA3 assuming that it was 100%.

(Table 3). By 60 minutes, eel pyruvate kinase was completely inactivated by iodoacetamide and this was accompanied by a 50% reduction in the amount of the cysteine residue, contained in peptide EN2, which was available for alkylation by iodo[2-^{3}H] acetic acid. There was also a 16% reduction in the amount of cysteine residue, contained in peptide EA1, which was available for alkylation by ^{3}H-IAA. The combination of Mg^{+2} and PEP could completely protect against enzyme inactivation and while there was no change in the amount of the cysteine residue, contained in peptide EA1, which was available for alkylation by ^{3}H-IAA, there was a 2 fold increase in the amount of the cysteine residue, contained in peptide EN2, which was available for alkylation by ^{3}H-IAA.

Similarly, rabbit pyruvate kinase was incubated with iodoacetamide at an inhibitor to enzyme subunit mole ratio of 354:1 and the number and location of the reactive cysteine residues was determined (Table 4). By 75 minutes, the rabbit enzyme was completely inactivated and this was accompanied by a 94% reduction in the availability of the cysteine residue contained in peptide RN2 and a 97% reduction in the availability of the cysteine residue contained in peptide RN3. The combination of Mg^{+2} and PEP could completely protect against enzyme inactivation and while there was no change in the availability of the cysteine residue contained in peptide RN3, the cysteine residue in peptide RN2 was completely protected from alkylation by iodoacetamide. Figure 4 indicates that a 1:1 stoichiometry existed between the percentage of the cysteine residue contained in peptide RN2 which was alkylated when the native enzyme was incubated with

TABLE 4

Determination of the Percentage of a Cysteine Residue Available for Carboxymethylation by ^{3}H-IAA Following Treatment of Native Rabbit Pyruvate Kinase with Iodoacetamide

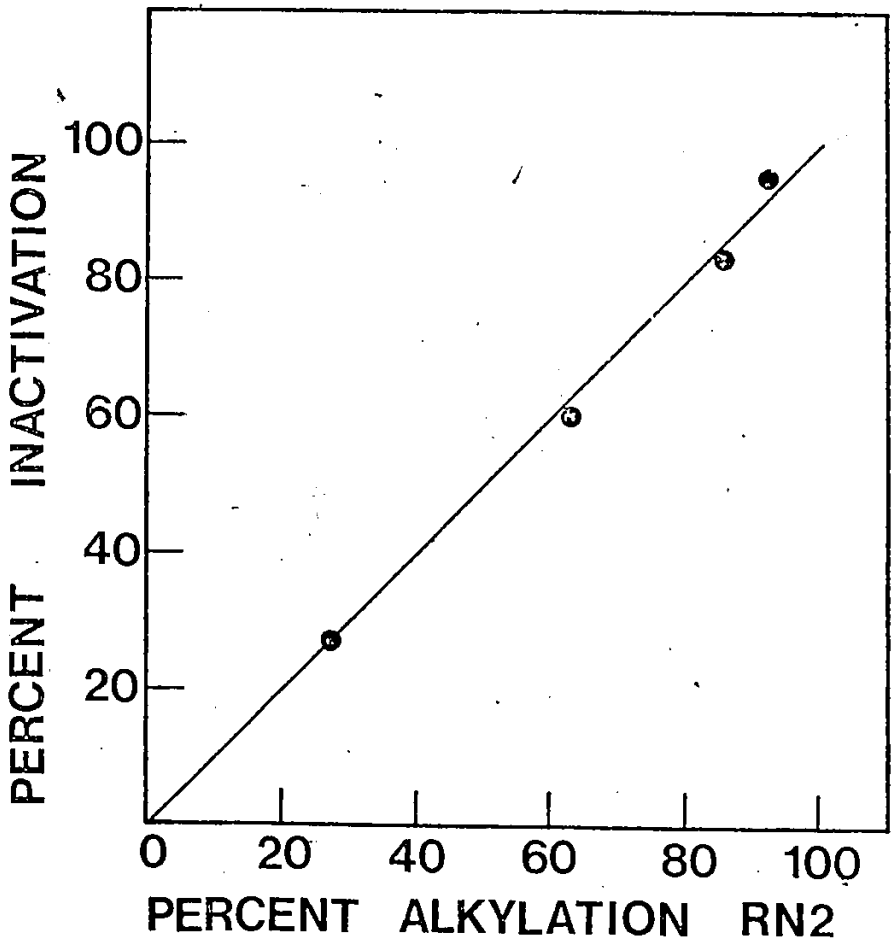
Sample	Cysteine Residue Location	Percentage Unreacted Cysteine Residue 5 min	15 min	30 min	50 min	75 min
Unprotected Enzyme	RN1	105.7	96.4	103.7	104.7	107.4
	RN2	73.6	36.4	15.2	7.8	5.8
	RN3	65.3	35.2	13.5	5.5	2.8
	RN4	106.1	114.2	116.3	107.4	109.2
	RN5	100.0	100.0	100.0	100.0	100.0
	RA1	101.8	95.8	97.6	95.7	98.1
	RA2	96.2	86.8	79.2	91.1	80.0
	RA3	99.3	89.9	92.7	95.6	87.9
	RA4	106.0	103.1	104.9	113.0	111.2
Mg^{+2}•PEP Protected Enzyme	RN1		95.4	100.9	93.3	93.3
	RN2		96.7	101.0	96.0	100.0
	RN3		36.4	15.8	6.6	4.6
	RN4		107.2	117.7	110.1	110.6
	RN5		100.0	100.0	100.0	100.0
	RA1		104.2	101.5	102.0	93.3
	RA2		111.1	102.5	105.3	99.7
	RA3		105.2	103.2	106.5	97.9
	RA4		115.0	113.3	108.2	108.6

Rabbit pyruvate kinase was incubated at pH 8.0, 30°C with iodoacetamide at an inhibitor to enzyme subunit mole ratio of 354:1 in the absence and presence of Mg^{+2}•PEP. At specific time points, the percentage of a cysteine residue which was available for carboxymethylation by ^{3}H-IAA was determined as described in the Methods. The percentages are expressed relative to that of the cysteine residue contained in peptide RN5 since the percentage availability of this residue was determined to be 100±9% throughout.

FIGURE 4

Relationship Between the Percent Inactivation of Rabbit Muscle Pyruvate Kinase due to Chemical Modification by Iodoacetamide and the Percent Alkylation of the Cysteine Residue Contained in Peptide RN2

Rabbit pyruvate kinase was incubated at pH 8.0, 30°C with iodoacetamide at an inhibitor to enzyme subunit mole ratio of 354:1 in the absence of Mg^{+2}•PEP. At specific time points, the percentage of the cysteine residue, contained in peptide RN2, which was available for carboxymethylation by ^{3}H-IAA was determined as described in the Methods. At the same time points, aliquots of the iodoacetamide-treated rabbit pyruvate kinase were assayed for residual catalytic activity at pH 7.5, 30°C as described in the Methods. Here the percent inactivation of the enzyme (equivalent to 100% minus the percent residual activity) is plotted as a function of the percent alkylation of the cysteine residue, contained in peptide RN2, by iodoacetamide.


PERCENT INACTIVATION
100
80
60
40
20
0
20
40
60
80
100
PERCENT ALKYLATION RN2

iodoacetamide and the percentage inactivation of the enzyme.

DISCUSSION

Several chemical modification studies have indicated that rabbit muscle pyruvate kinase contains at least one catalytically important cysteine residue (Flashner et al., 1972; Chalkley & Bloxham, 1976; Bloxham et al., 1978). A study of a related, but phylogenetically distant enzyme could provide more information on the role of the sulfhydryl groups in pyruvate kinase activity. An essential cysteine residue would be expected to be preserved throughout evolution whereas replacement by another amino acid is definite proof of the non-essential nature of a given residue. Eel white muscle pyruvate kinase appeared to be a promising candidate for such a study because amino acid analysis indicated that it contained fewer cysteine residues per subunit than rabbit muscle pyruvate kinase. Therefore an examination of the number, reactivity and location of the sulfhydryl groups of eel and rabbit muscle pyruvate kinases was undertaken.

Eel muscle pyruvate kinase contains 6 cysteine residues per enzyme subunit as determined by DTNB titration and ^{14}C-iodoacetic acid incorporation measurements while rabbit muscle pyruvate kinase contains 9 cysteine residues per enzyme subunit. This is in agreement with the total number of unique carboxymethylcysteine-containing peptides which were purified from the eel and rabbit enzymes. The value reported here for the cysteine content of rabbit muscle pyruvate kinase compares favourably with the values reported previously (Cottam et al., 1969; Flashner et al., 1972; Saheki et al., 1982b).

In the absence of glycerol, all 9 cysteine residues of the native rabbit enzyme reacted with DTNB as has been reported

previously (Flashner et al., 1972). In the presence of glycerol, 5 of the 6 cysteine residues of the native eel enzyme reacted with DTNB but only 1 of the 9 cysteine residues of rabbit muscle pyruvate kinase was reactive. Flashner et al. (1972) reported that a similar dramatic reduction in the number of reactive cysteine residues of the rabbit enzyme could be brought about by the addition of 1.0 mM PEP to the DTNB titration medium.

For the remaining studies, it was necessary to select a suitable sulfhydryl reagent which could irreversibly chemically modify the muscle isozymes at moderate inhibitor to enzyme subunit mole ratios. Iodoacetamide could fulfil these requirements and under identical conditions, eel pyruvate kinase was found to be 6.4 times more sensitive than the rabbit enzyme to inactivation due to chemical modification by this sulfhydryl reagent. Iodoacetamide has been used previously to investigate the role and reactivity of the sulfhydryl groups of rabbit pyruvate kinase. Jacobson & Black (1971) reported that rabbit muscle pyruvate kinase was insensitive to inhibition by iodoacetamide. However, it is impossible to compare their findings to those reported here because the authors did not indicate the inhibitor to enzyme subunit mole ratios they employed. Jursinic & Robinson (1978) reported that rabbit muscle pyruvate kinase was sensitive to inhibition by iodoacetamide but this study makes no mention of the apparent first-order rate constants of inactivation observed for specific inhibitor to enzyme subunit mole ratios. However, the apparent first-order rate constant of inactivation reported here for the chemical modification of rabbit muscle pyruvate kinase by iodoacetamide is quite close to a value reported previously (Flashner et al., 1972).

Double labelling experiments have provided information on the location and reactivity of the sulfhydryl groups of eel and rabbit muscle pyruvate kinases. The following observations support the conclusion that the cysteine residues contained in eel pyruvate kinase peptide EN2 and rabbit pyruvate kinase peptide RN2 are located either near or within the active sites of these isozymes and are functionally important.

1) Eel and rabbit muscle pyruvate kinases were chemically modified by iodoacetamide in a specific manner such that only 2 of the 6 cysteine residues of the eel enzyme and 2 of the 9 cysteine residues of the rabbit enzyme were alkylated. As a result of chemical modification by iodoacetamide both eel and rabbit muscle pyruvate kinases were completely inactivated.

2) Enzyme inactivation can not be attributed to the chemical modification of a functional group other than a cysteine residue since radioactivity incorporation measurements indicated that only cysteine residues were specifically alkylated by iodoacetamide under the conditions employed here.

3) Both eel and rabbit muscle pyruvate kinases exhibited a very high specificity in terms of the types of substrates or cations which could provide protection against inactivation. The substrate PEP in combination with the divalent cation Mg^{+2} could specifically protect both isozymes from inactivation and simultaneously it could protect only 1 of the 2 reactive cysteine residues of both eel and rabbit muscle pyruvate kinases from alkylation by iodoacetamide. This would suggest that these substrate-protected cysteine residues are located either near or within the PEP binding site of the active site of pyruvate kinase.

4) The substrate-protected cysteine residues of eel and rabbit muscle pyruvate kinases are contained in the homologous peptides EN2 and RN2. In the case of the rabbit enzyme, a direct 1:1 stoichiometry could be demonstrated between the percent inactivation of the enzyme and the percentage of the cysteine residue, contained in peptide RN2, which was alkylated in the tetrameric enzyme.

5) Yeast (Burke et al., 1983) and sturgeon muscle (Anderson & Randall, 1975) pyruvate kinases contain regions of their polypeptide chains which are highly homologous to peptides EN2 and RN2. The fact that this particular structural feature has been preserved in these phylogenetically distant isozymes supports the conclusion that the cysteine residue contained in these sequences has an important function.

The complete structure of yeast pyruvate kinase is known (Burke et al., 1983) and extensive crystallographic data has been obtained for the M_1 isozyme of cat muscle (Levine et al., 1978; Stuart et al., 1979). Therefore predictions can be made regarding the locations of specific primary structures within the tertiary structure of pyruvate kinase. A portion of a 34 residue tryptic peptide, isolated from bovine muscle pyruvate kinase (Johnson et al., 1979), has been fitted to the electron density corresponding to helix 3 of domain A of cat muscle pyruvate kinase (Muirhead et al., 1981). This peptide has been reported to contain a catalytically important lysine residue (Johnson et al., 1979) which has been placed at the active site of the enzyme at the connection between the C-terminus of domain B and helix 3 of domain A

(Muirhead et al., 1981). Amino acid residue-337 of yeast pyruvate kinase (Burke et al., 1983) corresponds to this catalytically important lysine residue while amino acid residue-296 of the yeast enzyme corresponds to the cysteine residues of eel peptide EN2 and rabbit peptide RN2. Therefore, the corresponding cysteine residue of cat muscle pyruvate kinase (Harkins & Fothergill, 1977) may also be located 41 residues away, on the N-terminal side, of the catalytically important lysine residue. This would place this cysteine residue in the vicinity of alpha-carbon 50 of domain B of the subunit of cat muscle pyruvate kinase (Stuart et al., 1979). The sulfhydryl group would be between domains A and B of the enzyme close to the PEP binding site. Stammers & Muirhead (1975) have previously shown that a methyl mercury binding site, presumably a cysteine residue, is present in cat muscle pyruvate kinase close to the active site, 10 Å away from the ADP binding site. This interpretation of the tertiary structure of pyruvate kinase would support the conclusions of the chemical modification studies reported here. However, in order to definitively determine the relative importance of this cysteine residue of cat muscle pyruvate kinase, which corresponds to the cysteine residues of eel and rabbit peptides EN2 and RN2 respectively, it will be necessary to accurately place this residue within the tertiary structure of the enzyme. Once the complete primary structure of cat muscle pyruvate kinase has been determined (McAleese et al., 1982), the task of fitting the sequence of the protein to the crystallographic data can commence.

Several explanations can be presented in order to account for the inactivation of eel and rabbit muscle pyruvate kinases due to chemical modification by iodoacetamide. All the available evidence suggests that the cysteine residues located in peptides EN2 and RN2 are located either near or within the active sites of the 2 muscle isozymes. Therefore the alkylation of this specific cysteine residue could induce an unfavourable conformational change in the enzyme subunit resulting in inactivation. Alternatively, this cysteine residue might be located at the opening to the active site pocket therefore the introduction of the alkylating reagent into this structure might result in a blocking of the active site and inactivation. Finally this cysteine residue might be directly involved in the catalytic mechanism of the enzyme. In order to distinguish between these various possibilities, it will be necessary to precisely determine the spatial relationship of this cysteine residue with respect to the substrate binding sites within the tertiary structure of pyruvate kinase.

Eel and rabbit muscle pyruvate kinases exhibited different kinetics of inactivation in response to chemical modification by iodoacetamide. Complete inactivation of the rabbit enzyme correlated with the alkylation of 100% of the cysteine residues contained in peptide RN2 and there was a 1:1 stoichiometry between the percentage enzyme inactivation and the percentage of this cysteine residue which was alkylated. However alkylation of only 50% of the cysteine residues contained in peptide EN2 of tetrameric eel pyruvate kinase was required in order to bring about complete inactivation. The simplest explanation for these observations is

the alkylation of this important cysteine residue in 2 of the 4 subunits of eel pyruvate kinase is sufficient to bring about complete enzyme inactivation whereas modification of this residue in all 4 subunits of the rabbit enzyme is required in order to bring about complete inactivation. This would suggest that at the alkylation reaction conditions employed here, eel pyruvate kinase consisted of non-identical subunits. This conclusion is supported by kinetic data obtained for the eel enzyme at pH 8.0. In the absence of FDP, the eel enzyme exhibited 2 apparent affinities for the substrate PEP. A Hill plot of the data obtained at pH 8.0 and in the absence of FDP exhibited an inflection point between two linear regions. Such kinetic behaviour can be attributed to the presence of 2 kinetically different forms of the same enzyme which exhibit different affinities for the substrate PEP (Van Berkel et al., 1973; Van Berkel, 1974; Van Berkel et al., 1977; Berglund & Humble, 1979).

Structural studies have indicated that eel pyruvate kinase is a homotetrameric enzyme. Therefore, if eel pyruvate kinase consists of non-identical subunits, these subunits can only differ in their conformations. On the basis of kinetic measurements, Berglund & Humble (1979) have suggested that the homotetrameric M_2 isozyme from pig kidney also consists of subunits which differ in their conformations at pH 8.0. According to the model proposed by Berglund & Humble (1979), pig kidney M_2 type pyruvate kinase consists of 2 subunits of the "tense" or T type conformation and 2 subunits of the "relaxed" or R type conformation at pH 8.0. The R subunit exhibits a higher affinity binding site for the substrate PEP than the T subunit. This accounts for the fact that the enzyme

exhibits two apparent K_m values for PEP in the absence of FDP. However in the presence of FDP, only the lower apparent K_m value is observed. Therefore it is proposed that the allosteric effector FDP can transform the pig kidney enzyme into a homotetramer consisting of 4 subunits of the R conformation.

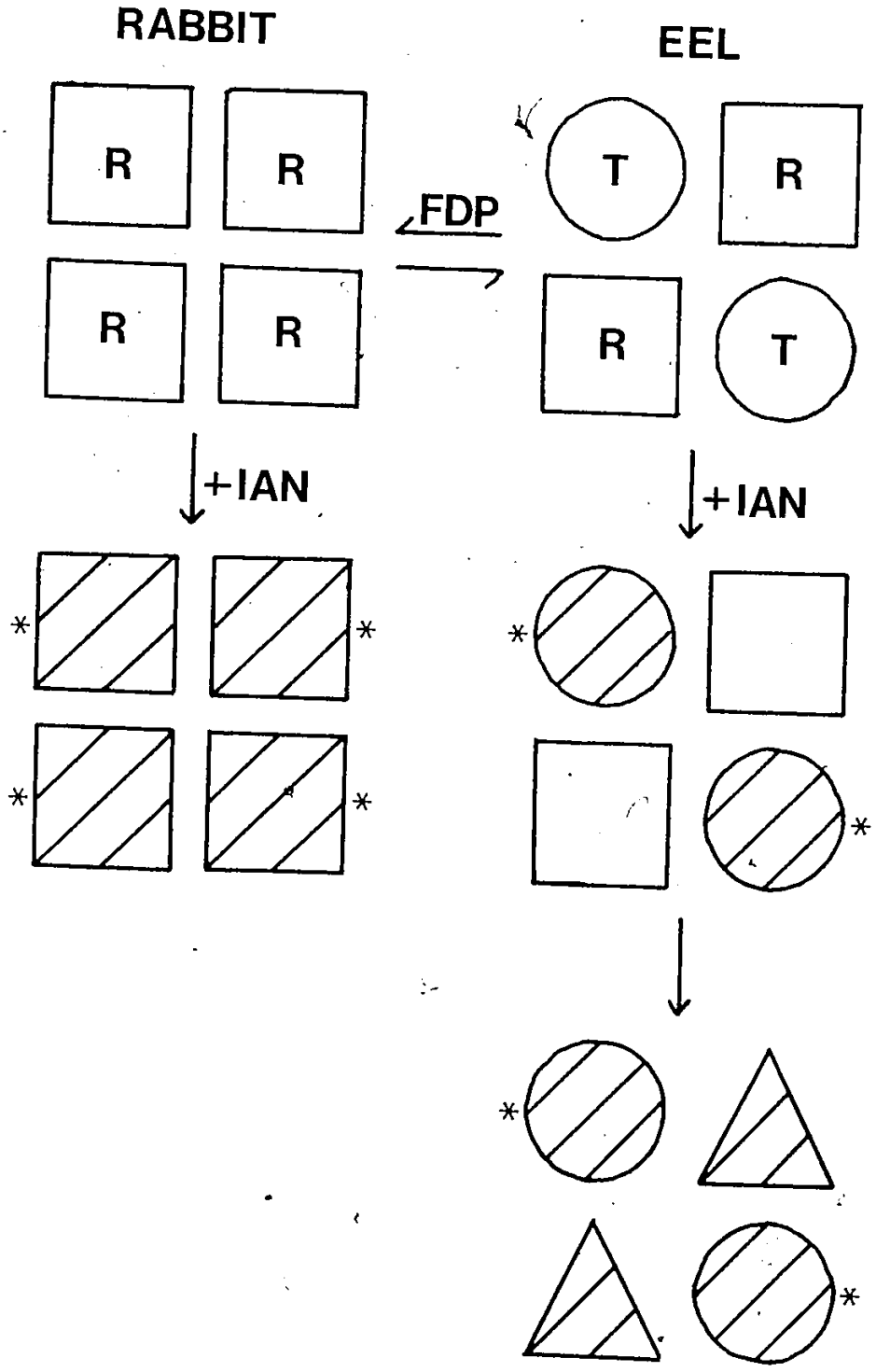
This model can account for the inactivation kinetics exhibited by eel and rabbit muscle pyruvate kinases in response to chemical modification by iodoacetamide (Fig. 5). The presence of 2 types of subunits, which differ in conformation, in the structure of tetrameric eel pyruvate kinase could account for the fact that 2 of the 4 functionally important cysteine residues of the enzyme were more susceptible to alkylation. A cysteine residue located either near or within the active site of pyruvate kinase might be expected to be more susceptible to chemical modification in subunits of the T conformation since these subunits exhibit a low affinity binding site for the substrate PEP. However, this does not account for the fact that eel pyruvate kinase was completely inactivated by the alkylation of only 2 of the 4 functionally important cysteine residues. Presumably the alkylation of the 2 most reactive cysteine residues, contained in peptide EN2, resulted in an unfavourable conformational change which led to complete enzyme inactivation.

According to this model, the allosteric effector FDP can transform the eel muscle enzyme into a homotetramer consisting of 4 subunits of the R conformation. Subunits of the eel enzyme which undergo a T to R conformational change due to the presence of FDP, might be expected to exhibit a reduction in the reactivity of a functionally important cysteine residue located in the vicinity of the active site. This may account for the fact that FDP

FIGURE 5

Proposed Model for the Inactivation
of Eel and Rabbit Muscle Pyruvate Kinases

The model of Berglund and Humble (1979) has been modified here in order to account for the inactivation kinetics of eel and rabbit muscle pyruvate kinases. At pH 8.0 and in the absence of the allosteric effector FDP, tetrameric eel pyruvate kinase consists of 2 subunits of the tense or "T" conformation and 2 subunits of the "R" or relaxed conformation. The R subunit exhibits a higher affinity binding site for the substrate PEP than the T subunit. Hence eel pyruvate kinase exhibits 2 apparent affinity values for PEP at pH 8.0. Rabbit muscle pyruvate kinase consists of 4 subunits of the R conformation and exhibits only one apparent affinity value for PEP. FDP can promote the conversion of the eel enzyme from the R_2T_2 form to the R_4 form. Alkylation by iodoacetamide of a functionally important cysteine residue located either near or within the active site of the rabbit enzyme leads to inactivation(as denoted by the diagonal lines in the figure). Rabbit pyruvate kinase is completely inactivated by the chemical modification of this cysteine residue in all 4 subunits of the enzyme (denoted by the *). Chemical modification of the corresponding cysteine residue in the eel enzyme leads to enzyme inactivation. However, since the eel enzyme consists of non-identical subunits, the functionally important cysteine residue is more susceptible to chemical modification in subunits of the T conformation. The chemical modification and inactivation of the subunits of the T conformation results in an unfavourable conformational change in the unmodified subunits of the R conformation. Hence the chemical modification of the functionally important cysteine residue in just 2 of the 4 subunits of the eel enzyme can lead to complete enzyme inactivation.


RABBIT
EEL
R
R
R
R
T
R
R
T
FDP
+IAN
+IAN

could partially protect eel pyruvate kinase from inactivation due to chemical modification by iodoacetamide.

According to the model, rabbit muscle pyruvate kinase exists as a homotetrameric enzyme at pH 8.0 consisting of 4 subunits of the "active" R conformation. This would account for the fact that in the absence of FDP, rabbit muscle pyruvate kinase was much less sensitive to inhibition by iodoacetamide than the eel enzyme. Since rabbit muscle pyruvate kinase would be in its fully active state at pH 8.0, this could account for the fact that FDP could not activate the rabbit enzyme at this pH and FDP could provide no protection against inactivation due to chemical modification by iodoacetamide. Since the subunits of rabbit pyruvate kinase are considered to be structurally and conformationally identical at pH 8.0, this model can account for the fact that the cysteine residues, contained in peptide RN2 of the tetrameric enzyme, exhibited identical reactivities.

Therefore this model, suggesting the possibility of a conformational R-T pair as first described by Monod et al. (1965), can account for the observations of the kinetic and chemical modification studies reported here. Two separate observations suggest that eel pyruvate kinase is in fact subject to FDP-dependent interconversion between conformationally different forms.

1) In the absence of the allosteric effector, FDP, eel pyruvate kinase exists as multiple isoelectric forms. However, in the presence of FDP, the most acidic isoelectric form predominates. Therefore eel pyruvate kinase is subject to FDP-dependent changes in its surface charge properties.

2) Kwan et al. (1980) reported that in the presence of 1.0 mM

PEP and 8 mM Mg^{+2}, sturgeon and rabbit muscle pyruvate kinases exhibited distinctly different optical rotary dispersion spectra at pH 7.7 in the absence of FDP. While FDP had no effect on the kinetic and optical properties of the rabbit enzyme, it converted the sturgeon muscle enzyme into a form which was kinetically and optically equivalent to the rabbit enzyme. Sturgeon and eel muscle pyruvate kinases are very similar both kinetically and structurally. Preliminary CNBr peptide mapping studies have indicated that sturgeon muscle pyruvate kinase, like the eel enzyme, resembles a mammalian M_2 type isozyme. Therefore eel and sturgeon muscle pyruvate kinases may be subject to similar FDP-dependent conformational changes, as manifested by O.R.D. spectra perturbations.

Numerous chemical modification studies have sought to determine the role and reactivity of the sulfhydryl groups of pyruvate kinase but few have tried to identify the reactive residues and their locations. Flashner et al. (1972) reported that DTNB modified a single catalytically unimportant sulfhydryl group of rabbit muscle pyruvate kinase in the presence of Mg^{+2}-PEP. Harkins & Fothergill (1977) reported that cat muscle pyruvate kinase contained a cysteine residue which was likely to be equivalent to the catalytically unimportant cysteine residue of rabbit pyruvate kinase (Flashner et al., 1972). This "non-essential" cysteine residue was reported to be contained within a cyanogen bromide peptide which is homologous to cyanogen bromide peptides EX1 and RX1 isolated from eel and rabbit muscle pyruvate kinases respectively. However, Harkins & Fothergill (1977) assumed that the most reactive cysteine residue of cat

muscle pyruvate kinase to the sulfhydryl reagent iodoacetic acid in the presence of Mg^{+2}•PEP was equivalent to the most reactive cysteine residue of rabbit muscle pyruvate kinase to DTNB in the presence of Mg^{+2}•PEP. Also the authors assumed that the most reactive cysteine residue of cat muscle pyruvate kinase was, by definition, catalytically unimportant and they failed to determine whether the chemical modification of this residue resulted in enzyme inactivation. Therefore this report gave rise to some misconceptions concerning the role and location of the sulfhydryl groups of pyruvate kinase.

Stammers & Muirhead (1975) reported that cat muscle pyruvate kinase contained a sulfhydryl group which was equivalent to the chemically reactive but catalytically unimportant cysteine residue of rabbit muscle pyruvate kinase (Flashner et al., 1972). This cysteine residue was reported to be located between domains A and C of the cat enzyme subunit (Stammers & Muirhead, 1975). Muirhead et al. (1981) assumed that this "non-essential" cysteine residue was contained within the sequence of the cyanogen bromide peptide which had been previously isolated from cat muscle pyruvate kinase (Harkins & Fothergill, 1977). However Muirhead et al. (1981) encountered considerably difficulty when they attempted to fit this cyanogen bromide peptide to the electron density corresponding to the sequence surrounding the reactive cysteine residue located between domains A and C. This is not surprising since the chemical modification studies reported here, in combination with the sequence data reported for yeast pyruvate kinase (Burke et al., 1983),

indicate that it is likely that this cyanogen bromide peptide can be fitted to the electron density corresponding to the sequence surrounding alpha carbon 50 of domain B of cat muscle pyruvate kinase (Stuart et al., 1979). The chemical modification studies reported here indicate that in the absence of Mg^{+2}·PEP, rabbit muscle pyruvate kinase contains not one but 2 reactive cysteine residues. The chemically reactive but catalytically unimportant cysteine residue contained in peptide RN3, isolated from the rabbit muscle enzyme, may correspond to the reactive cysteine residue reported to be located between domains A and C of cat muscle pyruvate kinase (Stammers & Muirhead, 1975).

Bloxham & Chalkley (1976) reported that rabbit muscle pyruvate kinase could be inhibited due to the alkylation of a catalytically important cysteine residue by 5-chloro-4-oxopentanoic acid. This essential residue was reported to be contained within a specific tryptic pentapeptide isolated from the rabbit enzyme (Chalkley & Bloxham, 1976). However the authors were unable to clearly show that the alkylation of this cysteine residue was responsible for the inactivation of rabbit pyruvate kinase. The authors recognized the fact that the reagent 5-chloro-4-oxopentanoic acid could participate in Schiff's base formation with the ε-amino group of a reactive and catalytically important lysine residue. However they did not seriously consider the possibility that the modification of a lysine residue might be responsible for the inactivation of the rabbit enzyme by this reagent. This assumption was made in spite of the fact that a pK_a value of 9.2 was determined for the catalytically important functional group of rabbit pyruvate kinase in these chemical modification studies. This value is substantially

higher than the pK_a values determined previously for the modification of the sulfhydryl groups of rabbit pyruvate kinase (Flashner et al., 1972; Jacobson & Black, 1971). Also prior to this report, Hollenberg et al. (1971) had reported that rabbit muscle pyruvate kinase contained a catalytically important lysine residue which was susceptible to chemical modification.

In a subsequent paper, Bloxham et al. (1978) reported that chemical modification of rabbit muscle pyruvate kinase by methyl methanethiosulphonate resulted in enzyme inactivation. A catalytically important cysteine residue was reported to be located within the tryptic pentapeptide Cys-(Glx, Asx, Ser)-Arg. However a number of observations suggest that the inactivation of the rabbit enzyme due to chemical modification by methyl methanethiosulphonate was due to the formation of 2 disulphide bonds in the enzyme which induced an unfavourable conformational change.

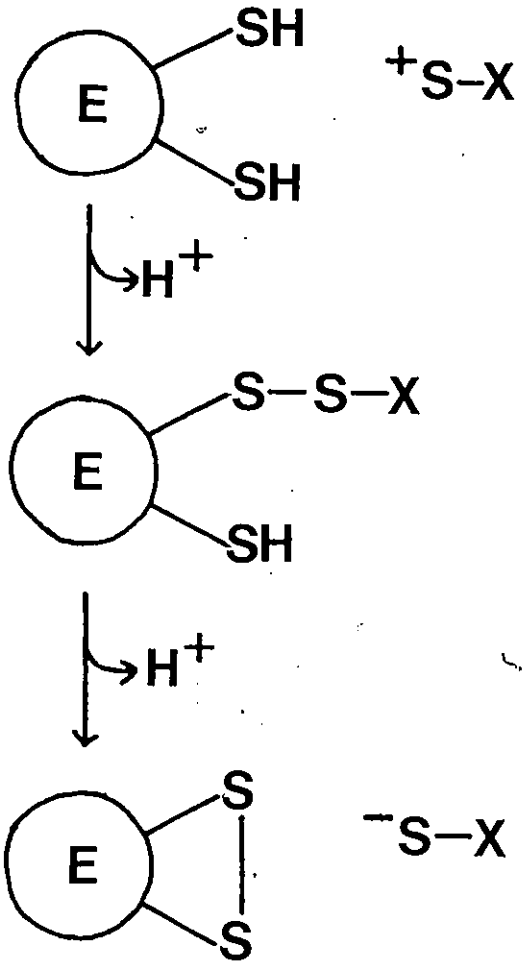
1) Previously Flashner et al. (1972) reported that sulfhydryl reagents which were covalently linked to the thiol groups of an enzyme via a disulfide bond were susceptible to displacement due to nucleophilic attack by neighbouring cysteine residues. This mechanism is illustrated in Figure 6.

2) Bloxham et al. (1978) reported that plots of the log percentage residual activity versus time were curved indicating that chemical modification of the cysteine residues of the rabbit enzyme by methyl methanethiosulphonate was reversible. This could be attributed to the displacement of the reagent due to disulphide bond interchange.

3) On the basis of radioactivity incorporation measurements, Bloxham et al. (1978) reported that rabbit muscle pyruvate kinase

FIGURE 6

Mechanism of Disulfide
Bond Interchange



The sulfhydryl reagent $^{+}$S-X chemically modifies a reactive cysteine residues of the enzyme E. However the reagent is susceptible to displacement due to nucleophilic attack by a neighbouring cysteine residue (Flashner et al., 1972). The net result is the sulfhydryl reagent catalyzes the formation of a disulfide bond within the structure of the enzyme.

contained 4 cysteine residues which were susceptible to chemical modification by methyl methanethiosulphonate when the reaction was taken to completion. This is a surprising observation since Flashner et al. (1972) reported that all 9 of the cysteine residues of rabbit muscle pyruvate kinase were susceptible to chemical modification by DTNB when the reaction was taken to completion. This has been confirmed in the present report. This apparent discrepancy can be accounted for, in part, by the displacement of radioactively labelled methanethiol groups from chemically modified rabbit pyruvate kinase due to disulfide bond interchange leading to the formation of 2 cystine residues per enzyme subunit. 4) The authors reported that treatment of chemically modified rabbit pyruvate kinase with dithiothreitol failed to restore the enzyme's catalytic activity in spite of the fact that the methanethiol group attached to the "catalytically important" cysteine residue was removed under these conditions. This suggests that the chemical modification of this particular cysteine residue was not responsible for the loss of catalytic activity. If enzyme inactivation was in fact due to the formation of 2 disulphide bonds within the structure of rabbit pyruvate kinase, treatment with dithiothreitol would not be expected to result in a restoration of catalytic activity if these disulphide bonds were buried within the structure and hence not accessible to the reducing reagent. Therefore, all these observations suggest that the cysteine residue reported to be contained within the tryptic pentapeptide Cys-(Asx, Glx, Ser)-Arg, isolated from rabbit muscle pyruvate kinase (Bloxham et al., 1978), is not catalytically important.

None of the 9 carboxymethylcysteine-containing peptides purified from rabbit muscle pyruvate kinase here appear to correspond to the tryptic pentapeptide Cys-(Asx, Glx, Ser)-Arg isolated previously from the rabbit enzyme (Bloxham et al., 1978). This apparent discrepancy can be accounted for if the tryptic pentapeptide actually corresponds to the tryptic tripeptide RN1 of sequence Cys-Asn-Arg. A number of observations suggest that this is in fact the case. The tryptic pentapeptide differs from peptide RN1 only in the presence of 2 extra residues. Since the tryptic pentapeptide was obtained in very low yield (Bloxham et al., 1978), the 2 additional amino acids could be contaminants. This is supported by the fact that the amino acids Ser and Glx were present in less than 1:1 stoichiometric amounts relative to the other amino acids. Also, the tryptic "pentapeptide" was reported to exhibit the mobility of a neutral peptide at pH 6.5 in some instances and the mobility of an acidic peptide carrying one negative charge at pH 6.5 on other occasions (Bloxham et al., 1978). If the tryptic "pentapeptide" is actually equivalent to the tryptic tripeptide RN1, this variation in electrophoretic mobility can be attributed to a single charge change resulting from the deamidation of the asparagine residue in peptide RN1.

If the tryptic "pentapeptide" isolated previously from rabbit muscle pyruvate kinase (Bloxham et al., 1978) is equivalent to the tryptic tripeptide RN1 reported here, this would support the conclusion that the "essential" cysteine residue studied by Bloxham et al. (1978) is in fact catalytically unimportant. The chemical modification studies reported here, indicate that the cysteine residue contained in peptide RN1 of the rabbit enzyme is not cataly-

tically important. Furthermore, Burke et al. (1983) reported that in yeast pyruvate kinase, this cysteine residue is replaced by a serine residue in a region of the polypeptide chain homologous to peptide RN1. This is definite proof of the non-essential nature of this functional group.

Therefore these series of studies have served to illuminate the role of the sulfhydryl groups in pyruvate kinase. The alkylation of a specific cysteine residue, contained within a highly conserved region of the pyruvate kinase polypeptide chain, led to the complete inactivation of the eel and rabbit muscle isozymes. This cysteine residue, which has been preserved throughout evolution, may be present either near or within the active site of pyruvate kinase. Whether this important cysteine residue plays a role in the catalytic mechanism of pyruvate kinase remains to be shown.

A model has been presented in order to account for the different inactivation kinetics exhibited by eel and rabbit muscle pyruvate kinases in response to chemical modification by iodoacetamide. The model proposes that at pH 8.0 and in the absence of FDP, eel muscle pyruvate kinase, unlike the M_1 isozyme from rabbit muscle, consists of 2 types of subunits which differ in conformation. This model has been employed previously to account for the kinetic differences exhibited by the M_1 and M_2 type isozymes of a mammal (Berglund & Humble, 1979). Eel muscle pyruvate kinase, has been shown to be structurally and kinetically similar to a mammalian M_2 isozyme. Therefore further examination of the role of the functional groups in the catalytic mechanisms of eel and rabbit muscle pyruvate kinases, might be expected to provide useful

information on the molecular basis for the kinetic differences exhibited by the mammalian M_1 and M_2 type isozymes.

GENERAL CONCLUDING REMARKS

The purification and characterization of a new pyruvate kinase isozyme from the white muscle of the American eel is reported here. Of the mammalian pyruvate kinase isozymes, the eel enzyme most closely resembles the M_2 type isozyme both kinetically and structurally. In mammals, the M_2 isozyme is a ubiquitous form present in many tissues of the adult (Imamura & Tanaka, 1972). While the M_1 type is the only pyruvate kinase isozyme in adult skeletal muscle, the M_2 type appears to be the predominant isozyme in fetal skeletal muscle. This suggests that a change-over in the expression of the pyruvate kinase isozymes occurs in mammalian skeletal muscle during development. Since the M_1 and M_2 type pyruvate kinases appear to be the products of the same gene (Saheki et al., 1982b; Hance et al., 1982), this change-over in isozyme expression may involve the synthesis of the M_1 type at the expense of the M_2 isozyme.

The M_2 type represents the predominant pyruvate kinase in all fetal tissues and in dedifferentiating cancer cells (Imamura & Tanaka, 1972). Therefore it has been proposed that the M_2 type represents the prototype of the pyruvate kinase isozymes and the M_1 type evolved from the M_2 type. It might be expected that a change-over in the expression of the pyruvate kinase isozymes might not be observed in the skeletal muscle of species more primitive than mammals. In this case, the M_2 isozyme would predominate in both adult and fetal skeletal muscle.

Previous studies have shown that in the skeletal muscle of the chicken (Strandholm et al., 1975) and the frog (Schloen et al., 1969), an M_2-like isozyme predominates in the fetus while an M_1-like isozyme predominates in the adult. Guderley & Cardenas (1979) reported that a similar change in the expression of the pyruvate kinase isozymes of the salmon occurred in skeletal muscle. However pyruvate kinase from adult salmon muscle, unlike the isozyme from eel white muscle, more closely resembles a mammalian M_1 isozyme both kinetically and structurally than a mammalian M_2 isozyme (Guderley & Cardenas, 1980a,b). Therefore it would be of value to conduct a study of the developmental changes in the expression of the pyruvate kinase isozymes in the tissues of the eel. Such a study might reveal that the fetal pyruvate kinase form persists in the skeletal muscle of the adult eel. This information would be useful in the elucidation of the evolution of pyruvate kinase isozymes.

On the basis of kinetic and chemical modification studies, a model has been presented here in order to account for the properties of eel and rabbit muscle pyruvate kinases (Fig. 5, Chapter 5). The most significant feature of this model is that it proposes that eel muscle pyruvate kinase can consist of 2 types of subunits which differ in conformation and FDP can effect the inter-conversion of these conformers. In order to test the validity of this model, it will be necessary to provide further evidence for the presence of conformationally different subunits in the structure of tetrameric eel pyruvate kinase. This information could come indirectly from further chemical modification studies. It should be possible to detect two diff-

erent reactivities for the important cysteine residue of eel muscle pyruvate kinase in the absence of FDP. Under these conditions, a biphasic inactivation curve would be obtained consisting of a rapid initial loss of catalytic activity correlating with the modification of the important cysteine residue in two subunits of the T type, followed by a slow loss of the remaining activity correlating with the modification of the important cysteine residue in 2 subunits of the R type conformation. Unfortuneately in the present study, a biphasic inactivation curve was not observed because an unfavourable conformational change resulted in complete enzyme inactivation following the modification of just 2 of the 4 important cysteine residues of the protein. However, preliminary experiments have shown that in the presence of 1.0 mM PEP, a biphasic inactivation curve is obtained. PEP may therefore prevent this unfavourable conformational change and it may be possible to detect two different reactivities for the important cysteine residue of the eel enzyme under these conditions. In addition, the model proposes that the inactivation kinetics of the eel enzyme should be similar to those of the rabbit enzyme in the presence of FDP. Under these conditions, it may be possible to demonstrate that complete enzyme inactivation correlates with the modification of the important cysteine residue in all 4 subunits of the eel enzyme.

Rabbit and sturgeon muscle pyruvate kinases have been shown to exhibit different optical rotary dispersion spectra in the absence of FDP at pH 7.7 (Kwan et al., 1980). However, while FDP does not alter the kinetic and optical properties

of the rabbit enzyme, it converts the sturgeon enzyme into a form which is kinetically and optically similar to the rabbit enzyme. A similar comparative study of eel and rabbit muscle pyruvate kinases could provide direct evidence for the presence of conformationally different subunits in the structure of eel pyruvate kinase.

The model can account for the effects of FDP on the kinetic and chemical properties of the eel and rabbit enzymes. One might ask whether the effects of other modulators of eel pyruvate kinase activity can be explained in terms of the interconversion of conformationally different enzyme subunits. For instance, eel pyruvate kinase exhibited a decreased dependency on FDP for activation with decreasing pH. Also inflection points in Hill plots were not as noticeable when the assay pH was decreased from 8.0 to 7.2 in the absence of FDP and at pH 6.3 a single straight line was obtained on a Hill plot. These observations could be accounted for by the pH-dependent interconversion of a heterotetrameric eel enzyme consisting of 2 subunits each of the R and T conformations into a homotetrameric enzyme consisting of 4 subunits of the R conformation. Similar pH-dependent interconversion between catalytic forms of the mammalian M_2 isozyme has been described previously (Berglund & Humble, 1979).

In order to test the validity of this model, it will be necessary to further characterize the kinetic behaviour of eel pyruvate kinase in response to various modulators of enzyme activity. Further chemical modification studies could assist in the correlation of kinetic properties to

enzyme structural features. This would be a worth-while endeavour since this model could potentially provide the basis for a molecular interpretation of the kinetic behaviour of the mammalian M_1 and M_2 type pyruvate kinases.

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APPENDIX

Abbreviations for the Names of Some Amino Acids

Ala	alanine
Asn	asparagine
Asp	aspartic acid
Asx	asparagine or aspartic acid
Arg	arginine
Cys	cysteine
Cmc	carboxymethylcysteine
Gln	glutamine
Glu	glutamic acid
Glx	glutamine or glutamic acid
Gly	glycine
His	histidine
Hser	homoserine
Hserl	homoserine lactone
Ile	isoleucine
Leu	leucine
Lys	lysine
Phe	phenylalanine
Pro	proline
Ser	serine
Thr	threonine
Tyr	tyrosine
Val	valine