AN ABSTRACT OF THE THESIS OF

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Title: TRINITROPHENYLATION OF BOVINE MUSCLE PYRUVATE KINASE

Abstract approved: Redacted for privacy

Janet M. Cardenas

Bovine muscle pyruvate kinase (E.C. 2.7.1.40) was inactivated by treatment with 2,4,6-trinitrobenzenesulfonate through the covalent modification of 0.75-0.80 lysyl residues per subunit of enzyme. The rate of trinitrophenylation was reduced in the presence of ADP, ATP and high concentrations of AMP but not by PEP. Analysis of the concentration dependence of the protection provided by ADP and ATP showed both nucleotides provided complete protection of the enzyme against modification. Dissociation constants derived from these experiments were 0.84 and 0.49 mM for ADP and ATP in the presence of Mg^{++}. The high concentrations of AMP required to decrease the inactivation rate were consistent with the relatively low affinity of the enzyme for this nucleotide. No protection was seen in the presence of saturating amounts of PEP or Mg^{++}. The protection results were consistent with the reactive lysyl residue being located in or very near the nucleotide binding site of the enzyme.

The rate of trinitrophenylation was greatly influenced by ionic strength. The activating potassium, the poorly activating sodium and the non-activating tetramethylammonium salts were equally effective in decreasing the rate of enzyme inactivation.

The rate of the trinitrophenylation reaction increased with increasing pH. The dependence of the rate of the reaction on the pH was consistent with the involvement of the ε-amino group with a pK of 9.6. The deprotonation of this reactive lysyl residue did not appear to be responsible for the loss of the catalytic activity of the enzyme at high pH. The decrease in catalytic activity was consistent with an absolute dependence upon the protonated state of a residue with a pK of 8.3. The presence of PEP did not affect the pH profile of the trinitrophenylation reaction.

Bovine muscle pyruvate kinase was shown to catalyze the decarboxylation of oxalacetate in the presence of Mn^{++}. The specific activity of the decarboxylation was only about 0.15 percent of the enzyme's kinase specific activity. The two activities of the enzyme have very different sensitivities to trinitrophenylation. When 85-90 percent of the kinase activity had been lost through the modification, the enzyme still maintained over 80 percent of its decarboxylase activity.

The trinitrophenylated protein was treated with trypsin and the modified peptide was isolated by gel filtration on Biogel P-6. The peptide was shown to be homogeneous by high voltage electrophoresis and by high pressure chromatography. Amino acid analysis and sequence determination revealed the presence of an acidic peptide 34 residues long and containing ε-trinitrophenylated lysine.

Attempts to determine the amino terminal sequence of the native and S-carboxylamidated pyruvate kinase using automated Edman liquid phase sequencing techniques and dansyl chloride modification were unsuccessful. Isolation of residues with no free amino group from a thorough enzymatic digestion of pyruvate kinase resulted in the isolation

of amino blocked serine.

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Associate Professor of Biochemistry and Biophysics

Redacted for privacy

Chairman of Department of Biochemistry and Biophysics

Redacted for privacy

Dean of Graduate School

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Typed by Linda M. Johnson for Stephen C. Johnson

To Linda

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ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
CD	Circular dichroism
EDTA	Ethylenediaminetetraacetate
EPR	Electron paramagnetic resonance
FdP	Fructose 1,6-diphosphate
NAD^+	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
NMR	Nuclear magnetic resonance
PTH-	Phenylthiohydantoin-
PEP	Phosphoenolpyruvate
SDS	Sodium dodecyl sulfate
tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
TNBS	2,4,6-Trinitrobenzenesulfonate
TNP	2,4,6-Trinitrophenyl
UV	Ultroviolet

TRINITROPHENYLATION OF BOVINE MUSCLE PYRUVATE KINASE

I. INTRODUCTION

Pyruvate Kinase

The transfer of the phosphoryl group from PEP to ADP resulting in the formation of pyruvate and ATP is catalyzed by the enzyme pyruvate kinase (ATP: pyruvate phosphotransferase, E.C. 2.7.1.40). This reaction has an absolute requirement for a monovalent and divalent cation, K^+ and Mg^{++} being the most effective. Since the energy derived from the hydrolysis of PEP is much greater than the required energy for phosphorylating ADP, the production of ATP and pyruvate is favored. Using the equilibrium constant of 6.45×10^3 determined by McQuate and Utter (1959), an apparent ΔG of -5.2 kcal/mole can be derived.

Since the reaction is only slightly reversible and is the last step of glycolysis, regulation of pyruvate kinase is important in controlling the relative rates of glycolysis and gluconeogenesis (Scrutton and Utter, 1968). Under physiological conditions which require high levels of gluconeogenesis, pyruvate kinase is inhibited and the conversion of pyruvate to PEP is carried out by pyruvate carboxylase and PEP carboxykinase. When glycolysis is the preferred pathway, pyruvate kinase is activated.

At least three distinct isozymes of pyruvate kinase have been identified in mammalian tissues (Hall and Cottam, 1978). As with many isozyme groups, the different forms of pyruvate kinase have specialized

regulatory and kinetic properties which make them particularly suited to the metabolic requirements of the tissues in which they are located.

Tissues that are capable of large fluxes through glycolysis and have little gluconeogenesis have the type M isozyme. This isozyme has a high specific activity and low apparent sensitivity to metabolic control. It is the major isozyme of cardiac muscle and brain and the only form found in skeletal muscle. The M isozyme follows the classical Michaelis-Menten kinetics with a K_m for PEP of 0.4-0.09 mM and a K_m for ADP of 0.3 mM (McQuate and Utter, 1959; Tanaka et al., 1967; Cardenas et al., 1973).

Type L isozyme is found in the liver, kidney and intestinal mucosa. These tissues utilize both the glycolytic and gluconeogenic pathways and thus require a pyruvate kinase sensitive to metabolic control. This isozyme is inhibited by high concentrations of ADP ($>$0.5 mM), ATP and phenylalanine (Cardenas et al., 1975). Ljungstrom et al. (1974) demonstrated that type L pyruvate kinase could be inhibited by phosphorylation. The phosphorylation was later shown to occur in vivo (Ljungstrom and Ekman, 1977) demonstrating hormonal control of enzyme activity. Type L is allosteric with respect to PEP with a Hill coefficient greater than 2 and a $K_{0.5}$ of 0.5-0.83 mM (Cardenas and Dyson, 1973; Tanaka et al.,1967; Kutzbach et al., 1973). Upon addition of FdP the saturation curve for PEP becomes hyperbolic with the $K_{0.5}$ dropping to values similar to those seen for type M. The K_m for ADP is 0.1-0.4 mM and is unaffected by FdP.

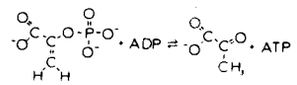
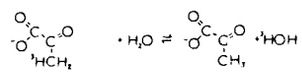
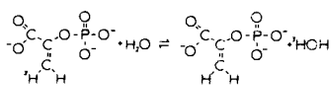
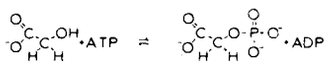
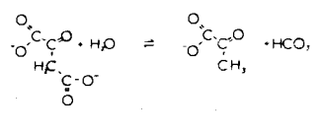
Several tissues contain a third isozyme called type K. It is the main isozyme found in rapidly proliferating tissues such as fetal and tumor tissues. During liver regeneration cells which normally produce

type L pyruvate kinase will produce type K (Tanaka et al., 1967). Liver cells placed in cell culture will also revert to the production of the type K isozyme (Weinhouse, 1972). Several adult tissues not undergoing rapid turnover, such as kidney, lung and adipose retain type K along with type M or L. The metabolic pressures which cause isozymic shifts are not understood. The presence of K in several types of tissues may mean that this form is the least specialized and amenable to a wider variety of metabolic conditions. Type K pyruvate kinase has allosteric kinetics with respect to PEP with a $K_{0.5}$ for PEP of 0.3-0.4 mM (Imamura et al., 1972; Corcoran et al., 1976). The $K_{0.5}$ for PEP drops slightly in the presence of FdP. The K_m for ADP is 0.2-0.4 mM (Corcoran et al., 1976).

Erythrocytes contain a form of the enzyme with very similar kinetic and regulatory properties as type L but with slightly different electrophoretic mobility and amino acid content (Chern et al., 1972; Blume et al., 1971;Imamura and Tanaka, 1972). There is currently a great deal of debate as to whether the erythrocyte pyruvate kinase (type R) is a separate gene product and therefore a different isozyme or whether some type of post-translational modification differentiates it from type L (Marie et al., 1977).

Several reactions have been shown to be catalyzed by pyruvate kinase (Table 1). All the reactions require the presence of a divalent cation. However, the relative abilities of the different cations to activate the catalysis varies with the reaction. The relative abilities of the monovalent cations remains constant in all except the oxalacetate decarboxylase reaction, which does not appear to have a monovalent cation requirement. There is also a constant requirement for a carboxyl group.

Table 1 **Reactions Catalyzed by Pyruvate Kinase**

Reaction	Cation Requirement: Divalent Cation	Cation Requirement: Monovalent Cation	Required Cofactors: Vertebrate Muscle	Required Cofactors: Yeast
I. Pyruvate phosphotransferase [a] 	pH<6.0 $Co^{++} > Mg^{++} > Mn^{++} > Ni^{++}$ pH>7.5 $Mg^{++} > Mn^{++} > Co^{++} > Ni^{++}$	pH 6.0 $Mg^{++} > Co^{++} > Mn^{++} \gg Ni^{++}$	$K^+ > NH_4^+ > Rb^+ > Tl^+ > Cs^+, Li^+, Na^+$	None	None
II. Pyruvate enolase [b] 	$Mn^{++} > Zn^{++} > Mg^{++} > Ca^{++}$	K^+	ATP or P_i	ATP
III. PEP enolase [c] 	$Co^{++} > Ni^{++} > Mn^{++} > Mg^{++}$	$NH_4^+, K^+ > Rb^+ > Cs^+ > Na^+$	ADP	ADP
IV. Fluorokinase [d] $F^- + ATP \rightleftharpoons ADP + F\text{-}O\text{-}P(O)(O^-)\text{-}O^-$	$Mg^{++} > Mn^{++} > Zn^{++}$	K^+	HCO_3^-	HCO_3^- FdP required in the presence of Mg^{++} but not Mn^{++}
V. Hydroxylamine Kinase [e] $ATP + H_2NOH \rightleftharpoons ADP + H_2N\text{-}O\text{-}P(O)(O^-)\text{-}O^-$	at 1.3 mM divalent cation $Zn^{++} > Co^{++} > Mn^{++} > Mg^{++}, Cu^{++}$ at 6.7 mM divalent cation $Co^{++} > Zn^{++} > Mn^{++} > Mg^{++}$	$K^+, NH_4^+ > Na^+$	HCO_3^-	HCO_3^- FdP required in the presence of Mg^{++} but not Mn^{++}
VI. Glycolate Kinase [f] 	$Mn^{++} > Co^{++} > Zn^{++} > Mg^{++} \gg Ca^{++}$	K^+	None	FdP
VII. Oxalacetate Decarboxylase [g] 	Mn^{++}, Mg^{++} Ca^{++} Inhibitory	None	None	—

a Boyer, 1962; Kayne, 1973; Kwan et al., 1975; Kayne, 1971; Solvonik and Colier, 1955

b Dunaway-Mariano et al., 1979; Rose, 1960; Robinson and Rose, 1972; Ford and Robinson, 1976.

c Robinson and Rose, 1972; Ford and Robinson, 1976

d Tietz and Ochoa, 1958; Kupiecki and Coon, 1960; Flavin et al, 1957; Leblond and Robinson 1976.

e Cottam et al., 1968; Kupiecki and Coon 1959 and 1960; Leblond and Robinson, 1976.

f Kayne, 1974; Dunaway-Mariano et al., 1979; Leblond and Robinson 1976.

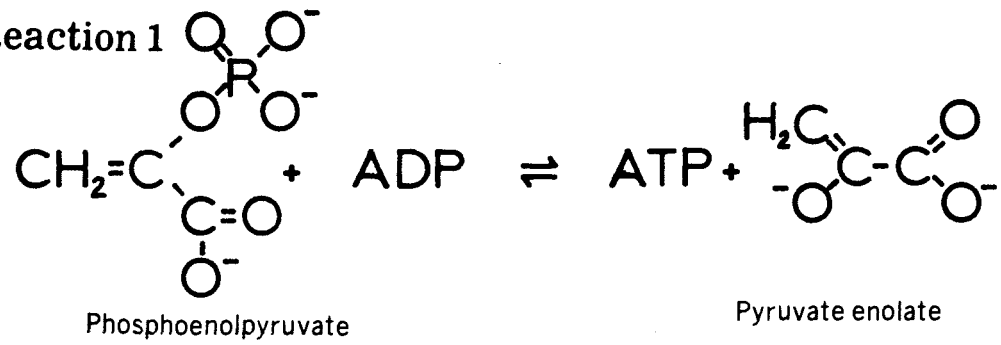
g Kosicki, 1978; Creighton and Rose, 1976 a and b

When the substrate does not have a carboxyl group bicarbonate is required as a cofactor.

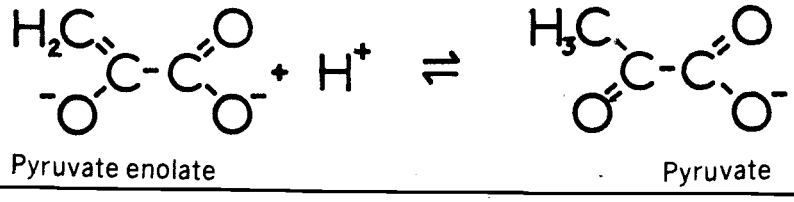
The overall reaction of pyruvate kinase can be divided into two distinct half reactions as follows:

Figure 1

Reaction 1



Reaction 2



Examples of the enzyme's ability to catalyze the phosphoryl transfer independently of enolization (Reaction 1) are seen in its ability to phosphorylate fluoride, hydroxylamine and glycolate (Table 1).

Evidence for the existence of Reaction 2 was presented by Rose (1960) when he found that the enzyme would catalyze the enolization of pyruvate in the presence of monovalent and divalent cations and a "phosphate-like" moiety. The enolization was later found to be rapid compared with the rate of release of pyruvate. This indicates that Reaction 2 is not rate limiting and provides evidence further for the presence of an enolate intermediate (Robinson and Rose, 1972).

Wolfenden (1972) proposed that oxalate was a transition state analog for enzyme reactions having an enolate intermediate. Transition state

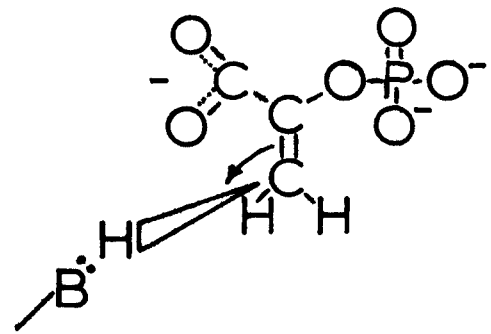
theory as proposed by Wolfenden (1972) states that an analog of the transition state of the substrate would be a very potent competitive inhibitor of that substrate. The enzyme would in fact have a greater affinity for this inhibitor than for the substrate. Any factors which affect the normal catalysis of the enzyme would also affect the binding of the transition state analog. Reed and Morgan (1974) studied the kinetic and magnetic resonance properties of the interaction of oxalate with pyruvate kinase and found that oxalate was indeed a strong inhibitor, having a K_I of 6μM. Despite this ability to bind tightly to the enzyme, not all the factors affecting PEP binding affected the binding of oxalate. Whereas substituting tetramethylammonium ion for K^+ increases the dissociation constant for PEP by a factor of 5 (Nowak, 1976) it has no effect on the binding of oxalate.

Recent evidence suggests that the release of the products from the enzyme determines the rate of the reaction. Rao et al. (1979) found that the equilibrium constant for enzyme bound substrates and products was approximately one. The interconversion of ADP and PEP to ATP and pyruvate must be, therefore, rapid compared to the release of either substrates or products. It is also interesting to note that the free energy difference on the enzyme must be small despite the large free energy difference between the substrates and products in solution.

Stereochemical analysis of the pyruvate kinase reaction have shown that the addition of the proton in conversion of the enolate intermediate to pyruvate comes from the si face as shown in Figure 2 (Rose, 1970). The C-3 group of PEP undergoes attack from the same side in reactions involving PEP carboxykinase and enolase (Nowak and Mildvan, 1970).

There is currently some debate as to whether the phosphate transfer

Figure 2



proceeds via a S_n1 or S_n2 type reaction (Lowe and Sproat, 1978; Blattler and Knowles, 1979). The S_n1 mechanism suggests the presence of a highly reactive metaphosphate intermediate. Blattler and Knowles (1979) questioned this mechanism based on the difficulty of an enzyme to control any reaction involving such a reactive intermediate. The debate as to whether the phosphoryl group actually leaves PEP before it becomes bound to ADP or after probably will come down to semantics. The lifetime of a free metaphosphate intermediate would have to be extremely short and tightly controlled by the enzyme to prevent phosphatase activity.

Enzyme Structure

All isozymes of pyruvate kinase form tetramers with a molecular weight of around 220,000, each subunit being approximately the same size. Bovine and rabbit muscle pyruvate kinase have four PEP binding sites per tetramer (Cardenas *et al.*, 1973; Kayne, 1971) suggesting the presence of one active site per subunit.

Types M and K are immunologically cross-reactive, as are types L and R, while the two pairs are immunologically distinct. Homology of primary structure of the isozymes was compared by peptide mapping using limited proteolysis on four isozymes from rat (Saheki *et al.*, 1978). This study revealed little similarity between M, K and L but a high degree of homology between L and R.

Despite these differences, no great secondary and ternary structural differences have been reported in the different isozymes. All of the isozymes are able to form completely active hybrids. K-M and K-L hybrids are found *in vivo*. No L-M hybrids are found *in vivo*, but this is probably because no cell seems to synthesize types L and M subunits

simultaneously. Completely active L-M hybrids, however, can be made *in vitro* (Dyson and Cardenas, 1973).

A high degree of homology exists between muscle pyruvate kinases isolated from different sources. In addition to very similar amino acid contents (Hall and Cottam, 1978), sequencing results support homology between the muscle isozyme from very divergent vertebrates. Of the 8 residues sequenced from a CNBr peptide containing a highly reactive sulfhydryl from cat, rabbit and sturgeon muscle, five were identical (Anderson and Randall, 1975; Harkins and Fothergill, 1977).

The amino terminal serine of rabbit muscle pyruvate kinase is blocked by an N-acetyl group (Brummel *et al.*, 1976). It appears that all eukaryotic forms of the enzyme also have blocked amino terminals. Hydrazinolysis, carboxypeptidase digestion and tritium exchange have failed to reveal a carboxylterminal residue of the rabbit muscle isozyme, suggesting that this end may be also blocked (Cottam *et al.*, 1969; Brummel *et al.*, 1976).

Crystals of cat muscle pyruvate kinase are well ordered and suitable for high resolution x-ray structure analysis (Stammers and Muirhead, 1975). Extensive x-ray analysis has been carried out on these crystals down to 0.26 nm (Stammers and Muirhead, 1975; Levine *et al.*, 1978). The overall shape of the subunit is elliptical with dimensions of 4.5 by 7.5 nm. The subunit interfaces are at one end of the elliptical monomer so that large areas of each subunit within the tetramer are exposed to solvent. Each subunit binds to two others. Pairs of subunits across one axis have more contact points than the other pair. This difference in subunit interaction could lead to the dimer formation seen in 2 M urea (Cottam and Mildvan, 1971).

The ternary structure of each subunit forms three domains, denoted

A,B and C by Stammers and Muirhead (1975). The largest domain, A, contains around 220 residues and is made up of a cylindrical beta sheet of eight parallel strands interconnected by alpha helices forming an outer cylinder coaxial to the first. A highly homologous structure is found in triose isomerase (Stammers and Muirhead, 1977; Levine et al., 1978). Domain B extends furthest from the center of the tetramer and contains about 100 residues. The electron density of this domain is much weaker than that of the rest of the structure, indicating more flexibility in the polypeptide backbone (Stammers et al., 1977). Domain C, containing approximately 120 residues, is located in the interior core of the tetramer. Regions involved in the subunit interaction are found in this domain.

Substrate Binding

High resolution x-ray crystallography has been used to study substrate binding to cat muscle pyruvate kinase (Stammers and Muirhead, 1975). Domain C of the protein subunit contains the characteristic mononucleotide binding fold. At pH 6.0, ATP and ADP bind in this region in the presence of either Mg^{++} or Mn^{++} and high ionic strength. This, however, is far removed from the PEP binding site, which is found in the A domain. At pH 8.0 the binding site for ADP in the presence of Mn^{++} shifts to the A domain. This site partially overlaps the PEP binding site and extends into the cleft between the A and C domains. This later nucleotide binding site is thought to be the binding site involved in catalysis.

High resolution x-ray analysis of nucleotide binding to the catalytic site reveals no significant density associated with the base portion of the molecule (Stammers and Muirhead, 1975). The authors attributed this to the base being in free rotation, suggesting it plays no large role in

binding. Catalytic studies with a wide variety of nucleotide analogs support this conclusion. Hohnadel and Cooper (1973) showed that the enzyme could accept wide variations within the adenine base with little effect upon the K_m or V_m of the reaction. Other purine and pyrimidine dinucleotides can replace ADP as a substrate (Plowman and Krall, 1965) or are very effective competitive inhibitors (Janson and Cleland, 1974). Affinity labeling reagents for adenine nucleotide binding enzymes fail to inactivate pyruvate kinase when the reactive moiety is on the base (Hampton and Slotin, 1975; Hampton et al., 1978). Apparently there are insufficient adenine base-protein interactions to allow modification reactions to occur.

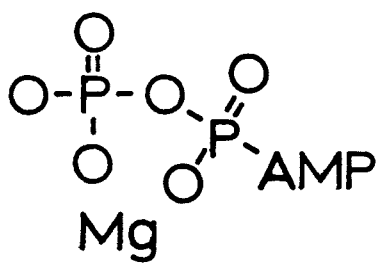
The enzyme is much more sensitive to changes within the phosphate or ribose portion of ADP. Any alterations in the 2' or 3' hydroxyls of the ribose greatly reduces the binding and ability of the analog to serve as a substrate (Hohnadel and Copper, 1973). Changes in the phosphate of the nucleotide shows a greater sensitivity in the beta position than in the alpha (Hampton et al., 1973; Eckstein and Goody, 1976). Affinity labels with reactive groups in the phosphate positions have proven to be effective inactivators (Berghauser and Geller, 1974; Wyatt and Colman, 1977).

It has been suggested that there is a stereospecificity for the metal · ATP complex on the enzyme. Using thio-phosphate analogs (Jaffe and Cohn, 1978) and CrATP stereoisomers (Dunaway-Mariano et al., 1979) it was shown that the enzyme preferred the A isomer over the B (Figure 3).

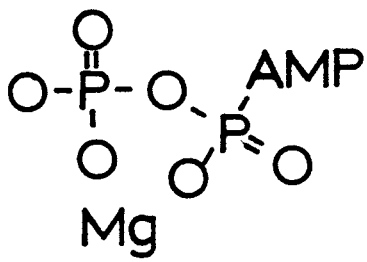
Several analogs of PEP have been synthesized to test the specificity of the enzyme for various portions of this substrate (Woods et al., 1970;

Stubbe and Kenyon, 1972; Soling et al., 1971; James and Cohn, 1974; Nowak and Mildvan, 1972). Figure 4 shows the structures of those analogs that will replace PEP as a substrate for the pyruvate kinase reaction. The intact carboxyl and phosphoryl groups are absolute requirements. While all these PEP analogs bind with an affinity close to that of PEP, all are extremely poor substrates. The requirements for catalysis must then be much more stringent than for binding.

Figure 3



MgATP - A Isomer



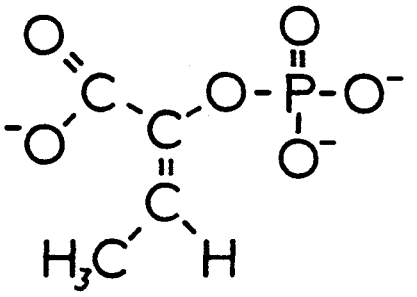
MgATP - B Isomer

Activating Cations

Pyruvate kinase was the first enzyme which was shown to have an absolute requirement for monovalent cations (Boyer et al., 1942). For several years it was believed that the general role of the monovalent cation was to induce the proper conformation of the enzyme. Support was lent to this idea by the relatively high concentrations of monovalent cations (5-100 mM) which were required to gain the maximal catalytic activity and the observations that spectroscopic changes were induced by the activating cations (Suelter et al., 1966).

It wasn't until Suelter (1970) made a survey of all the enzymes

Figure 4

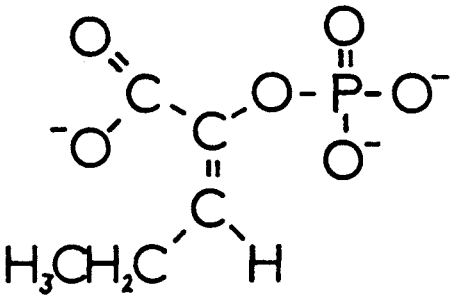


Stubbe and Kenyon, 1971

Phosphoenol α ketobutyrate

Km 0.045 mM

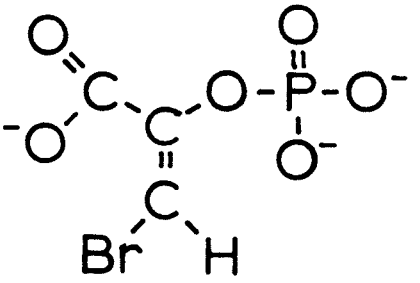
$\frac{Vm}{Vm \text{ with PEP}}$ 0.001



Stubbe et al., 1971

Phosphoenol α ketovalerate

$\frac{Vm}{Vm \text{ with PEP}}$ 0.001

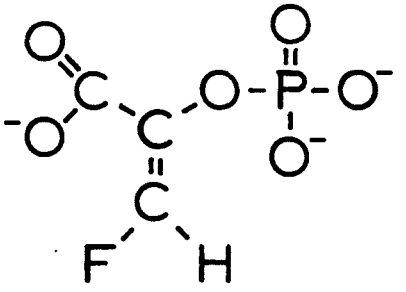


Stubbe and Kenyon, 1971

Phosphoenol 3-bromopyruvate

Km 0.044 mM

$\frac{Vm}{Vm \text{ with PEP}}$ 0.003



Stubbe and Kenyon, 1971

Phosphoenol 3-fluoropyruvate

Km 0.040 mM

$\frac{Vm}{Vm \text{ with PEP}}$ 0.0023

known to require monovalent cations that the model implicating K^+ in stabilizing enolate intermediates was purposed. Kayne and Reuben (1970) observed that TI^+ binds within 0.5 to 0.7 nm of enzyme bound Mn^{++}. Observations that K^+ decreases the affinity of the enzyme· Mn^{++} complex for PEP analogs lacking a free carboxyl group (Nowak and Mildvan, 1972b) suggests the carboxyl group of PEP is coordinated on the enzyme by K^+.

The relative abilities of various monovalent cations to activate rabbit muscle pyruvate kinase are: $K^+ \gtrsim NH_4^+ > Rb^+ > TI^+ > Cs^+$, Li^+, Na^+ (Kayne, 1971).

Pyruvate kinase has also an absolute requirement for divalent cations (Table 1). The relative catalytic efficiency of the different divalent cations tends to vary with pH (Kwan et al., 1975) with the order $Mg^{++} > Mn^{++} > Co^{++}$ at pH 7.4 (Solvonik and Colier, 1955). Binding of the divalent cation yields a shift in the EPR (Mildvan and Cohn, 1965) and UV spectra (Suelter and Melander, 1963) of the enzyme, suggesting that divalent metal binding causes a conformational change. Recent experiments using the stable, substitution inert CrATP complex suggest two divalent metal ions are required for catalysis, one complexed with the phosphate ions of the nucleotide and the other bound to the enzyme. CrATP activates the enolization of pyruvate (Gupta et al., 1976a) and phosphorylation of glycolate (Dunaway-Mariano et al., 1979) only in the presence of a second cation, Mn^{++} or Mg^{++}.

The specific role of divalent cations in catalysis is unclear. One possible role is to properly orient the substrate on the enzyme. Robinson and Rose (1972) found that the relative rate of tritium loss from (3-^{3}H) PEP is related to the electronegativity of the divalent cation used, suggesting that the metal affects the pK of the base involved in the

proton transfer step.

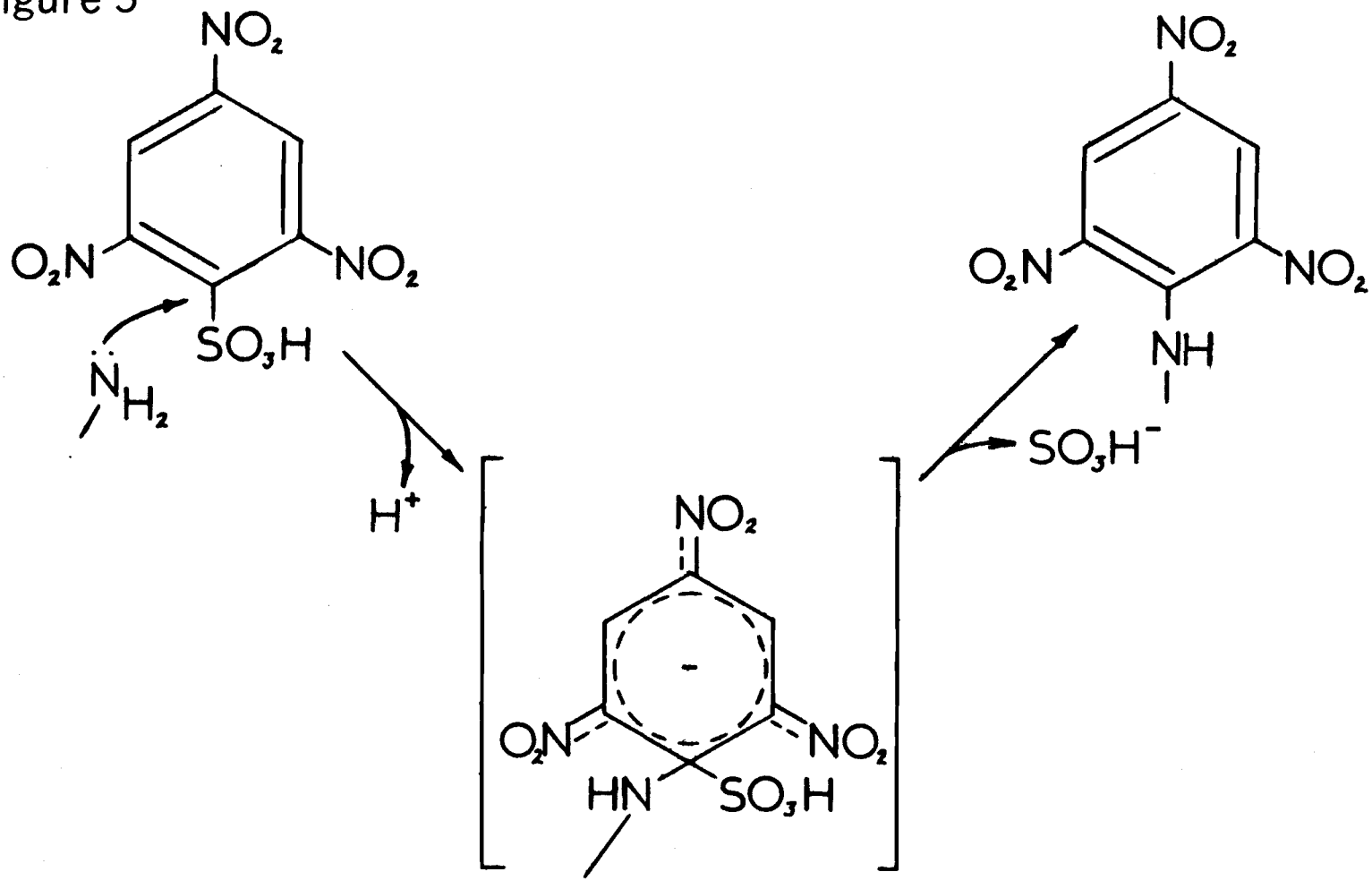
Chemical Modification

Lysine modification

2,4,6-Trinitrobenzenesulfonate has been extensively used to specifically modify primary amino groups in peptides and proteins. The reaction involves the nucleophilic substitiution of an unprotonated primary amino group for the sulfonate group (Figure 5). Since sulfonate is a relatively poor leaving group, only the strongest nucleophilic groups in a protein are able to replace it. The phenolic hydroxyl group of tyrosine and the imidazole imino group of histidine do not react (Okuyama and Satake, 1960). The sulfhydryl group of cysteine is able to replace the sulfonate but the resultant S-trinitrophenyl linkage is very labile at the alkaline pH (Kotaki et al., 1974).

Trinitrobenzensulfonate has been used to inactivate pyruvate kinase from rabbit skeletal muscle (Hollenberg et al., 1971), bovine skeletal muscle (Hubbard and Cardenas, 1975) and yeast (Roschlau and Hess, 1972). In all cases, this reagent inhibits catalytic activity by modifying a single lysine residue per subunit. The inactivation, however, is not complete. Hollenberg et al. (1971) and Roschlau and Hess (1972) were only able to obtain 95 to 60 percent inactivation, respectively. Roschlau and Hess (1972) suggested two possible reasons. First, the modified subunit might retain some of its catalytic activity. Second, the modification of some lysine residues may affect the ability to modify others. This could be caused by direct steric interference or by a conformational change within the protein. No experimental evidence presented to date has explained the inability to obtain 100 percent inactivation.

Figure 5



The rate of inactivation is decreased in the presence of ADP but not affected by PEP (Hollenberg et al., 1971; Roschlau and Hess, 1972; Hubbard and Cardenas, 1975). Using rabbit skeletal muscle pyruvate kinase, Hollenberg et al. (1971) looked at the affect of substrates, substrate analogs and metal activators on the rate of inactivation. The nucleotides ADP and ATP at 5 mM concentrations provide the best protection while the nucleoside, adenosine, also at 5 mM, provides no protection. The metal activators K^+ and Mg^{++} at 100 mM and 4 mM concentrations, respectively, also provide some protection. Magnesium provides greater protection than K^+ at these concentrations. Protection by cations is eliminated in the presence of 1 mM PEP. Neither FdP or pyrophosphate at 10 mM concentrations protect the enzyme against inactivation by trinitrobenzenesulfonate.

Pyruvate kinases from other sources have been reported to have the same pattern of substrate protection. ADP also protects pyruvate kinases from bovine skeletal muscle and yeast against trinitrophenylation (Hubbard and Cardenas 1974; Roschlau and Hess, 1972). PEP does not protect against inactivation but may even increase the rate of inactivation of the bovine muscle enzyme. As in the case of rabbit muscle pyruvate kinase, Roschlau and Hess (1972) found that Mg^{++} alone will protect the yeast enzyme.

Using the concentration dependence of ADP protection against trinitrophenylation, Hollenberg et al. (1971) calculated the dissociation constant of ADP to be 2.7×10^{-4} M for the enzyme·ADP complex in the presence of 100 mM KCl, 10 mM $MgCl_2$ and 0.1 M phosphate, pH 8.3. This constant agrees very well with the ADP K_m of 0.3 mM (McQuate and Utter, 1959). They also showed that the ADP·enzyme complex provided complete

protection against modification.

Using NMR, Flashner et al. (1973) examined the ability of the trinitrophenylated enzyme to bind Mn^{++} and substrates. By following the proton relaxation rates of water, they found no evidence for the formation of an enzyme·Mn^{++}·ADP complex with modified enzyme. They could also find no difference between native and trinitrophenylated enzymes in the formation of the enzyme·Mn^{++}·PEP complex.

Only those activities that require the binding of an adenine nucleotide are affected by the trinitrophenylation of pyruvate kinase. Detritiation of (3-^{3}H) pyruvate was followed by Flashner et al. (1973) by the trinitrophenylated enzyme. They found that ATP mediated detritiation is greatly reduced or eliminated but the phosphate-mediated detritiation is unaffected. The K_m of pyruvate and V_m for the detritiation reaction are also unchanged. Jursinic and Robinson (1978) followed the ability of the enzyme to decarboxylate oxalacetate as the rabbit muscle enzyme was trinitrophenylated. They demonstrated that the majority of the kinase activity is inhibited by one modification per subunit while the decarboxylase activity does not appreciably decrease until more than one site per subunit is modified.

Trinitrophenylation does not significantly alter ternary and quaternary structure of pyruvate kinase. Hubbard and Cardenas (1975) studied the hydrodynamic properties and subunit interactions of the trinitrophenylated bovine muscle pyruvate kinase and found little or no change in its overall shape and size upon modification. The sedimentation coefficient decreases slightly from 9.85 to 9.45 upon trinitrophenylation. However, the native tetrameric structure is retained, as sedimentation equilbrium studies give a molecular weight of 226,000±2,000 for the modified enzyme. This is within experimental error of the 230,000

value determined for the native protein.

Trinitrophenylation does not alter the ability to form tetramers with other isozymers of pyruvate kinase (Hubbard and Cardenas, 1975). When the trinitrophenylated bovine muscle isozyme is denatured in the presence of the native type L isozyme and then renatured, a complete set of isozyme hybrid tetramers is formed.

Johnson and Deal (1970) used pyridoxal 5'-phosphate to eliminate 90 percent of rabbit muscle pyruvate kinase activity by covalently modifying a single lysine per subunit. In contrast to trinitrophenylation this modification is inhibited by PEP along with ADP and ATP.

Davidoff et al. (1973) achieved 50 percent inactivation of rabbit muscle pyruvate kinase through the modification of two lysines per tetramer with 3.5 dimethylpyrazolecarboxamidine. The reagent appears to be competitive with respect to Mn^{++}, Ca^{++}, K^{+} and NH_4 reduced the rate by which the enzyme is covalently modified. The effect of the substrates on the inactivation rate was not reported.

Sulfhydryl Modification

Pyruvate kinase is very sensitive to inactivation by sulfhydryl alkylating agents (Mildvan and Cohn, 1966; Jacobson and Black, 1971; Flashner et al., 1972; Chalkley and Bloxham, 1976; Bloxham et al., 1978). Despite extensive efforts to identify a catalytically essential cysteine, no clear picture has emerged to explain the data. Mildvan and Cohn (1966) used p-chloromercuribenzoate to inactivate rabbit muscle enzyme, finding ADP, ATP, PEP, and pyruvate all protected against inactivation. Stammers and Muirhead (1975) found that p-chloromercuribenzoate causes a loss in crystallinity of cat muscle pyruvate kinase, indicating a disruption in

the regular structure of the molecule.

Iodoacetamide inactivates rabbit muscle enzyme by alkylating two groups, one with a pK less than 6.8 and the other with a pK of 7.8. The alkylation of the more acidic group is inhibited by K^+ or MgADP but not by PEP, ADP or Mg^{++} along (Jacobson and Black, 1971). Flashner et al. (1972) used 5,5'dithiobis-(2-nitrobenzoate) to induce an intramolecular disulfide bond resulting in the loss of catalytic activity. The modification is prevented by PEP, Mg^{++} or MgADP but also causes a shift in the CD spectrum of the protein. Bloxham used two reagents 5-chloro-4-oxopentanoate and methyl methanethiosulfonate to specifically label the same site in rabbit muscle pyruvate kinase (Bloxham et al., 1978; Chalkley and Bloxham, 1976). However, the relative abilities of the substrates to protect against the inactivation is different in each case (Bloxham and Chalkley, 1976; Bloxham et al., 1978). Bloxham et al. (1978) concluded that modification by the latter reagent destablized the enzyme structure. Crystallographic work failed to find a reactive sulfhydryl near enough to the substrate binding sites to play a direct role in catalysis in cat muscle. The nearest methylmercury sites are 1.0 and 1.6 nm away (Stammers and Muirhead, 1975). It therefore appears possible that no cysteine is directly involved in catalysis but may instead be important in maintaining the structural integrity of the enzyme.

Histidine Modification

The dependence of catalytic activity on pH indicates the involvement of a group with a pK around that of histidine (Mildvan and Cohn, 1965; Wieker and Hess, 1971). The inactivation of rabbit muscle (Dann and

Britton, 1974) and of yeast (Bornmann and Hess, 1974) pyruvate kinase with the histidine specific reagent, diethylpyrocarbonate, supports the conclusion. Inactivation of pyruvate kinase by diethylpyrocarbonate results in the modification of 2 histidine residues per subunit of rabbit muscle enzyme. In both cases, the rate of the inactivation is reduced in the presence of activating divalent cations and the phosphorylated substrates PEP, ADP and ATP suggesting that the essential histidine (or histidines) is involved in phosphoryl transfer.

Arginine Modification

All glycolytic enzymes except triose isomerase are inactivated by arginine modifying reagents (Riordan et al., 1977). Kinetic analysis of 2,3-butanedione and phenylgloxal inactivation of rabbit muscle pyruvate kinase reveals that modification of a single arginine per subunit is sufficient to completely inactivate the protein (Berghauser, 1977; Cardemil and Eyzaguirre, 1979). The presence of ATP and PEP decreases the rate of inactivation while pyruvate and lactate have little or no effect. These findings suggest that an arginine is located near the phosphate binding site of PEP.

Affinity Labeling

Affinity labels designed to specifically modify the PEP binding site have not been successful. Yun and Suelter (1979b) used bromopyruvate to inactivate yeast pyruvate kinase. The presence of PEP fails to protect against inactivation and the properties of the inactivated enzyme are similar to the properties of the enzyme modified by the sulfhydryl modifying reagent, 5,5'-dithiobis-(2-nitrobenzoic acid) (Yun and Suelter, 1979a).

The authors concluded that the incorporation of bromopyruvate was not in the catalytic site of the enzyme.

Wyatt and Colman (1977) have used 5-fluorosulfonylbenzoyladenosine to specifically inactivate rabbit muscle pyruvate kinase. The reactive sulfonyl fluoride moiety is thought to be located in a position analogous to the gama-phosphoryl group of ATP. The inactivation is biphasic with two moles of label incorporated per mole of enzyme subunit. However, modification of the nucleotide binding site cannot explain the protection of substrates. High concentrations (10mM) of ATP or ADP alone do not protect against inactivation in spite of the fact that they should bind to the enzyme even in the absence of Mg^{++}. However, PEP, Mg^{++}, or MgADP are effective protectors. Another affinity analog used by Berghauser and Geller (1974), adenosine-5-(2,3-dibromohydrogensuccinate), inactivated pig heart pyruvate kinase. Here again, 10 mM ATP did not protect.

While most analogs with reactive groups on the base do not inactivate the enzyme (Hampton _et al_., 1978; Hampton and Slotin, 1975), those with reactive groups which extend outside the active site will inactivate some pyruvate kinases. Using an iodoacetamido alkyl N^6 ADP, Hampton _et al_. (1978) inactivated the L isozyme of rate but not type M or K. Alkyl groups less than eight carbons long have no effect on any of the isozymes, indicating the alkylation occurs outside the active site.

Mechanism of Catalysis

From chemical modification experiments, the same functional groups are involved in both allosteric and hyperbolic forms of the enzyme. In addition, physical resonance studies using paramagnetic metal ions suggest similar if not identical quaternary enzyme·PEP·ADP·divalent cation complexes

in both types of pyruvate kinase (Mildvan and Cohn, 1970). There is considerable debate as to the order in which the substrates bind to form this quaternary structure and if the order is the same in the allosteric forms as in the hyperbolic ones. Most studies on the kinetics of muscle pyruvate kinase indicate that the binding of the substrates is random (Ainsworth and MacFarlane, 1973; Mildvan and Cohn, 1966; Reynard et al., 1961). Reynard et al. (1961) found that the K_m for each substrate is independent of the concentration of the other substrate. These findings were questioned by Dann and Britton (1978). They failed to detect ADP binding in the absence of PEP using equilibrium dialysis and isotope trapping and suggested that the enzyme prefers to bind PEP before ADP.

The binding mechanism is much less clear with the allosteric enzyme. Kinetic analysis of an FdP activated brewer's yeast and E. coli indicates an independent ramdom formation of the quaternary complex (Boiteux et al., 1979). Analysis of the kinetics of FdP activated baker's yeast, however, reveals an ordered addition of PEP, ADP and then Mg^{++} to the enzyme with pyruvate released before MgATP (Macfarlane and Ainsworth, 1972).

Based on analysis of the kinetics of FdP activated pig liver pyruvate kinase Macfarlane and Ainsworth (1974) proposed ping pong mechanism with pyruvate leaving the enzyme before ADP binding. This reaction scheme would require a phosphorylated enzyme intermediate. Such an intermediate has not been found. Giles et al. (1976) could not find evidence for its existence on the pig liver enzyme using isotope exchange studies. Studies on other liver pyruvate kinases have not supported a ping pong mechanism. Dann and Britton (1977) could find no evidence for a phosphorylated enzyme intermediate on rabbit liver pyruvate kinase. Results from the kinetic

analysis of the human liver enzyme were consistent with a sequential mechanism. At present, the majority of the evidence supports either a random or sequential binding of the substrates to the liver enzyme. Further kinetic analysis of the pig liver pyruvate kinase is required to explain its inconsistent behavior.

Like most kinases, pyruvate kinase appears to undergo a conformational change upon binding of substrates. Binding of activating monovalent and divalent cations and PEP all alter in some way the conformation of the enzyme. These conformational changes were first detected by UV difference spectroscopy (Suelter and Melander, 1963; Kayne and Suelter, 1965; Suelter et al., 1966; Wilson et al., 1967). Analysis of the protein difference spectra suggest that the environment of one or more trytophan residues becomes more hydrophilic upon addition of either monovalent or divalent cations or PEP (Kayne and Suelter, 1965). Despite these conformationa changes sedimentation velocity experiments indicated that there are no major changes in the structural integrity of the enzyme (Wilson et al., 1967; Kayne and Suelter, 1968).

The use of paramagnetic divalent cations has greatly increased our understanding of the binding of substrates to pyruvate kinase. The EPR spectrum of the enzyme bound paramagnetic Mn^{++} monitors structural changes on the protein which involve ligand substitutions or alterations in the coordination sphere of the cation. Structural changes that alter the accessibility of the cation to the bulk solvent can also be monitored (Reed and Cohn, 1973). Upon formation of the binary Mn^{++}·enzyme complex, the EPR spectrum indicates moderate distortion of the electronic symmetry of the bound cation. However, formation of the ternary complex with PEP yields gross changes in the position and shape of the spectral lines.

Changes in the position of the lines indicate changes in the types of ligands which bind the cation to the enzyme while changes in the shape of the lines indicate changes in the exposure of the cation to the solvent (Reed and Cohn, 1973).

The number of water ligands in the primary coordination sphere of the bound paramagnetic divalent cation can be deduced from proton magnetic resonance relaxation rates (PRR). In the binary enzyme·Mn^{++} complex the mean number of water molecules coordinated to Mn^{++} was estimated to be three (Reuben and Cohn, 1970). When the ternary complex is formed with PEP the number of water molecules bound drops to 0.5 (James et al., 1973).

Direct evidence for conformational changes upon the formation of the ternary complex has come from NMR experiments which are able to calculate distances from the monovalent cation to the divalent cation in the active site. Thallium 205 which can be monitored in NMR will activate pyruvate kinase about 60 percent as effectively as K^+ (Kayne, 1971). Relaxation times of TI^+ are greatly affected by the electron nuclear interactions of the unpaired electronic spin of Mn^{++} and can be used to calculate the distance between the two cations. Using this technique, Reuben and Kayne (1971) observed a change in distance between the two cations from 0.82 to 0.49 nm upon addition of PEP to the binary enzyme·Mn^{++} complex. Similar results are seen when TI^+ is replaced by monomethylamine (Nowak, 1976) or Li^+ (Hutton et al., 1977). The structural shift is not seen when the non-activating dimethyl, trimethyl and tetramethyl ammonium salts are used (Nowak, 1976). Oxalate, phosphoglycolate and pyruvate can replace PEP in inducing this conformational change (Nowak, 1978b). The shift induced by pyruvate is smaller suggesting the enzyme·Mn^{++}·ATP·pyruvate

structure may be more open, facilitating the release of products.

No conformational changes have been detected upon the binding of either ADP or ATP to the enzyme (Nowak, 1978b; Reed and Cohn, 1973; Kwan et al., 1975). The inability to perturb the electronic environment of the enzyme bound Mn^{++} not only suggests a lack of conformational change but indicates that the nucleotide is not directly coordinated to the enzyme bound divalent cation (Sloan and Mildvan, 1976).

The conformational change induced by PEP binding appears to be doing more than creating the proper orientation of substrates in the active site. Recent work done by Rao et al. (1979) using ^{31}P NMR has shown large shifts in the phosphate resonance peak of PEP upon formation of the enzyme ternary and quaternary complexes. Such a shift can be explained by a distortion in the O-P-O bond angle (Gorenstein and Kari, 1975). This distortion could destabilize the phosphate linkage, facilitating the catalysis of its transfer.

II. METHODS

Materials

Biochemical reagents, such as substrates, TNBS, lactate dehydrogenase, trypsin and 2-mercaptoethanol were purchased from Sigma Chemical Corporation. Iodoacetamide was from Aldrich Chemical Co. Chemicals used in the synthesis of PTH-ε-TNP lysine and dansyl chloride modification were from Pierce Chemical Co. Ammonium sulfate was purchased from Schwartz/Mann. Biogel P-6, Biogel A-1.5 m and AG50W-2X were from Bio-Rad Labs while carboxylmethyl Sephadex was from Sigma. All reagents were of reagent grade or highest grade available.

Solvents and Standard Solutions

Deionized, distilled water was used in making all solutions. KOH and HCl were used to adjust pH unless otherwise stated. KOH was used in the place of NaOH in washing resins due to the inhibitory effect of Na^+ on pyruvate kinase.

Pyridine was distilled over ninhydrin and stored in dark containers at 4° for up to 3 months. Constant boiling HCl was prepared by mixing equal volumes of concentrated HCl and H_2O before distilling. The first 10-15 percent of the distillate was discarded. The later distillate having a boiling point of 108° was saved.

Phenol red indicator used in peptide mapping was made up by dissolving 0.1 g phenol red in 28.2 ml freshly prepared 0.1 N NaOH and 221.8 ml H_2O. The ninhydrin protein stain was prepared by dissolving 0.25 g

ninhydrin in 50 ml acetone and 2.5 ml acetic acid. Ninhydrin-collidine was prepared by mixing 60 ml n-butanol, 20 ml acetic acid, 8 ml collidine and 0.1 g ninhydrin.

Bovine Muscle Tissue

Bovine muscle used in the preparation of pyruvate kinase was obtained from freshly slaughtered cows. The muscle, usually from the neck, was either used the same day or frozen at -80°. Muscle from the jaw was also used but found to contain more fat and connective tissue, resulting in lower yields.

Isolation of Bovine Muscle Pyruvate Kinase

Bovine skeletal muscle pyruvate kinase was purified as described by Cardenas *et al*. (1973). The homogenization buffer was changed to 0.02 M tris-HCl, pH 7.5, 1 mM EDTA and 10 mM 2-mercaptoethanol, resulting in better yields during the extraction step.

Fractions with the highest specific activities from the carboxymethyl Sephadex column were rechromatographed on Biogel A-1.5 m in 50 mM potassium phosphate and 10 mM 2-mercaptoethanol. The enzyme fractions were precipitated by dialysis against saturated ammonium sulfate, 10 mM 2-mercaptoethanol and approximately 5 mM $MgCl_2$, pH 7.0 at 4°. The enzyme could be stored 2-3 months under these conditions. The homogeneity of the enzyme was confirmed by SDS polyacrylamide gel electrophoresis.

Enzyme Assays

All enzyme assays were performed using a Beckman Acta III spectrophoto-

meter equipped with a Lauda circulating water bath that maintained the temperature at 25°. The pyruvate kinase reaction was coupled to that of lactate dehydrogenase (Bucher and Pfleiderer, 1955). The standard assay was carried out in 0.05 M imidazole-HCl, pH 7.0, 0.1 M KCl, 0.01 M $MgCl_2$, 2 mM ADP, 1 mM PEP, 0.16 mM NADH and approximately 6 units (μmoles per min) of lactate dehydrogenase. Pyruvate kinase catalyses the production of pyruvate from PEP which is converted to lactate by the lactate dehydrogenase. The production of one mole pyruvate to lactate involves the conversion of one mole NADH to NAD^+. The loss of NADH is followed by monitoring the decrease in absorbance of the assay medium at 340 nm. Therefore, upon the addition of a rate limiting amount of pyruvate kinase to the standard assay medium in a 1.0 cm path length cuvette, the activity can be determined using the equation:

$$\frac{\Delta A}{min} \times \frac{0.161 \times 10^{-6} mole}{ml} \times \frac{\text{Volume of assay}}{\text{Volume of sample}} = \frac{\text{Unit}}{ml}$$

A unit of enzyme is that amount of enzyme that will convert one micromole of substrate per min.

The decarboxylation of oxalacetate to pyruvate was also assayed coupled to lactate dehydrogenase. Fresh oxalacetate solutions were prepared daily by dissolving solid oxalacetate in H_2O (4 mg/ml) and incubating for 10 min at room temperature. An equal volume of 0.1 M triethanolamine-HCl, pH 7.6 was added and the mixture was allowed to sit for 15 min. The pH was adjusted to 7.6 and the solution was kept on ice until used. In the standard assay, 0.1 ml oxalacetate was added to 1.0 ml 0.05 M triethanolamine-HCl, pH 7.6, 0.1 M KCl, 1 mM $MnSO_4$, 0.2 mM NADH and 6 units of lactate

dehydrogenase and the decrease in absorbance at 340 nm was monitored. From the immediate decrease of the absorbance of NADH at 340 nm upon the addition of oxalacetate, it was determined that less than 5 percent of the oxalacetate, had already decarboxylated to pyruvate. This remained constant throughout the experiment. The non-enzymatic decarboxylation in the presence of 1 mM Mn^{++} was determined in each assay. These values which averaged 5-6 pM, were substracted from the decarboxylation rates in the presence of enzyme.

Determination of Protein Concentration

The concentration of pyruvate kinase was usually determined using the extinction coefficient of 0.55 ml/mg/cm at 280 nm or using the ratio of absorbance at 280 to that at 260 nm of 1.71 (Cardenas *et al.*, 1973). When determining protein concentration in a hetereogeneous mixture, the Folin-Ciocaltequ method was used as described by Clark (1964), using bovine serum albumin as the standard.

Determination Of Substrate Concentration

ADP concentrations were determined from the absorbance difference at 340 nm upon the addition of less than 1.5×10^{-7} moles ADP to 1.0 ml 0.05 M imidazole-HCl, pH 7.5, 0.1 M KCl, 0.01 M $MgCl_2$, 0.5 mM PEP, 0.16 mM NADH and approximately 6 units each lactate dehydrogenase and pyruvate kinase. The difference in absorbance in a 1.0 cm pathlength cuvette can be converted to moles per liter of ADP using

$$\frac{\Delta A}{6.22 \times 10^{3}\ 1/\text{mole}} \times \frac{\text{Volume of assay}}{\text{Volume of sample}} = \frac{\text{moles}_{ADP}}{l}$$

PEP concentrations were determined using the same procedure with 2 mM ADP replacing 0.5 mM PEP in the assay medium.

SDS Polyacrylamide Gel Electrophoresis

Analysis of protein by SDS electrophoresis was carried out using the system described by Laemmli (1970). Discontinous gels were run with either a 15 or 7.5 percent separating gel with a 3 percent stacking gel. Protein samples containing ammonium sulfate were dialyzed against H_2O prior to electrophoresis in order to prevent the precipitation of the SDS. A micro-slab gel apparatus was used (Matsudaira and Burgess, 1978), which resolved the proteins at 200 volts at 4° in 10-50 min. The stained and then destained gels were dried between sheets of Bio-Rad gel slab cellulose backing membrane. The dried gels could then be permanently stored.

Isolation Of Blocked N-Terminal Residue

Isolation of the N-terminal blocked amino acid was done using a modified scheme as described by Yoshida (1972). Approximately 15 mg S-carboxyamidated pyruvate kinase was digested with 0.5 mg pronase in 10 mM tris-HCl, pH 7.0, 1 mM $CaCl_2$ at 37° for 24 hr. The pH of the digest was adjusted to 3.0 with formic acid and then placed on a 1.5 x 10 cm AG50W-2X (H^+) column. The column was washed with cold H_2O. The first 75 ml which eluted from the column was neutralized with freshly prepared 0.5 N NaOH and lyophilized. This material was then dissolved in 5 ml 25 mM tris-HCl, pH 7.5 containing 0.1 M NaCl and digested with 1 mg carboxypeptidase A for 8 h at 37°. The digest was again acidified to pH

3.0 with formic acid and washed through a fresh AG50W-2X (H^+) column, collecting the first 25 ml, neutralizing and lyophilizing the sample as described above. The dried sample was dissolved in 2.0 ml H_2O, of which 0.25 ml was applied directly to the amino acid analyzer, 0.25 ml was hydrolyzed in constant boiling HCl for 3 h at 110° before analysis and 0.25 ml was digested with hog kidney acylase before analysis. The analysis of the unhydrolyzed sample revealed no free amino acids present.

Sulfhydryl Modification

S-carboxyamidation and S-carboxymethylation were carried out as described by Means and Feeney (1971). A typical modification involved denaturation of 230 mg enzyme in 50 ml 0.5 M tris-HCl, pH 8.5,5 mM dithiothreitol, 2 mM EDTA and 8 M urea. The lyophilized protein was found not to be readily soluble, so whenever possible, native protein dialyzed overnight against cold H_2O was used. The solution was flushed with N_2, capped tightly and incubated for 2 h at 45°. Enough iodoacetamide (or iodoacetate) was added to bring the solution to 10 mM. After 30 min at room temperature, the reaction was stopped by the addition of sufficient solid dithiothreitol to bring its concentration to 55 mM. The protein was dialyzed at 4° for at least 24 h against several changes of H_2O. Care was taken to exclude light from the reaction and dialysis to prevent the formation of iodine which could modify tyrosine and histidine residues.

Amino Terminal Analysis Using Dansyl Chloride

Amino terminal analysis using dansyl chloride (Gray, 1972) was carried out on native bovine muscle pyruvate kinase that had been dialyzed 24 h against several changes of H_2O. Approximately 125 μg in 50 μl

10 percent SDS and denatured by boiling for 3 min. Once cooled to room temperature, 50μl of sequential grade N-ethyl morpholine was added and mixed thoroughly. The protein was then reacted for 1 h at room temperature with 15μl dansyl chloride in anhydrous dimethylformamide (25 mg/ml). The labeled protein was precipitated with 0.5 ml acetone and pelleted using a clinical centrifuge. The precipitate protein was washed with 80 percent acetone before drying under N_2 in a 50° bath. After addition of 0.5 ml constant boiling HCl to the dried protein, the tube was sealed *in vacuo* and then placed in a 110° oven for 18 h. The hydrolysate was taken up in 10μl 50 percent aqueous pyridine.

Polyamide sheets (5x5 cm) coated on both sides were used in the separation and identification of the dansyl amino acids. On one side, 1μl of the pyruvate kinase hydrolyzate was spotted while on the opposite side 0.05 nmole of a mixture of standard dansylated amino acids was applied. Ascending chromatography with 1.5 percent formic acid was carried out in a 150 ml glass beaker covered with parafilm. After drying, the sheet was turned 90° and developed with benzene/acetic acid (9:1). Using an ultraviolet lamp to visualize the spots on the dried sheet the unknown dansylated amino acids were identified by referring to the location of the standard dansylated amino acids on the opposite site. Further resolution could be achieved by developing the chromatograph with ethyl acetate/acetic acid/ methanol (20:1:1) in the same direction as the benzene/ acetic acid development.

Trinitrophenylation

Approximately 100-150 mg pyruvate kinase in 10 ml was dialyzed against three changes, one liter each, of 50 mM potassium phosphate,

pH 8.0. The enzyme was inactivated by titration at 0° in the dark with a one percent solution of TNBS. When the enzymatic activity had decreased to about 10 percent of the original value, one ml 1.0 M tris-HCl, pH 7.0 was added. The protein was was washed by precipitation at least three times with saturated ammonium sulfate, redissolving the protein after each precipitation with 0.1 M tris-HCl, pH 7.0 containing 10 mM 2-mercapto-ethanol. The final precipitate was dissolved in a minimum volume of distilled water and dialyzed against several changes of water overnight at 4°. The number of TNP-lysine residues per mole of protein was determined from the protein absorbance at 280 and 346 nm. The extinction coefficient for TNP-lysine used was 1.45×10^{4} l/mole/cm (Okuyama and Satake, 1960).

Rate constants for the trinitrophenylation reaction were determined on enzyme dialyzed 2-4 h at 4° against inactivation buffer, typically 0.05 M imidazole-HCl, 0.1 M KCl, pH 7.8. The enzyme was then diluted to 10 units/ml (0.7 μM) with inactivation buffer and allowed to react with TNBS (5-100 μM) in a covered tube at room temperature. At appropiate times 10 μl was removed, deluted with 50 μl 0.10 M tris-HCl, pH 7.2 and assayed.

Precautions were made to maintain the ionic strength at 0.15 M when rate constants at different pHs were determined. From pH 7.0 to 8.5 0.1 M triethanolamine-HCl, 0.05 M KCl was used with the pH adjusted with triethylamine. Borate (0.1 M) buffers were used from pH 8.0 to 10.5. The pH was adjusted with KOH and ionic strength adjusted with KCl.

Peptide Mapping

Peptide mapping of bovine muscle pyruvate kinase was done as

described by Chernoff and Liu (1961). Pyruvate kinase was dissolved in 0.2 M ammonium bicarbonate, pH 8.5 giving a final protein concentration of 10 mg/ml. The protein was denatured by placing in a boiling H_2O bath for 3-5 min. After cooling to room temperature the pH was readjusted to 8.5 and a drop of phenol red indicator added. The protein was digested at 36° by the addition of 0.1 mg trypsin per 10 mg pyruvate kinase. After approximately 3 h the protein digest was divided into 0.5 ml portions, dried in a vacuum desiccator and stored at -20°.

Chromatography was done on Whatman 3 MM paper (36 x 56 cm). The protein digest was dissolved in a minimum volume chromatography buffer, n-butanol/pyridine/acetic acid/H_2O (15:10:3:12) and then spotted on the upper left hand corner of the folded chromatography paper as shown in Figure 6. The digest was spotted by applying 5-10 μl portions to the paper and then drying with warm air using a hair dryer. The spot was usually 0.5-1.0 cm in diameter. The location of the spot was marked lightly with a pencil and the sheet was placed in a standard chromatography cabinet which had contained 200-300 ml chromatography buffer for at least 3 h. The paper was placed so that the fold was immersed in the buffer tray and held there by a glass rod. Descending chromatography was run for approximately 21 h at room temperature. The sheet was dried in a 90° oven which was connected to the laboratory hood to provide adequate ventilation. The paper was then gently folded along a line between the origin and the yellow phenol red spot. A glass rod was placed under the fold as shown in Figure 7. The paper was wetted with electrophoresis buffer, pyridine/acetic acid/ H_2O (25:1:250), pH 6.5 from both sides of the raised fold. The peptides which chromatographed along this fold were washed into a sharp line by the advancing buffer fronts

Figure 6

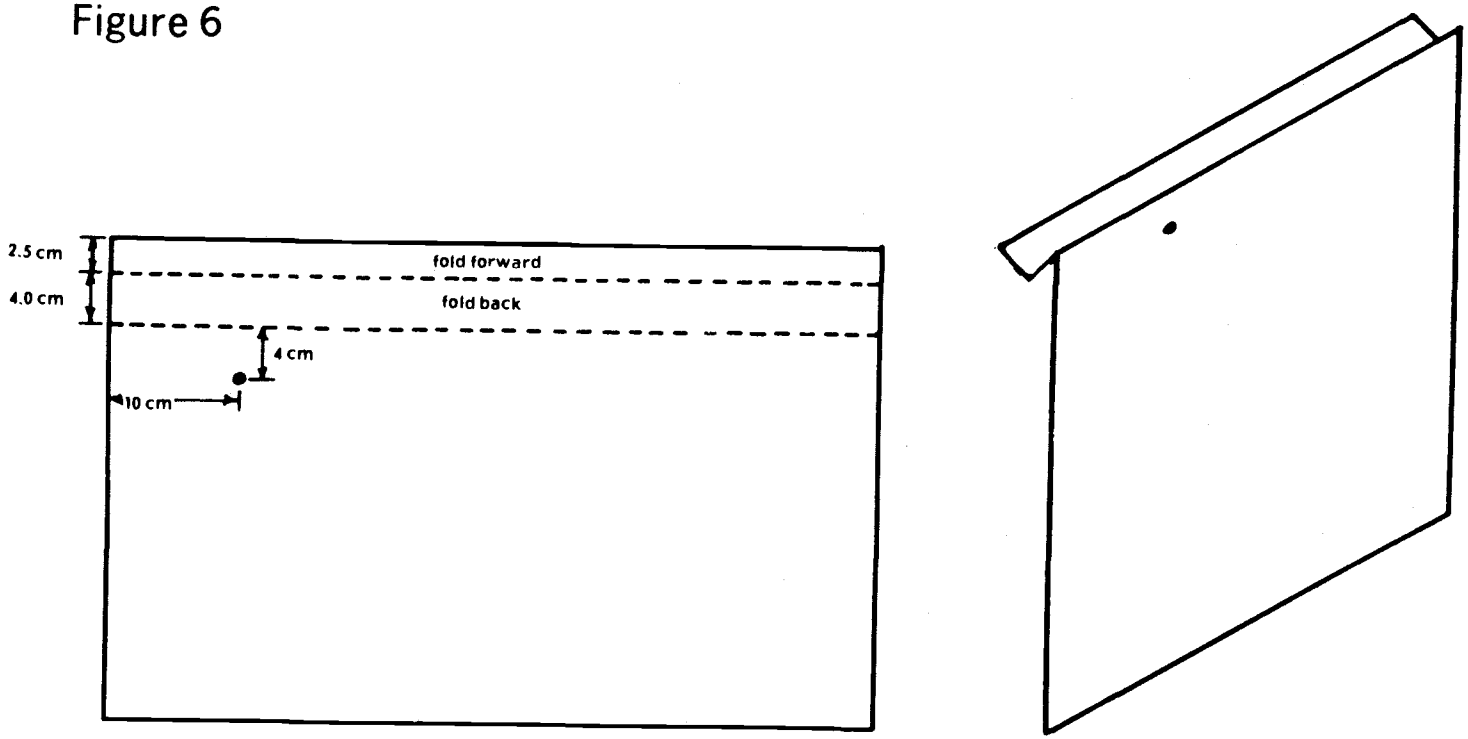
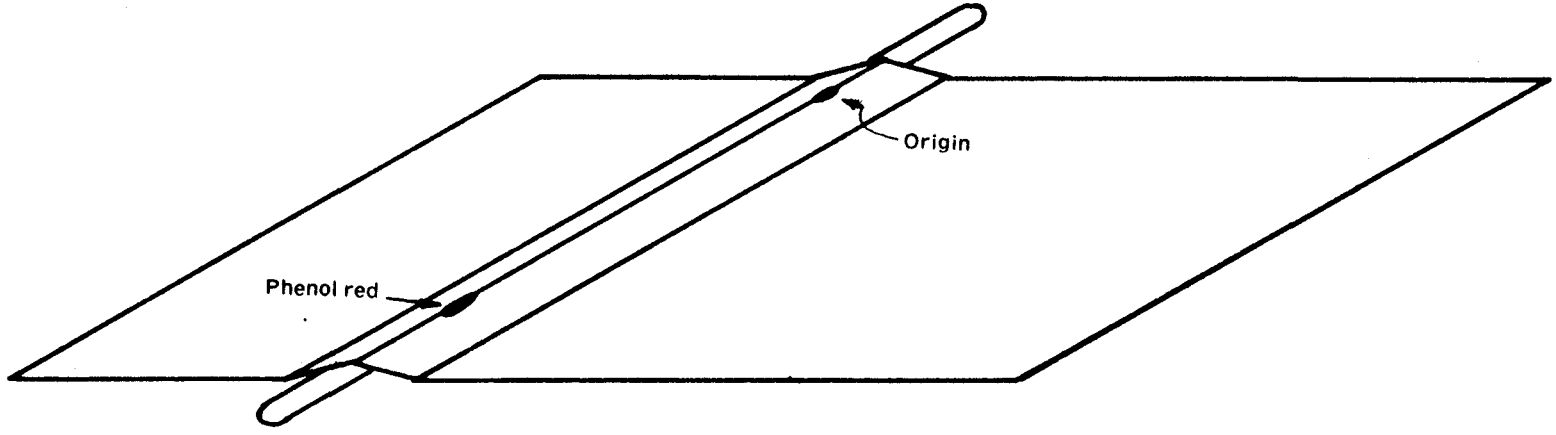


Figure 6. The 36 x 57 cm Whatman 3mm sheet was first folded forward 2.5 cm from the top along its long axis, then folded back 4 cm lower. The digest was spotted 10 cm from the left edge and 4 cm below the second fold.

Figure 7



from each side. Excess buffer was mopped up with a paper towel. Electrophoresis was carried out in a Savant HVE paper electrophoresis tank. The sheets were hung over the electrophoresis rack with the origin and indicator spots on the side going into the anode bath. The electrophoresis was run for 90 min at 2000 volts. The sheet was dried in a 90° oven for 30 min or overnight at room temperature. The best staining results were obtained by using freshly prepared ninhydrin-collidine. The sheet was stained either by spraying or by gently applying the stain with a Pasteur pipet. The chromatographs were developed at 90° for 10-15 min. The spots were then immediately marked with a pencil as they tend to fade with time. Polaroid photographs were also taken as soon as possible. The best results were obtained using Polaroid positive/negative type 55 film.

High Voltage Electrophoresis

High voltage electrophoresis was carried out using the FP22B Savant flat plate system cooled with a circulating water bath at 4°. Peptides were spotted on 5 x 57 cm strips of Whatman 3 MM paper. The following electrophoresis buffers were used:

pH 3.5	100 ml pyridine 1000 ml acetic acid 1890 ml H_2O
pH 6.4	200 ml pyridine 8 ml acetic acid 2792 ml H_2O

The strips containing the dried peptides were wetted with electrophoresis buffer around the spotted sample so that the advancing buffer fronts concentrated the sample into a very small spot. Electrophoresis was first run for 10 min at 500 volts to desalt the sample then increased

to 2000 volts for usually 30-45 min. The strip was then dried and stained with ninhydrin, ninhydrin-collidine or o-phthaldaldehyde. The ninhydrin stained strips were developed by heating in a 110° oven for 10 min. Staining by o-phthaldaldehyde was done as described by Lai (1977). The strips were first sprayed with a one percent triethylamine solution in acetone containing 0.05 percent 2-mercaptoethanol. After briefly (5 min) drying in a hood the strips were sprayed with 0.03 percent o-phthaldaldehyde solution in acetone. The peptides were visualized using an ultroviolet lamp.

Isolation of the TNP-Peptide

Approximately 100 mg trinitrophenylated protein was S-carboxymethylated, suspended in 0.5 percent ammonium bicarbonate, pH 8.0 and digested with 2 mg trypsin at 36°. The protein was solubilized after about one h. After three h the digestion was boiled and lyophilized. The lyophilized digest was dissolved in a minimum volume 0.5 percent ammonium bicarbonate and chromatographed on a 1.8 x 100 cm Biogel P-6 column at room temperature. Peptide elution was monitored at 230 nm and by fluoresence production with o-phthaldaldehyde. The absorbance of the TNP-peptide at 346 nm was used to locate the labeled peptide.

Detection of Peptides Using o-Phthaldaldehyde

Detection of peptides in the column effluent was carried out using o-phthaldaldehyde (Lai, 1977). From each tube of effluent to be analyzed, 50 μl was removed and placed in a clean test tube. The samples were then dried in an oven at 110°. Once cooled, 1.6 ml of 0.5 M sodium borate, pH 9.7 and 0.4 ml o-phthaldaldehyde (0.3 g/l), 0.015 percent 2-mercapto-

ethanol was added. Upon vigorous mixing, the fluoresence intensity was measured with excitation at 340 nm and emission at 455 nm.

Synthesis of PTH-ε-TNP-Lysine

ε-TNP-lysine was synthesized as described by Hubbard and Cardenas (1975). Polylysine (15 mg) in 1.0 ml 0.1 M potassium phosphate, pH 8.0 was added to 1.0 ml 20 mM TNBS and incubated overnight at room temperature in a covered test tube. The TNP-polylysine was precipitated by the addition of 1.0 ml 2 N HCl. The precipitate was collected by centrifugation and then washed three times by suspension in 2 N HCl followed by centrifugation. After the final centrifugation the pellet was suspended in 1.0 ml constant boiling HCl and hydrolyzed *in vacuo* at 110° for 24 h. The hydrolyzate was diluted with 5.0 ml H_2O and washed with several volumes diethyl ether. The aqueous phase was saved and dried. The PTH derivative was produced by dissolving 0.6 mole of the TNP-lysine in 0.5 ml 50 percent aqueous pyridine under N_2. Twenty μl of phenyl isothiocyanate was added under N_2, the solution capped tightly, mixed vigorously, and incubated for 20 min at 50°. The solution was washed with 400 μl heptane/ethyl acetate (10:1) twice and heptane/ethyl acetate (2:1) three times. The organic phases were discarded and the final aqueous phase was dried *in vacuo*. The dried product was then converted to the PTH form by adding 40 μl 1 N HCl in methanol and incubating for 10 min at 50°. The product was dried *in vacuo*.

Amino Acid Analysis

Amino acid analysis was carried out on lyophilized samples hydrolyzed in constant boiling HCl for 24 h *in vacuo* at 110°. The amino acid

concentrations were determined from automated integration of individual peaks using known standards.

III. RESULTS

N-terminal Analysis

Attempts to determine the amino terminal residue of bovine muscle pyruvate kinase using automated Edman liquid phase sequencing techniques were unsuccessful. Several cycles were run on both unmodified and S-carboxylamidated enzyme with no detectable PTH-amino acid released. The limiting factor in applying the lyophilized protein to the cup was its low solubilitiy in 50 percent acetic acid. Sufficient molar quantities, however, were applied to exceed the sensitivity of the detection system used. Dansyl chloride modification of the whole muscle protein resulted in only the dansylation of the ϵ-amino group of lysine and the hydroxyl group of tyrosine.

Since no primary amino group was detected using the above techniques, attempts were made to isolate the blocked amino terminal amino acid. The isolation was based on the ability of amino acids and peptides with a free amino terminal to bind to a cation exchange resin at low pH (Yoshida, 1972). The enzyme was thoroughly digested with pronase and those peptides with a blocked amino terminal were washed through a column of AG50W-2X at pH 2. The fragments eluting in the void volume were redigested with carboxypeptidase A. After chromatographing again on AG50W-2X the eluting residues were digested by acid hydrolysis or by hog kidney acylase. As shown in Table 2, the predominate amino acid found was serine. The isolation procedure will induce the formation of pyroglutamate whenever there is a free amino terminal glutamyl residue (Narita *et al.*, 1975). Since pyroglutamate is not a substrate for acylase (Birmbaum, 1955)

the large amount of contaminating glutamate was not seen when the pool was hydrolyzed with acylase.

Table 2

α Amino Blocked Residues from Bovine Muscle Pyruvate Kinase

Amino Acid	Acid Hydrolysis	Acylase Digestion
Asx	.41	.21
Ser	1.00	1.00
Glx	.66	.20
Gly	.20	.43
Ala	.20	.20

Table 2 Amino acid analysis of AG50W-2X (H^+) pool after acid hydrolysis or hog kidney acylase digestion. The molar ratios relative to serine are listed. All other amino acids present in amounts that were less than 10 percent that of serine.

TNBS Inactivation of Muscle Pyruvate Kinase

Determination of the rate constants of inactivation.

A plot of the logarithm of pyruvate kinase activity versus the time incubated with TNBS is linear down to about 40 percent of the initial activity (Figure 8). The slope of the linear portion of the line can be used to determine a first order rate constant and half-life for the inactivation. As seen in Figure 8 the inactivation is not complete. Approximately 10 percent of the initial activity remains after prolonged treatment with TNBS. In order to compare rate constants using a wide variety of TNBS concentrations and therefore following the reaction over different extents of the inactivation, the data was analyzed as described

by Levy et al. (1963). The rate at which active enzyme, E_a, is modified to modified enzyme, E_m, can be described as

$$\frac{dE_m}{dt} = -\frac{dE_a}{dt} = kE_a \qquad (1)$$

where t is time, k is the first order rate constant and E_a TNBS. Integration gives the value of E_a at any time during the course of the reaction.

$$E_a = E_a^o e^{-kt} \qquad (2)$$

where E_a^o is the initial concentration of active enzyme. When the inactivation is stopped at some particular time, t, the activity of the enzyme is equal to the activity of the unmodified enzyme plus the residual activity of the modified enzyme. The initial enzyme activity A^o is equal to the rate constant, k_a, for the reaction times the total enzyme concentration.

$$A^o = k_a E_a^o \qquad (3)$$

When all the enzyme has been modified, the activity, A^∞, is

$$A^\infty = k_m E_a^o \qquad (4)$$

where k_m is the rate constant for the enzymatic reaction with the modified enzyme. At any time, t, the activity will be

$$A = k_a E_a + k_m E_m \qquad (5)$$

Since $E_m = E_a^o - E_a$ we can substitute and obtain

$$A = k_a E_a - k_m E_a + k_m E_a^o \qquad (6)$$

Transposing and substituting equations 2,3,4 and 6 we obtain

$$A - A^{\infty} = (A^{o} - A^{\infty})e^{-kt} \qquad (7)$$

Taking the logarithm of this equation yields the general first order rate equation

$$\ln(A - A^{\infty}) = \ln(A^{o} - A^{\infty}) - kt \qquad (8)$$

With A^{∞} being 10 percent of the A^{o} the plot of log $(A - A^{o})$ versus time results in a straight line with a slope equal to k (Figure 8).

Ionic Strength Effects

The rate of trinitrophenylation was found to be greatly influenced by the ionic strength of the solution. Table 3 gives the rate constant and half-life for the trinitrophenylation reaction at various ionic

Table 3

Salts Added	Ionic Stength(M)	Rate Constant (min^{-1})	half-life (min)
None	0.01	.235	2.9
0.1M KCl	0.11	.056	12
0.2M KCl	0.21	.029	24
0.1M TMACl	0.11	.047	15
0.1M KCl plus 0.1 M TMACl	0.21	.031	22
0.1M NaCl	0.11	.071	9.7

Table 3. Effect of monovalent cations on the first order rate constant and half-life of trinitrophenylation. Pyruvate kinase (0.02 mg/ml) was incubated in a solution containing 0.05 M imidazole-HCl pH 7.8 1 x10^{-5} M TNBS and the indicated concentration of salts.

Figure 8

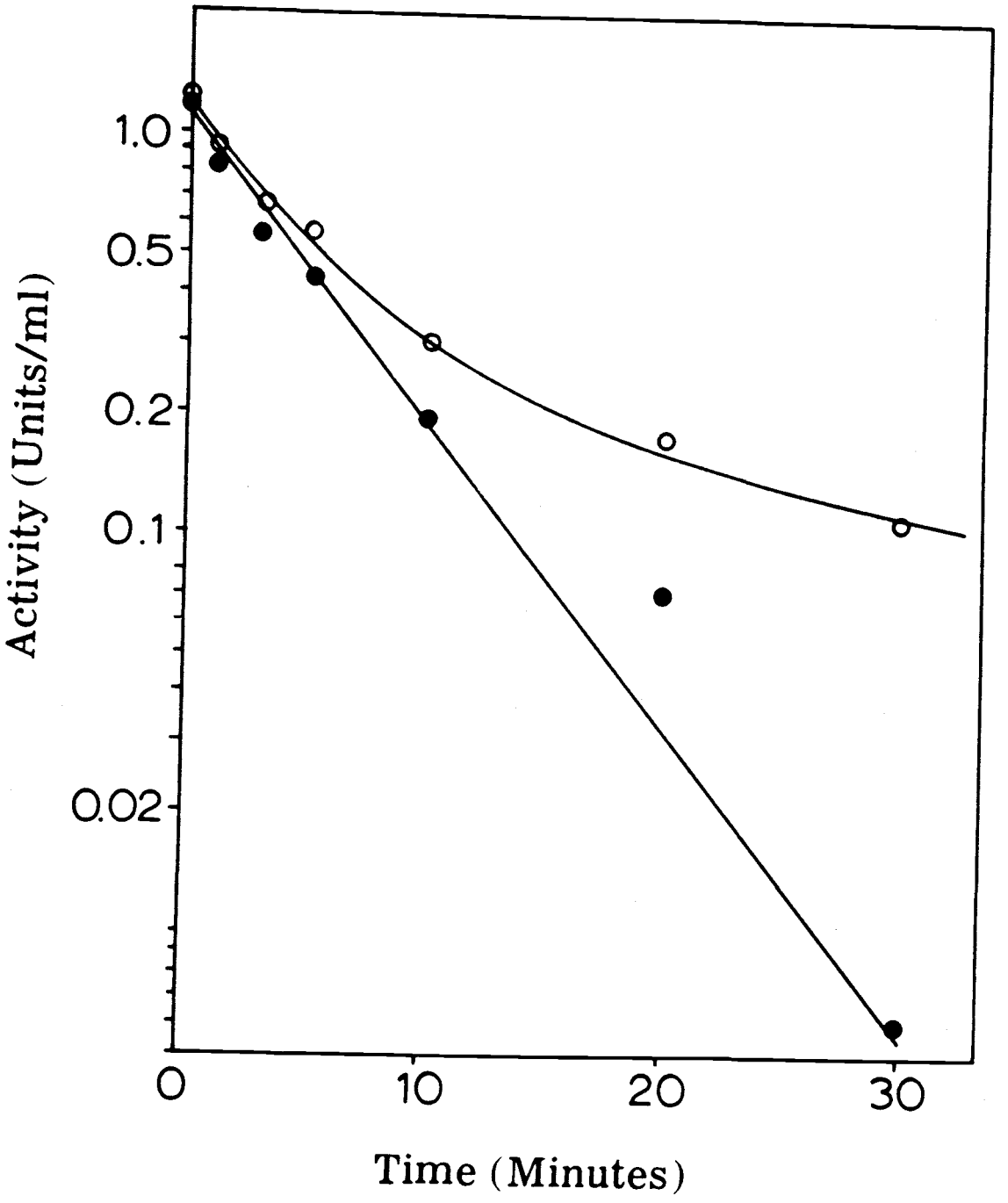


Figure 8: Irreversible inactivation of pyruvate kinase by TNBS. Pyruvate kinase at a concentration of 0.04 mg/ml was incubated at room temperature in the dark in 1.0 ml 0.05 M imidazole·HCl, pH 7.8, 0.1 M KCl and 3.7×10^{-5} M TNBS. Aliquots were removed at the indicated times and assayed as described in "Methods". The activities are plotted versus time in the upper curve. The activity minus the residual activity is plotted versus time in the lower curve. The residual activity was equal to 10 percent of the initial activity. The linearity of the lower curve fits Equation 8.

strengths. The activating potassium chloride, the poorly activating sodium chloride (Cardenas et al., 1973) or the non-activating and non-competitive tetramethylammonium chloride (Nowak, 1976) are equally effective in decreasing the rate of enzyme inactivation, suggesting that the effect is not a specific cation-enzyme interaction but rather a general ionic strength effect.

Protection by Substrates

Both ADP and ATP effectively protect the enzyme, (E), against inactivation by TNBS, (I). The protection in the presence of these substrates, (S), can be quantitated as described by Scrutton and Utter (1965).

$$E + I \xrightarrow{k_1} EI \qquad (9)$$

$$E + S \overset{K_d}{\rightleftharpoons} ES \qquad (10)$$

$$ES + I \xrightarrow{k_2} EIS \qquad (11)$$

$$E_T = E + ES \qquad (12)$$

where k_1 and k_2 are the rate constants for the inactivation without and with substrate present, respectively, K_d is the dissociation constant for the enzyme substrate complex and E_T is the total unmodified enzyme. The loss of unmodified enzyme with time, t, in the presence of excess I will be

$$-\frac{dE}{dt} = k_1E + k_2ES \qquad (13)$$

Equations 9 - 12 can be rearranged to yield

$$E = E_T \left[\frac{K_d}{K_d+S}\right] \tag{14}$$

$$ES = E_T \left[\frac{S}{K_d+S}\right] \tag{15}$$

Substitution of these into equation 13 yields

$$-\frac{dE_T}{dt} = k_1 E_T \left[\frac{K_d}{K_d+S}\right] + k_2 E_T \left[\frac{S}{K_d+S}\right] \tag{16}$$

Integration of 16 with an excess of substrate yields

$$-\ln E_T = \left[k_1 \frac{K_d}{K_d+S} + k_2 \frac{S}{K_d+S}\right] t - \ln E_T^o \tag{17}$$

where E_T^o is the initial enzyme concentration.

The slope (v) of a plot of log E_T versus t is

$$v_o = \frac{1}{2.303} k_1 \tag{18}$$

with no substrate present and

$$v_s = \frac{1}{2.303}\left[k_1 \frac{K_d}{K_d+S} + k_2 \frac{S}{K_d+S}\right] \tag{19}$$

when the substrate is present. Equations 18 and 19 can be combined

$$\frac{v_s}{v_o} = \frac{K_d}{K_d+S} + \frac{k_2}{k_1}\frac{S}{K_d+S} \tag{20}$$

and rearranged to

$$\frac{v_s}{v_o} = \frac{k_2}{k_1} + K_d \frac{1 - v_s/v_o}{S} \tag{21}$$

Therefore, the plot of the ratio v_s/v_o versus $\frac{1 - v_s/v_o}{S}$ yields a line with a slope K_d and an intercept of k_2/k_1.

Figures 9, 10 and 11 are plots showing the protection of ADP, MgADP and MgATP, respectively. In each, the y-intercept approaches zero indicating that the ability of TNBS to modify the enzyme·nucleotide complex is small compared to its ability to modify the uncomplexed enzyme. The dissociation constants calculated from the plots for ADP, MgADP and MgATP were 5.5 mM, 0.84 mM and 0.49 mM, respectively.

The ability of other phosphate compounds to protect against inactivation by TNBS was also investigated. The reduction in the rate of inactivation by AMP (Figure 12) appears to be consistent with its relatively low affinity for the nucleotide binding site. Kinetic analysis of AMP competition for the ADP site results in a K_I of approximately 0.05 M (Figure 13). This figure is close to the K_I for AMP of 0.055 M reported by Plowman and Krall (1965). Phosphate, pyrophosphate and tripolyphosphate each reduce the rate of inactivation but because of the high concentrations required and the sensitivity of the inactivation to changes in ionic strength the results of these experiments are difficult to interpret. If it is assumed that the phosphate ion is completely dissociated in solution and thereby overestimating the ionic strength most of the effect of the phosphate ion on the rate of the inactivation can be accounted for by the change in ionic strength. The The half-time of the inactivation doubles in the presence of 25 mM pyrophosphate. Assuming complete dissociation, the ionic strength increases by 20 percent (from 0.30 to 0.36 M) which does not seem sufficient to account for the doubling of the half-life (Table 3). The half-life doubles in the presence of approximately 20 mM tripolyphosphate,

Figure 9

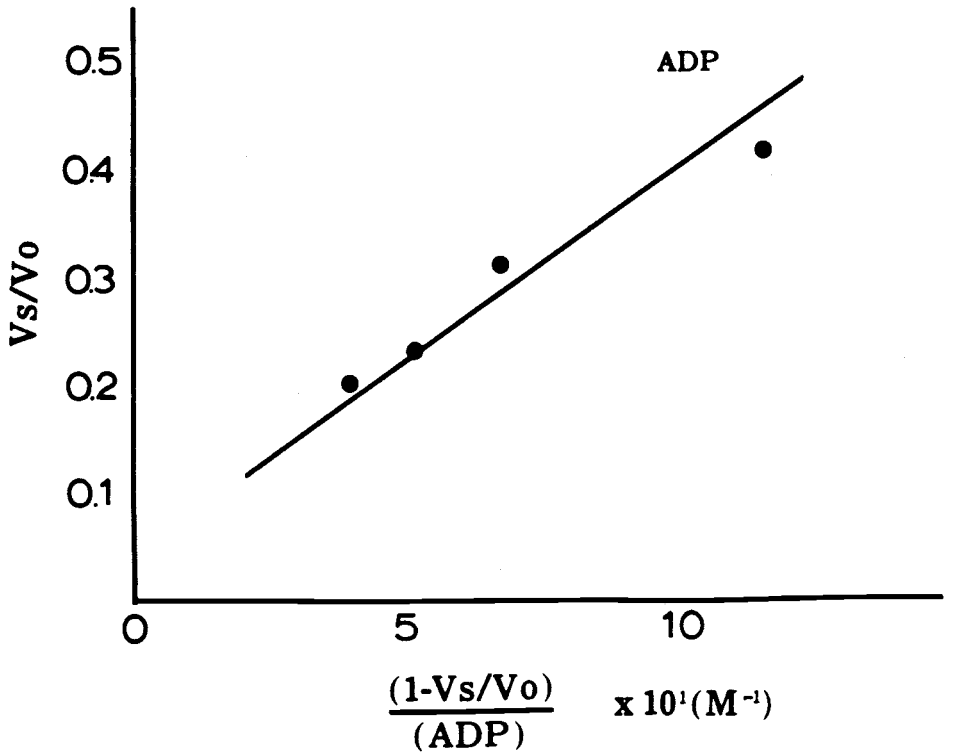


Figure 9. Determination of the dissociation constant for the pyruvate kinase·ADP complex. Pyruvate kinase at a concentration of approximately 0.01 mg/ml in 1.0 ml 0.05 M imidazole HCl, pH 7.8 and 0.1 M KCl was inactivated with 1.4×10^{-5} M TNBS in the presence of various amounts of ADP. Rate constants were determined using Equation 8 and plotted according to Equation 21. The slope of the plot gives a dissociation constant of 5.5 mM.

Figure 10

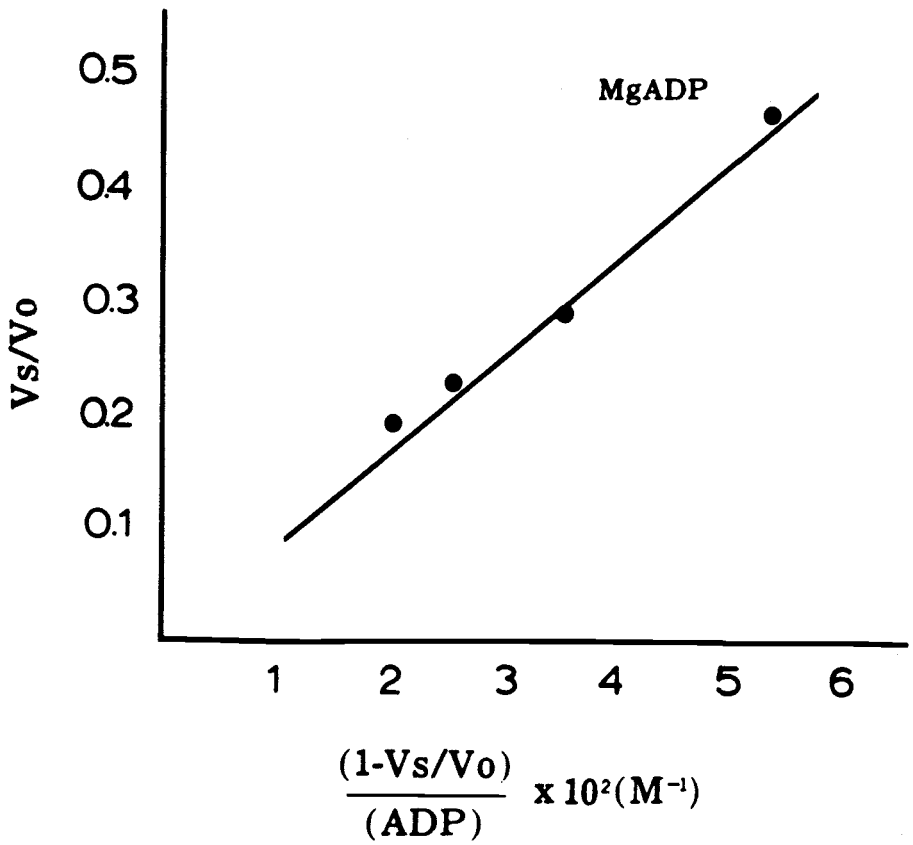
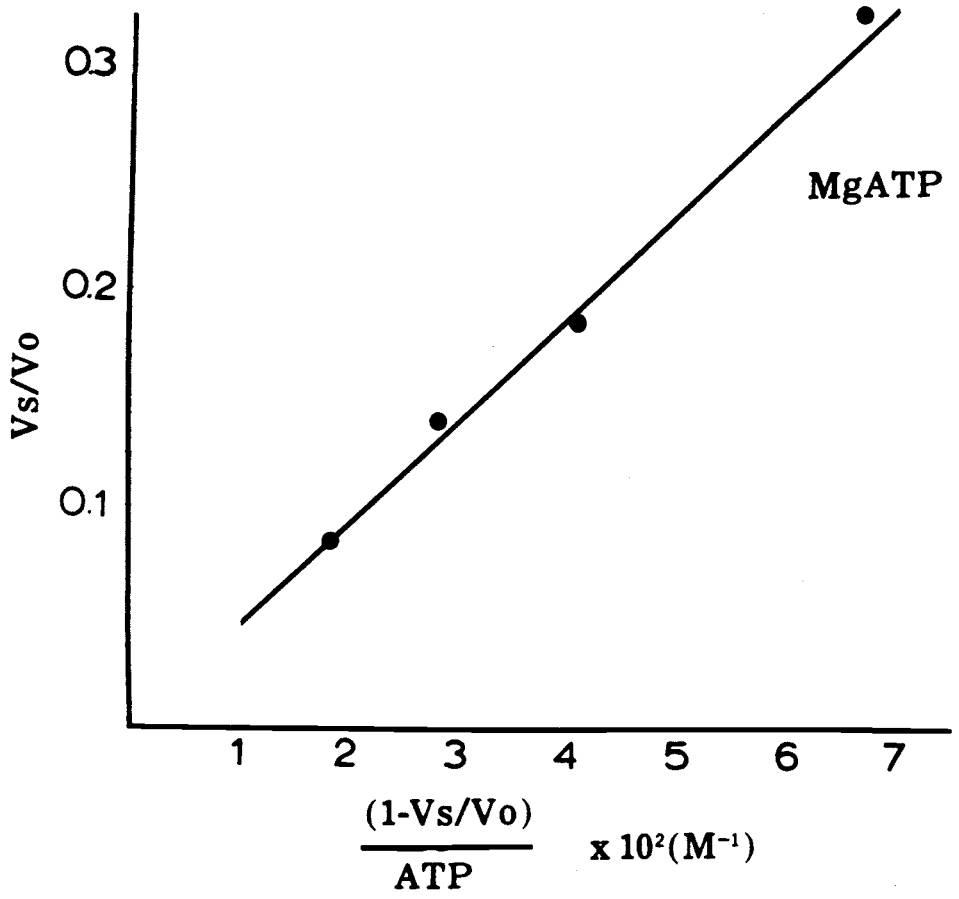


Figure 10. Determination of the dissociation constant for the pyruvate kinase · Mg^{++} · ADP complex. Pyruvate kinase at a concentration of approximately 0.01 mg/ml in 1.0 ml 0.05 M imidazole · HCl, pH 7.8, 0.1 M KCl and 0.01 M $MgCl_2$ was inactivated with 2.0×10^{-2} M TNBS in the presence of various amounts of ADP. Rate constants were determined using Equation 8 and plotted according to Equation 21. The slope of the plot gives a dissociation constant of 0.84 mM.

Figure 11



Figuure 11. Determination of the dissociation constant for the pyruvate kinase · Mg^{++} · ATP complex. Pyruvate kinase at a concentration of approximately 0.01 mg/ml in 1.0 ml 0.05 M imidazole · HCl, pH 7.8, 0.1 M KCl and 0.01 M $MgCl_2$ was inactivated with 1.4 x 10^{-5} M TNBS in the presence of various amounts of ATP. Rate constants were determined using Equation 8 and plotted according to Equation 21. The slope of the plot gives a dissociation constant of 0.49 mM.

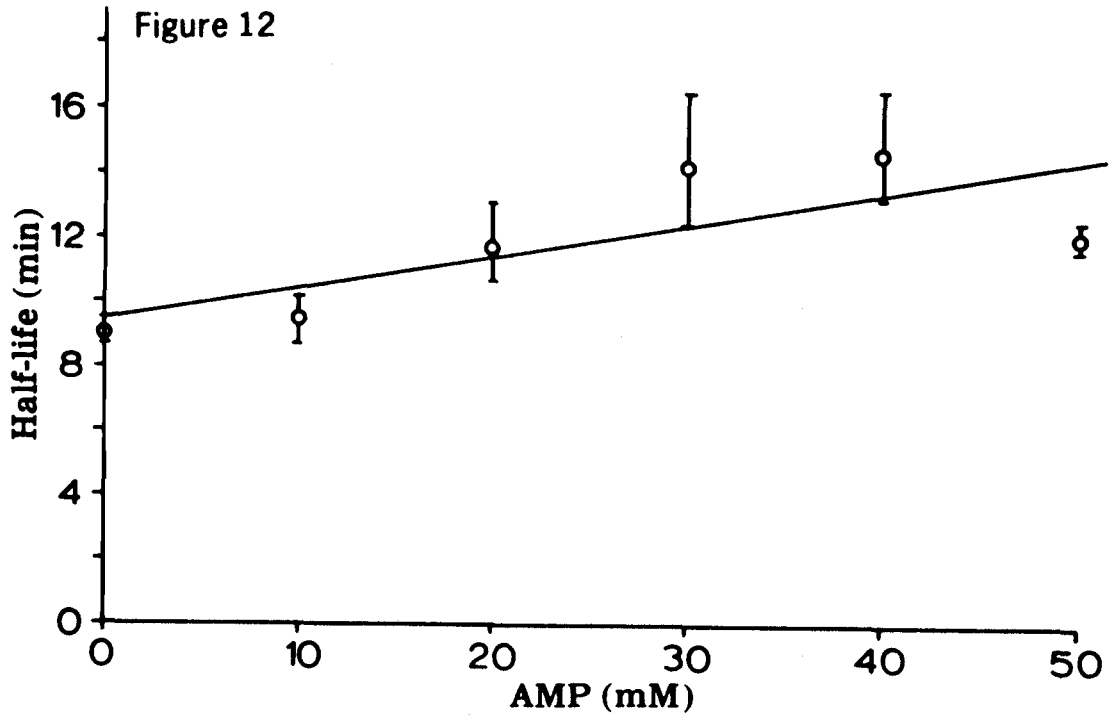


Figure 12 Effect of AMP on the rate of trinitrophenylation of pyruvate kinase. Pyruvate kinase at a concentration of approximately 0.01 mg/ml in 1.0 ml 0.05 M imidazole-HCl, pH 7.8, 0.1 M K^+ and 0.01 M free Mg^{++} was inactivated with 2.0×10^{-5} M TNBS in the dark at room temperature in the presence of varying amounts of AMP. The concentration of free Mg^{++} was determined using a dissociation constant of 2.04×10^{-2} M for MgAMP (Daniels and Alberty, 1975). The ionic strength was adjusted to 0.17 M with tetramethylammonium chloride. The half-lives of the trinitrophenylation reactions were determined from the rate constants of the reactions. The rate constants and 90 percent confidence intervals wre determined fitting the inactivation data to Equation 8.

Figure 13

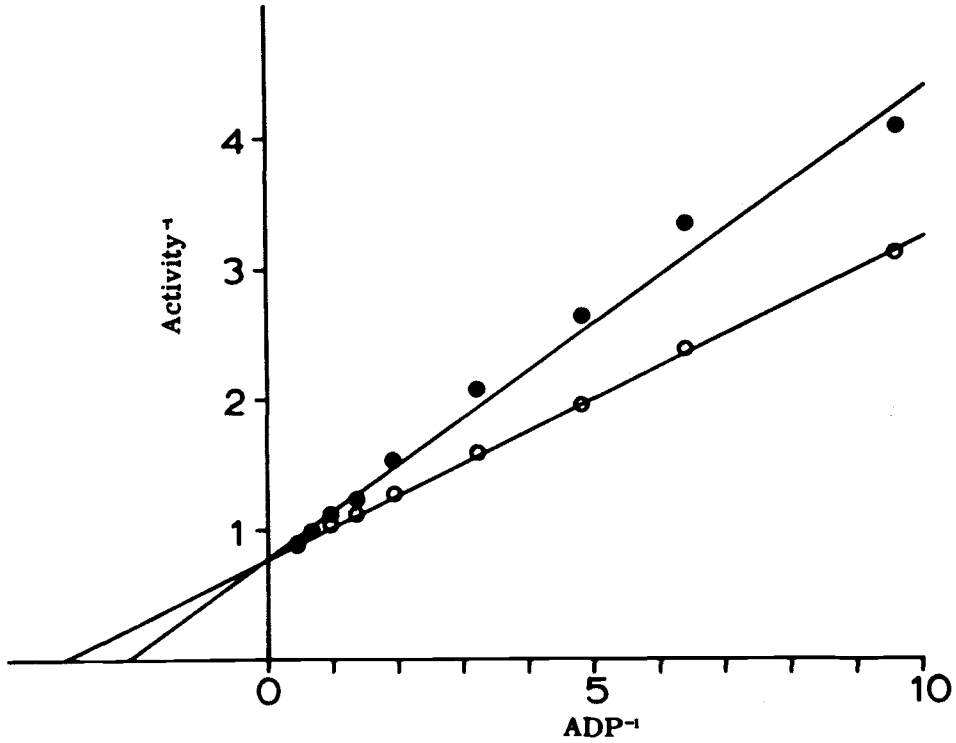


Figure 13. Inhibition of pyruvate kinase by AMP. Pyruvate kinase activity was measured as described in "Methods" in the presence of 0.05 M imidazole · HCl, pH 7.8, 0.10 M K^+ and 0.01 M free Mg^{++} (open circles) and plus the presence of 20 mM AMP (closed circles). The concentration of free Mg^{++} was determined using the dissociation constant of 2.04×10^{-2} M for MgAMP (Daniels and Alberty, 1975). The ionic strength was adjusted to 0.17 M with tetramethylammonium chloride.

which also increases the ionic strength a maximum of 30 percent.

The difficulty in interpreting the phosphate protection data is complicated by the inability to detect any kinetic inhibition of the enzyme by the phosphate ion. Rose (1960) using rabbit muscle pyruvate kinase reported that phosphate was a competitive inhibitor of ADP with a K_I of 18 mM. No inhibition was seen using bovine muscle pyruvate kinase at 20 mM phosphate and only 0.2 mM ADP. Pyrophosphate and tripolyphosphate were found to be too insoluble in the presence of Mg^{++} to allow kinetic inhibition studies.

Protection by Mg^{++}

There was no reduction in the rate of the inactivation seen in the presence of Mg^{++} alone. Figure 14 shows the plot of the inactivation in the presence and absence of 4 mM $MgCl_2$. In the presence of 10 mM $MgCl_2$ the slight decrease in the rate of the inactivation can be accounted for by the increase in ionic strength. It, therefore, does not appear that there is a specific divalent cation protection against trinitrophenylation of bovine muscle pyruvate kinase.

Kinetic Properties of Modified Enzyme

The effect of modification by TNBS on the binding of the substrates PEP and ADP is shown in Figures 15 and 16. Pyruvate kinase was inactivated to about 25 percent of its initial activity and then kinetically compared to the native enzyme. In both cases the K_m did not change after trinitrophenylation. The K_m for PEP and ADP were 6×10^{-5} M and 2.9×10^{-4} M, respectively, and in close agreement with those previously reported (Cardenas _et al._, 1973).

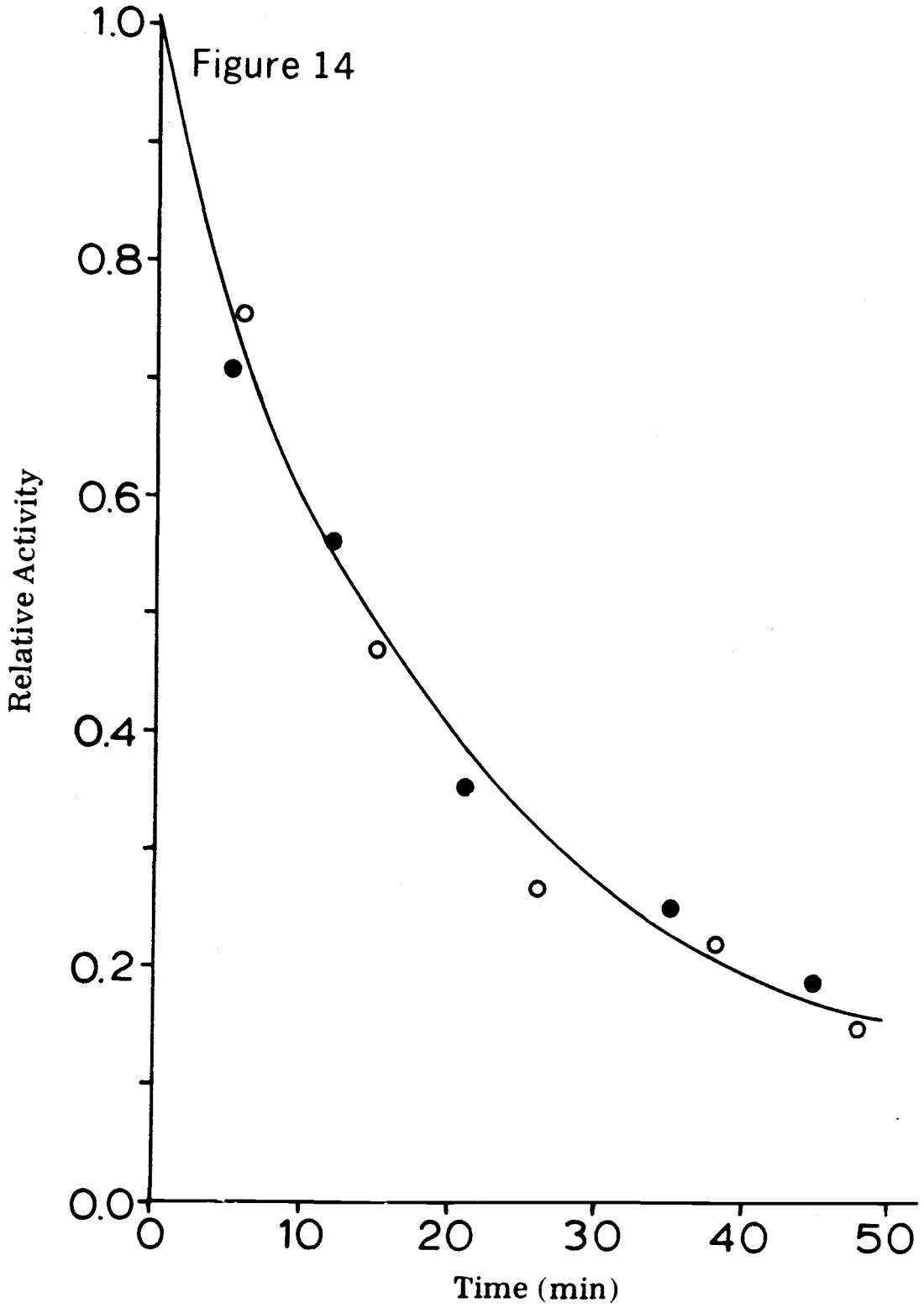


Figure 14 Effect of magnesium on trinitrophenylation of pyruvate kinase. Pyruvate kinase at a concentration of approximately 0.01mg/ml in 0.05 M imidazole · HCl, pH 7.8 and 0.10 M KCl was inactivated in the absence of Mg^{++} (closed circles) and the presence of 4.0 mM Mg^{++} (open circles) by 1.5 x 10^{-5} M TNBS. The inactivation was carried out at room temperature in the dark. The enzyme was assayed as described in "Methods".

Figure 15

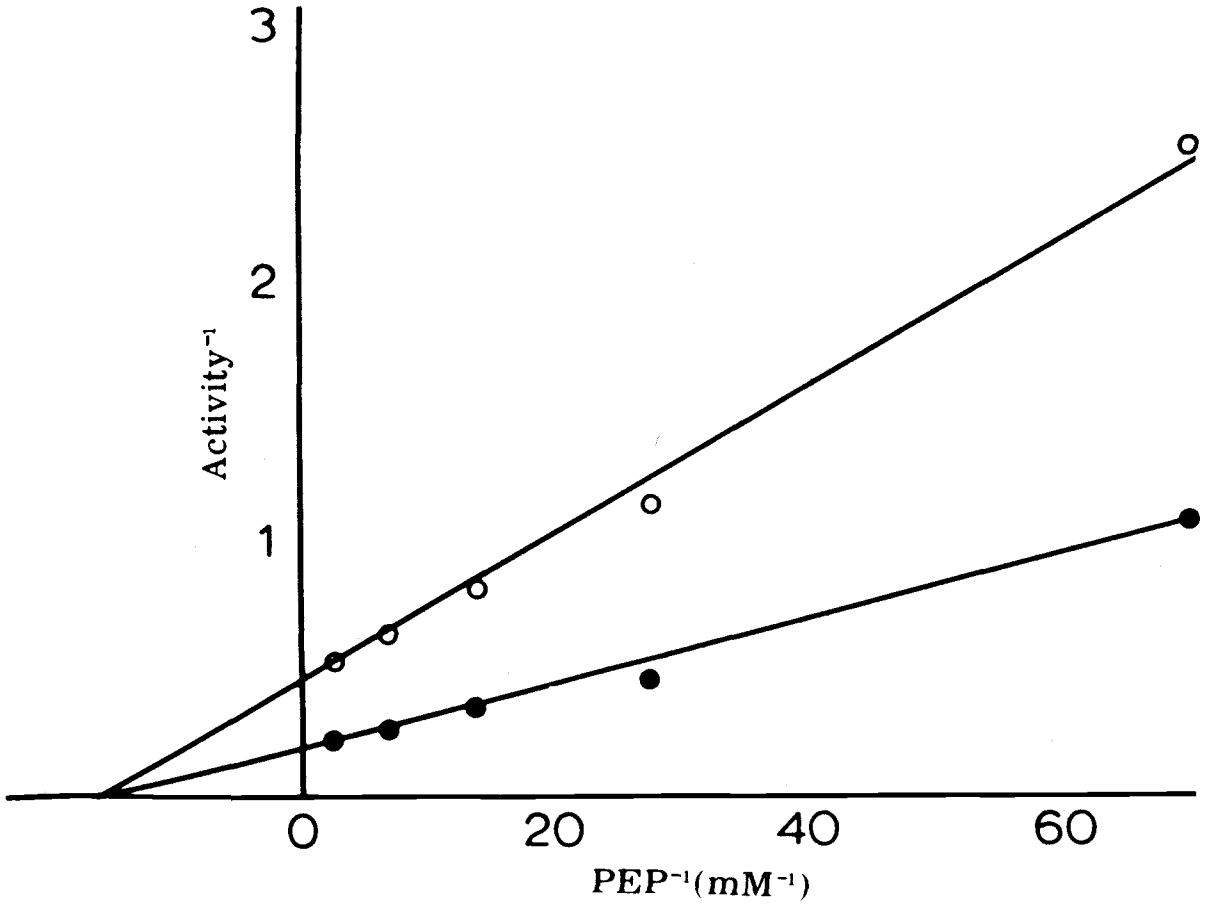


Figure 15. Effect of trinitrophenylation on the Km of PEP. Control (closed circles) and trinitrophenylated (open circles) pyruvate kinase were assayed as described in "Methods" at 2.0 mM ADP and varying amounts of PEP. The enzyme at a concentration of 0.01 mg/ml was trinitrophenylated by incubation for 1 h in 0.05 M imidazole HCl, pH 7.8, 0.10 M KCl, 0.01 M $MgCl_2$ and 1.2 x 10^{-5}M TNBS in the dark at room temperature. The protein was precipitated with saturated ammonium sulfate and then dialyzed for 3 h versus 0.05 M imidazole HCl, pH 7.0, 0.10 M KCl, 0.01 M $MgCl_2$ and 0.01 M 2 mercaptoethanol.

Figure 16

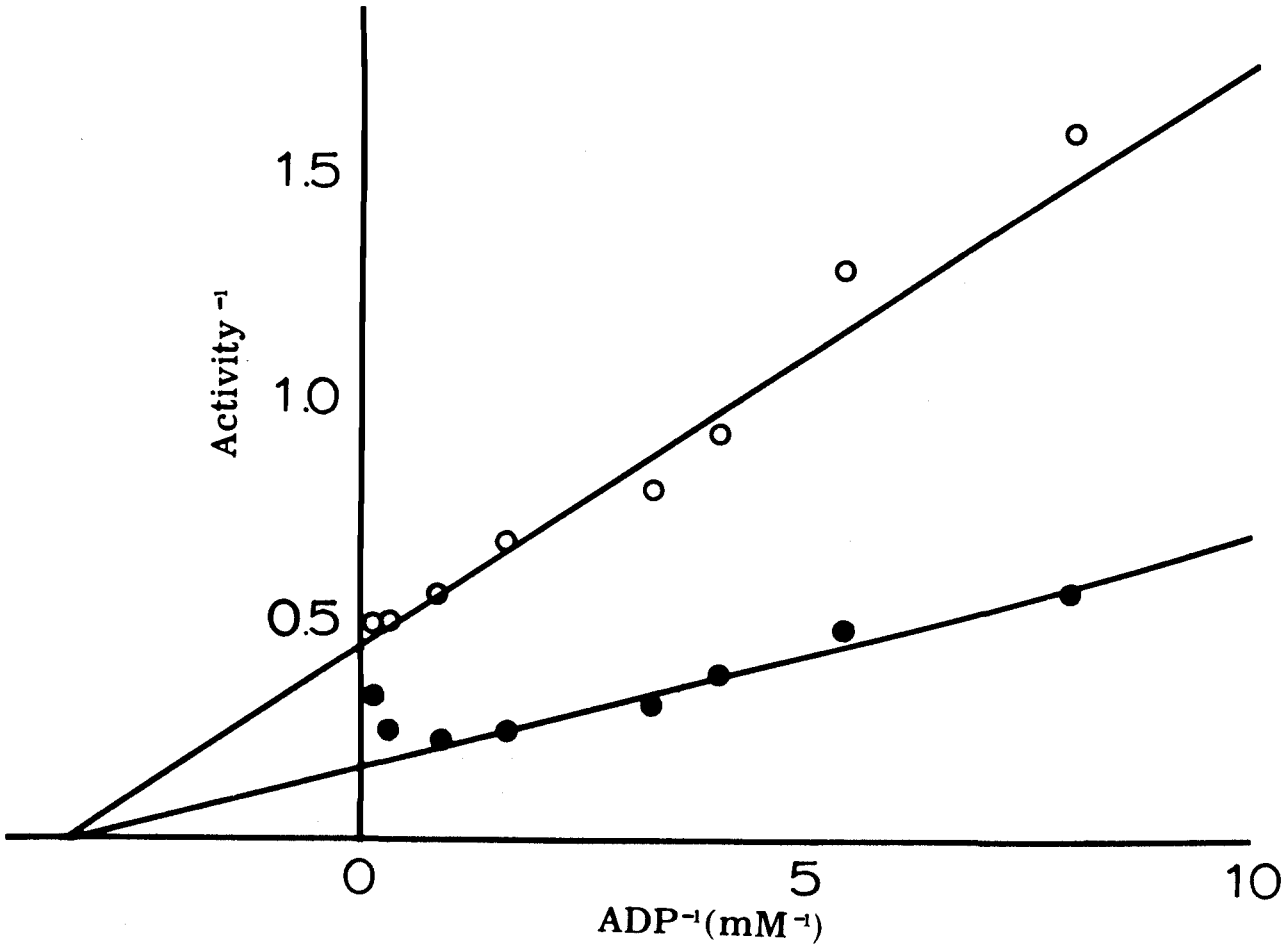


Figure 16 Effect of trinitrophenylation on the Km of ADP. Control (closed circles) and trinitrophenylated (open circles) pyruvate kinase were prepared as described in Figure 15. The enzyme was assayed as described in "Methods" with 0.25 mM PEP and varying amounts of ADP. The Km for ADP determined from this plot was 2.9×10^{-4} M.

Oxalacetate Decarboxylating Ability of Pyruvate Kinase

Like cod and rabbit muscle pyruvate kinase (Creighton and Rose, 1976b) bovine muscle pyruvate kinase catalytically decarboxylated oxalacetate in the presence of Mn^{++}. The oxalacetate decarboxylating activity of the enzyme was only about 0.15 percent of its kinase activity. Jursinic and Robinson (1978) reported that the rabbit muscle pyruvate kinase activity was much more sensitive to TNBS inactivation than the oxalacetate decarboxylase activity. Figure 17 shows the response of the two activities of bovine muscle pyruvate kinase to trinitrophenylation. Even when 85 - 90 percent of the kinase activity had been lost through modification, the enzyme still retained over 80 percent of its decarboxylase activity. The two activies, therefore, have different sensitivities to trinitrophenylation. The kinase activity which requires nucleotide binding is much more sensitive to modification than the decarboxylase activity which has no nucleotide requirement.

Effect of pH on Trinitrophenylation

The effect of pH on the rate of trinitrophenylation and on the catalytic activity of pyruvate kinase were compared and shown in Figure 18. The catalytic capacity of the enzyme decreases as the pH increases. This decreases in activity with increasing pH can be explained by a dependence upon a protonated residue with a pK around 8.3. However, this catalytically dependent residue does not appear to be the trinitrophenylated lysine. The rate of trinitrophenylation increases with pH, but the results are consistent with a pK of the ϵ-amino group of about 9.6. Since a conformational change has been reported upon binding of PEP (Reuben and Kayne, 1971), the effect of PEP upon the pH dependence of the

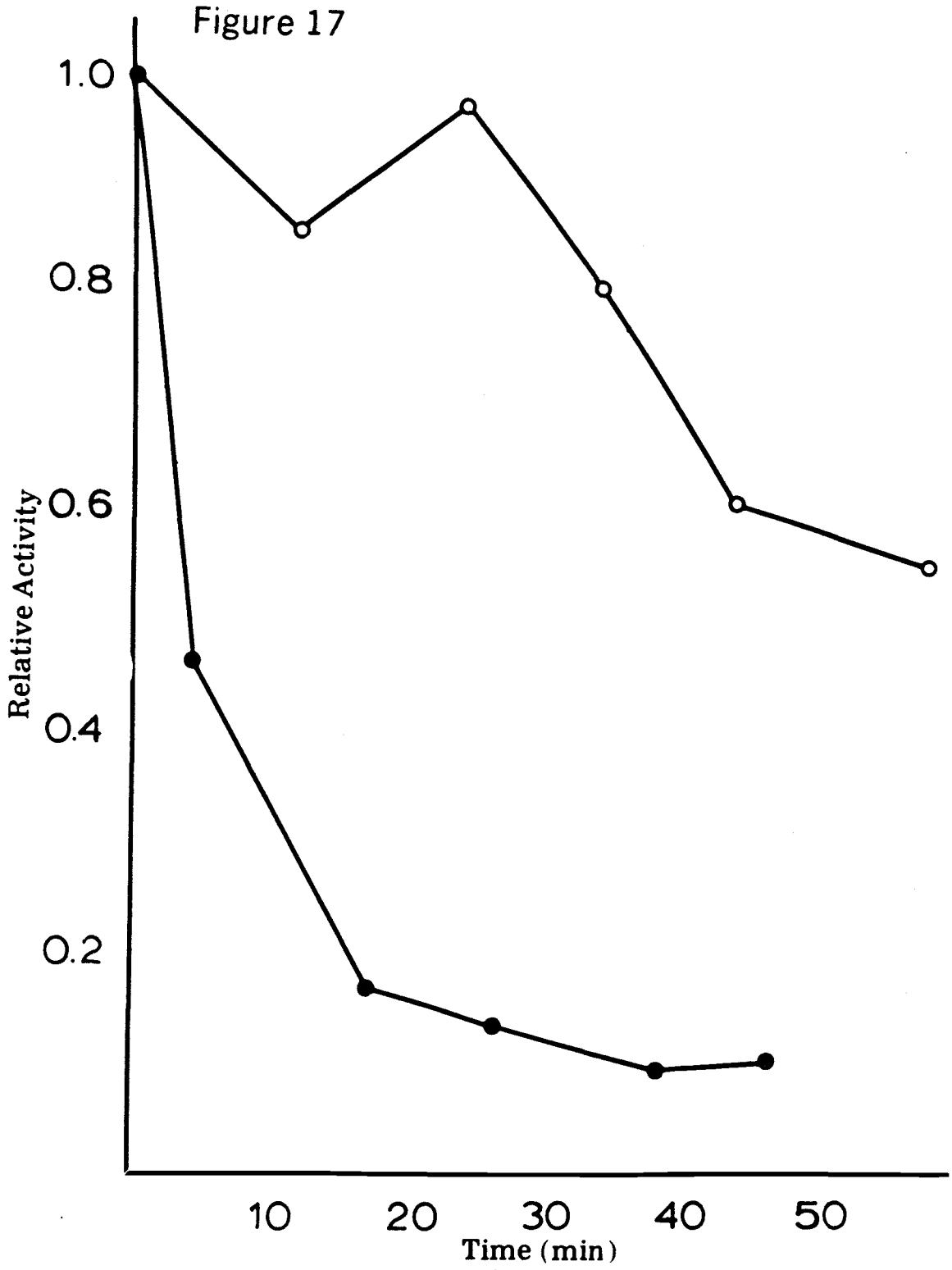


Figure 17. Inactivation of oxalacetate decarboxylase activity of pyruvate kinase by TNBS. Pyruvate kinase at a concentration of approximately 0.02 mg/ml in 0.05 M imidazole HCl, pH 7.8 and 0.10 M KCl was inactivated in presence of 2.6 x 10^{-5} M TNBS in the dark at room temperature. The pyruvate kinase activity (closed circles) and oxalacetate decarboxylase activity (open circles) were assayed as described in "Methods".

Figure 18

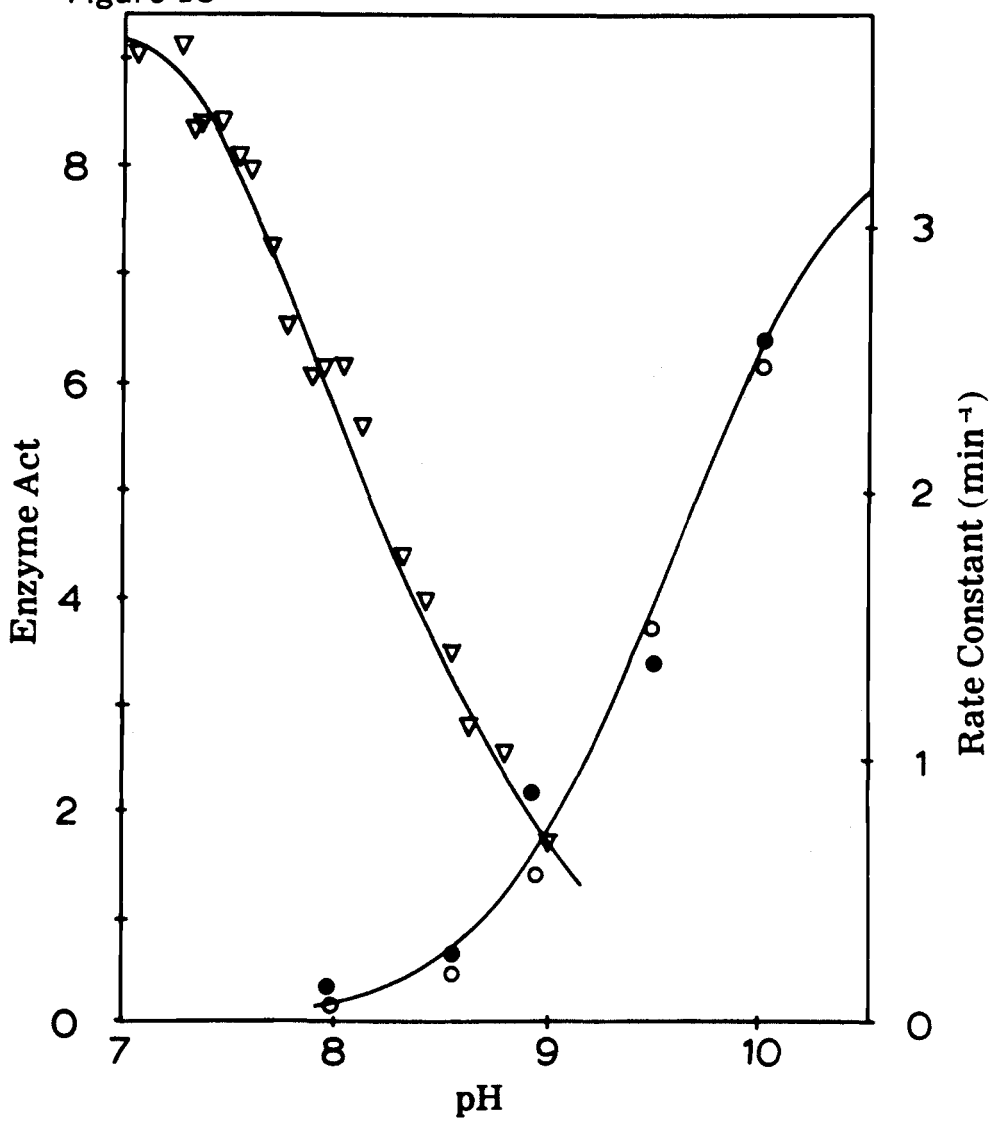


Figure 18. Effect of pH on pyruvate kinase activity and trinitrophenylation. Pyruvate kinase at a concentration of 0.01 mg/ml in 0.05 M borate, 0.15 M K^+ was inactivated with 6.5×10^{-4} M TNBS in the absence (open circles) and presence of 1.0 mM PEP (closed circles) at the indicated pH. The rate constants were determined using Equation 8. The activity of unmodified pyruvae kinase (open triangles) in 0.050 M triethanolamine · HCl, 0.1 M KCl, 2mM ADP, 1 mM PEP, 4 mM $MgCl_2$, .16 mM NADH and 5 units locate dehydrogenase was determined as described in "Methods". The pH was adjusted with triethylamine keeping a constant ionic strength of 0.2 M.

trinitrophenylation was studied. As shown in Figure 18 there was no significant shift in the pK of the lysine in the presence of PEP. Therefore any conformational change caused by the binding of PEP does not alter the pK of the trinitrophenylated lysine. The effect of pH on the binding of ADP was studied to see if the loss in catalytic activity was caused by a loss in the affinity for ADP. The K_m for ADP at pH 7.5 was 0.29 mM (Figure 16) and at pH it was 0.25 mM. Cardenas et al.(1973) reported the K_m for ADP to be 0.35 mM at the optimal pH 7.0. Therefore, the reduced activity of the enzyme at pH 7.8 is not due to a reduced affinity for ADP.

Peptide Mapping

An attempt was made to locate the trinitrophenylated tryptic peptide by peptide mapping. The two-dimensional paper chromatographic and electrophoretic pattern of the tryptic peptides is shown in Figure 19. Bovine muscle pyruvate kinase, having 4 identical subunits and 37 lysines and 33 arginines per subunit (Cardenas et al., 1973) could yield a maximum of 71 tryptic peptides. Approximately 60 spots could be resolved. Cottam et al. (1969) were able to distinguish 66-67 separate spots using rabbit muscle pyruvate kinase. No difference was ever detected between the maps using the TNBS modified and native enzyme.

Isolation of Trinitrophenylated Peptide

Bovine muscle pyruvate kinase was 90 percent inactivated by TNBS. Spectroscopic analysis of the trinitrophenylated protein at 280 and 346 nm indicated the presence of 0.75-0.80 trinitrophenyl groups per subunit.

Isolation of the TNP-peptide was done on Biogel P-6 in 0.5 percent

Figure 19

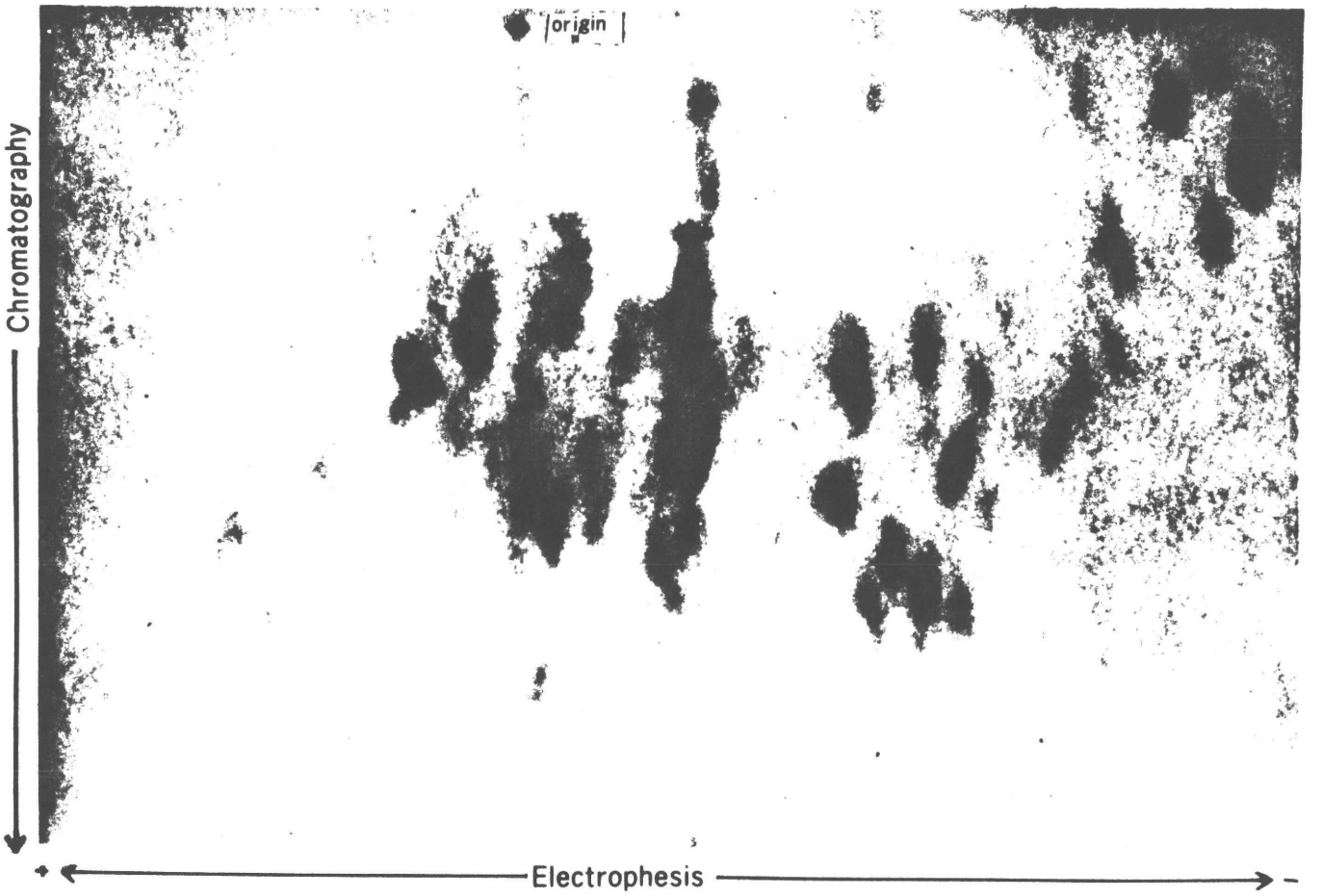


Figure 19. Tryptic peptide map of pyruvate kinase. Approximately 5 mg of the tryptic fragments of trinitrophenylated pyruvate kinase were separated by descending chromotography and high voltage electrophoresis as described in "Methods". The peptides were stained using freshly prepared ninhydrin - collidine.

ammonium bicarbonate (Figure 20). The TNP-peptide eluted just after the void volume and was found to be homogeneous by high voltage electrophoresis and high pressure liquid chromatography. The peptide failed to react with o-phthaldaldehyde which reacts with primary amines (Lai, 1977). Shown in Table 5 is the amino acid analysis of the isolated peptide. Being a tryptic peptide the ratios could be normalized to that of arginine. Despite the inability of the peptide to react with o-phthaldaldehyde, standard automated Edman liquid phase sequence analysis revealed an unblocked amino terminal alanine. The entire sequence is shown in Figure 21.

The PTH- -TNP-lysine was identified by thin layer chromatography by comparing its mobility to a synthesized standard. The sample, residue 25 of the peptide, and the standard were spotted on opposite sides of a polyamide sheet. The chromatographs were developed using water/acetic acid (2:1) or toluene/heptane/acetic acid (2:1:1). The sample and standard had identical mobilities in both solvent systems.

Figure 20

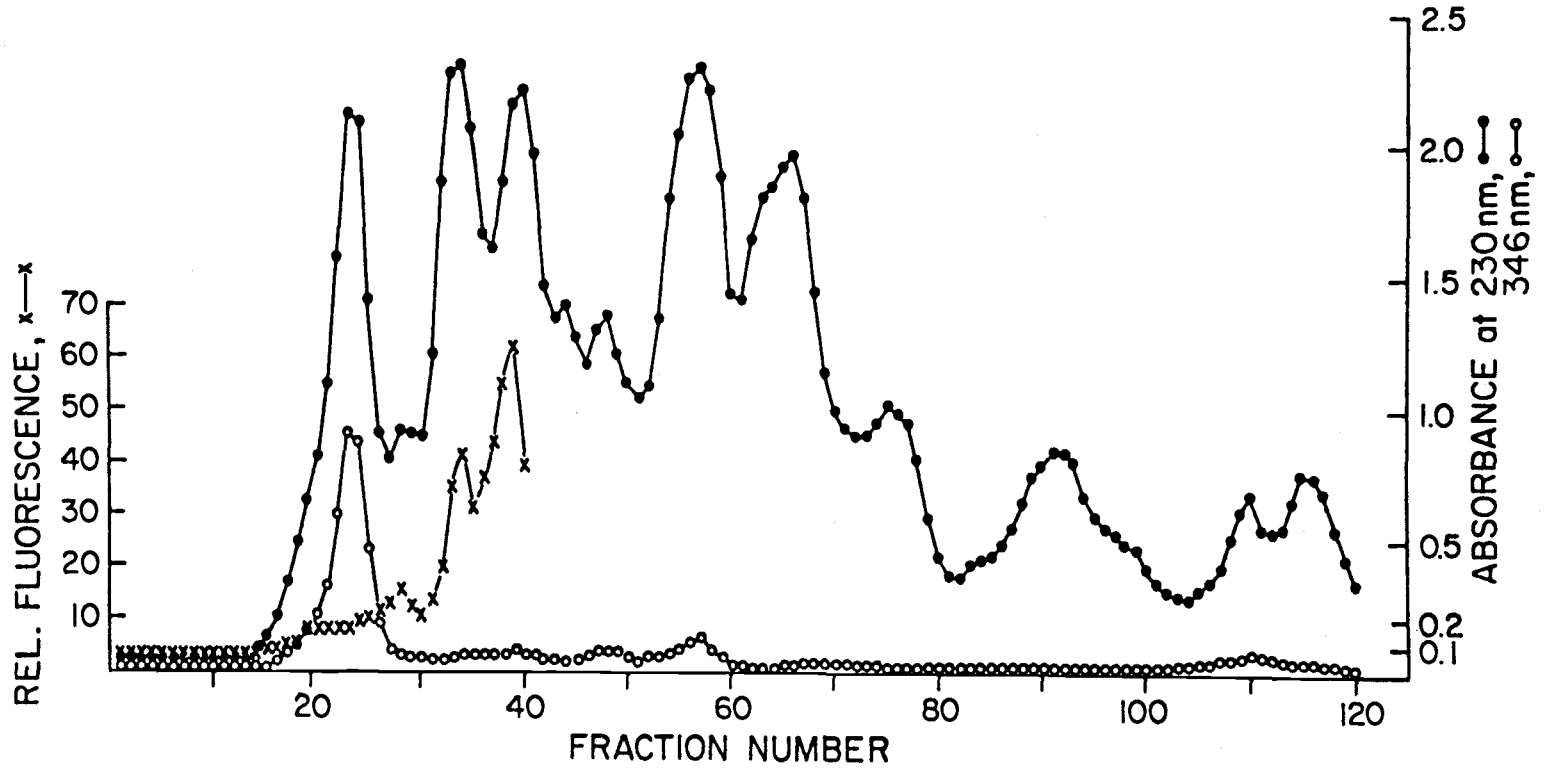


Figure 20. Elution pattern of tryptic peptides from trinitrophenylated bovine muscle pyruvate kinase as separated by chromatography on Biogel P 6. The absorbance at 230 nm is due to the presence of peptide bonds, the relative fluorescence values indicate the reactivity of eluted fractions to p phthaldialdehyde, and the absorbance at 346 nm was used to detect the trinitrophenyl peptide. Procedures are given in "Methods".

Table 4

Amino Acid	Nanomoles per Sample	Mole Ratio (Based on Arginine)
S-Carboxymethyl-Cysteine	2.81	0.80
Aspartate	16.68	4.76
Threonine	2.90	0.83
Serine	6.47	1.85
Glutamate	9.68	2.76
Proline	2.79	0.79
Glycine	13.31	3.80
Alanine	19.39	5.53
Valine	9.32	2.66
Methionine	2.73	0.78
Isoleucine	3.82	1.09
Leucine	10.97	3.13
Tyrosine	2.34	0.67
Phenylalaine	trace	—
Lysine	1.33	0.38
Arginine	3.51	1.00

Table 4. Amino acid analysis of the isolated trinitrophenylated peptide. The peptide was hydrolyzed in 6 N HCl at 110^{0} for 24 h *in vacuo*.

Figure 21

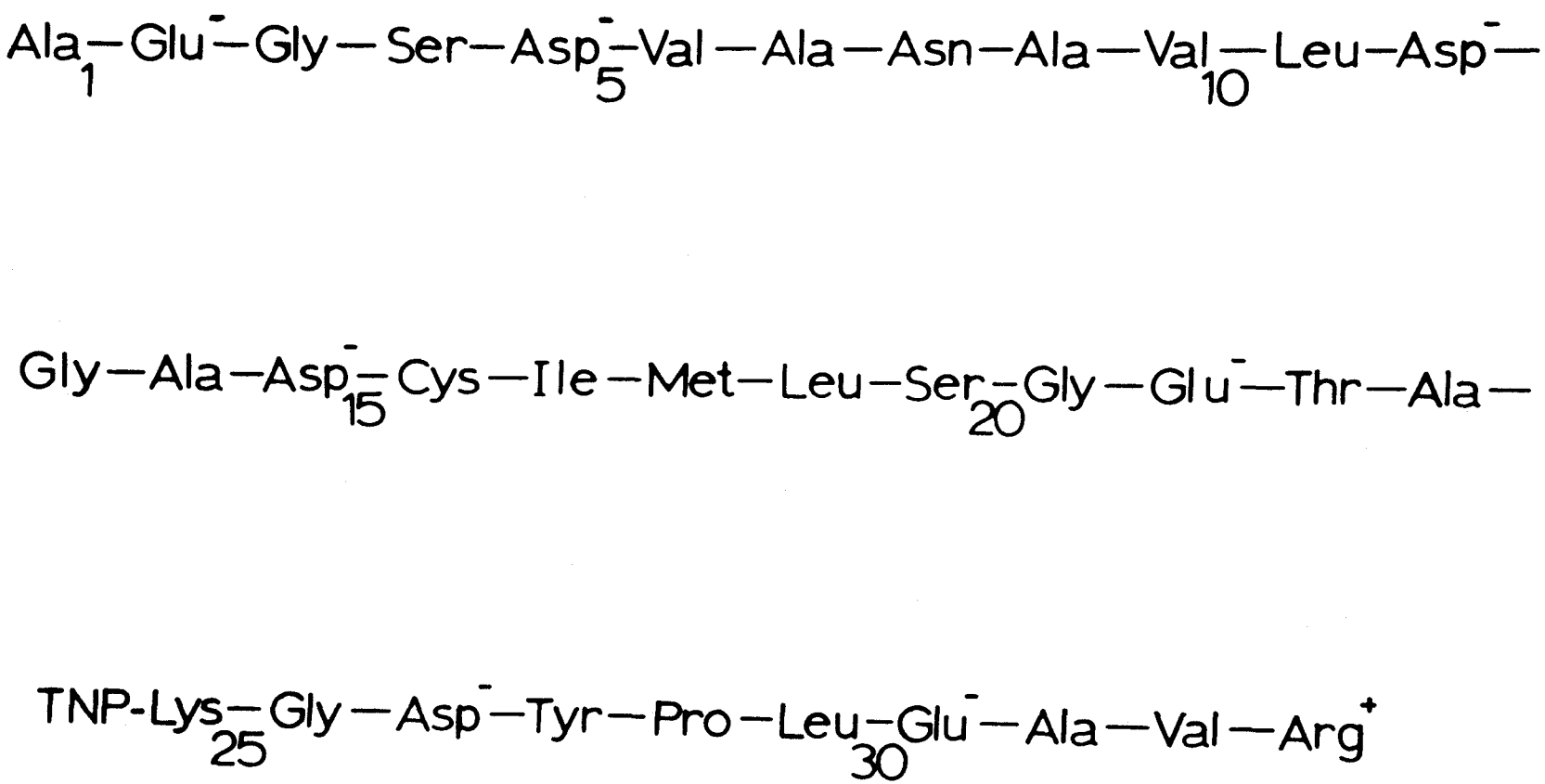


Figure 21. Sequenced of trinitrophenylated peptide isolated from labeled bovine muscle pyruvate kinase. Procedures are given in "Methods".

IV. DISCUSSION

Amino Terminal

No free amino terminal was detected on bovine muscle pyruvate kinase. Similar results from rabbit muscle (Cottam *et al.*, 1969), rat liver (Hall *et al.*, 1978) and human kidney (Harkins *et al.*, 1977) suggest the universal nature of this modification among eukaryotic pyruvate kinases. The prokaryotic bacterium *E. coli* has two forms of pyruvate kinase. The AMP activated form has an unmodified terminal serine (Somani *et al.*, 1972) but no free amino terminus was found on the FdP activated form (Waygood and Sanwal, 1974). Analysis of the amino terminal of pyruvate kinase from bovine muscle (Table 2) and rabbit muscle (Brummel *et al.*, 1976) has resulted in the isolation of an amino blocked serine. The serine from the rabbit muscle enzyme was shown to be -amino acetylated.

The biological significance of blocking the amino terminal of proteins is still unclear. Approximately 80 percent of the soluble proteins from Ehrlich Ascites cells are α-amino acetylated (Brown and Roberts, 1976). Bloemendal (1977) suggested that the blocked terminals may prevent rapid degradation by intracellular aminopeptidases. Brown (1979) attempted to test this hypothesis and found that proteins having an amino acetylated terminus were degraded at the same rate as those with an unmodified amino terminal valine. This evidence does not rule out acetylation as a protective mechanism. The possibility exists that only those proteins sensitive to aminopeptidase digestion are modified. Degradative rates of an individual protein with and without an α-amino acetyl modification need to be compared to provide a better test of this

hypothesis. Terminal acetylation does not appear to have an regulatory function, since removal of thegroup has only been shown to occur during protein catabolism (Gade and Brown, 1978). The modification of the positively charged α-amino group by acetylation may play an important role in protein folding or binding the polypeptide during translation to particular cellular organelles or membranes. With only a few exceptions, all α-amino acetylated proteins have a terminal serine, alanine, glycine, methionine or threonine (Bloemendal, 1977). These terminal residues alone are not enough to initiate modification, as several unblocked eukaryotic proteins have these residues at their amino terminal. An obvious pattern of amino acids, which could be part of a recognition site, has only been found for those proteins with an α-amino acetylated methionine. Sequence analysis of these proteins have always found a penultmate aspartic or glutamic acid (Tong, 1977). Bloemendal (1977) listed the four amino terminal residues of 37 proteins with α-amino acetylated terminals other than methionine and found no obvious pattern or homology.

Trinitrophenylation

Pyruvate kinase can be specifically modified by TNBS at a single site per subunit, resulting in the loss of most of its normal catalytic activity. The modified residue, shown to be a lysine (Hollenberg et al., 1971; Roschlau and Hess, 1972; Hubbard and Cardenas, 1975), appears to be located in or very near the nucleotide binding site on the enzyme. Rates of inactivation of both the hyperbolic (Hollenberg et al., 1971; Hubbard and Cardenas,1975) and allosteric (Roschlau and Hess, 1972) forms of the enzyme are greatly reduced in the presence of ADP but not in the presence of PEP. Analysis of the nucleotide protection of bovine muscle

pyruvate kinase reveals that MgADP, MgATP and ADP provide complete protection against inactivation with dissociation constants of 0.84 mM, 0.49 mM and 5.5 mM, respectively (Figures 9-11). AMP also appears to protect the enzyme. As seen in Figure 12, high concentrations of AMP will reduce the rate of inactivation. The requirement for high concentrations of AMP is consistent with the low affinity of the enzyme for AMP. The K_I of AMP was shown to be about 0.05 M (Figure 13) which is in close agreement with the value 0.055 M reported by Plowman and Krall (1965).

Protection against inactivation by various phosphate anions was carried out to clarify the role of the essential lysine in the binding of ADP. Phosphate, pyrophosphate and tripolyphosphate will each decrease the rate of the inactivation. However, the reason for this reduction is unclear. It requires high concentrations (20-50 mM) of these salts to see significant changes in the inactivation rate and it is very difficult to control for the ionic strength at these concentrations. No kinetic evidence for the binding of the phosphate ions to the ADP site is seen. The limiting factor in these kinetic studies is the low solubility of the phosphate anions in the presence of Mg^{++}. Therefore, no conclusion can be yet made as to the specific role of the essential lysine in the binding of the nucleotide, ADP, to pyruvate kinase.

As shown in Table 3,the presence of various salts greatly affects the rate of inactivation. The effect depended upon the concentration of the salt rather than the type. An approximate 4 fold reduction in the rate constant of the TNBS reaction occured upon addition of 0.1 M KCl, NaCl or tetramethylammonium chloride to the reaction mixture. Nowak (1976) showed that tetramethylammonium chloride would not compete for

K^+ sites on the enzyme and would not activate catalysis. Sodium inhibits K^+ activated catalysis but will only activate the enzyme approximately 9 percent as well as K^+ (Cardenas et al., 1973). These results show that the protective effects of the salts are probably due to increased ionic strength rather than to specific cation protection of the essential lysine. Increasing ionic strength also decreased the rate by which pyridoxal 5'-phosphate inactivated rabbit muscle pyruvate kinase (Johnson and Deal, 1970). Changes in the ionic strength may affect the sensitivity of the enzyme to TNBS by altering the conformation of the protein such that either the accessibility or the pK of the essential lysine is altered.

It was of interest to see if Mg^{++} alone, could affect the rate of trinitrophenylating bovine muscle pyruvate kinase. Hollenberg et al. (1971) found that 5 mM $MgCl_2$ decreased the rate of inactivation of rabbit muscle pyruvate kinase. Roschlau and Hess (1972) showed protection by 30 mM $MgSO_4$ against TNBS inactivation of yeast pyruvate kinase. Davidoff et al. (1973) found that 5 mM Mn^{++} strongly protected rabbit muscle pyruvate kinase against inactivation by 3,5-dimethylpyrazole-1-carboxamidine. This reagent was shown to cause 50 percent inactivation through modification of 2 lysines per tetramer.

As shown in Figure 14, the presence of 4 mM $MgCl_2$ did not affect the inactivation of bovine muscle pyruvate kinase by TNBS. With a dissociation constant of 9.1×10^{-4} M for the enzyme·Mg^{++} complex (Suelter et al., 1966) essentially all the Mg^{++} sites should be occupied at this concentration. The inactivation has been done in the presence of up to 10 mM $MgCl_2$ with only slight decreases in the rate of inactivation. These reductions in activation rates can be accounted for by the change in ionic strength.

The ionic strength effect was not considered by Hollenberg et al. (1971) and Roschlau and Hess (1972) and could be the reason for the Mg^{++} protection seen in the trinitrophenylation of rabbit muscle and yeast pyruvate kinase. The strong divalent cation protection against modification by 3,5-dimethylpyrazole-1-carboxamidine could not be duplicated in the trinitrophenylation of bovine muscle pyruvate kinase suggesting different sites of modification or different means of binding to the enzyme. Compounds like 3,5-dimethylpyrazole-1-carboxamidine have been shown to compete for the divalent cation binding site on pyruvate kinase and mimic Ca^{++} inhibition of the enzyme (Davidoff and Carr, 1972). 3,5-dimethylpyrazole-1-carboxamidine may be binding to the divalent cation binding site on the enzyme and then migrating to the nucleotide site, reacting with the essential lysine there.

As was the case for trinitrophenylation of rabbit muscle (Hollenberg et al., 1971) and yeast (Roschlau and Hess, 1972) pyruvate kinases, the loss of catalytic activity from the bovine muscle enzyme was not complete. Approximately 10 percent of the pyruvate kinase activity remained after prolonged incubation with TNBS. If trinitrophenylation does not completely eliminate ADP binding but only greatly reduces its affinity, one would expect the modified enzyme to have an increased K_m for ADP. As shown in Figure 16, this was not seen. The K_m for ADP was essentially the same for native and for 75 percent inactivated enzyme and only the V_m was affected.

The catalytic decarboxylation of oxalacetate by pyruvate kinase occurs at the PEP site and does not require the presence of a nucleotide (Creighton and Rose, 1976 a and b). As seen in Figure 17 the kinase

activity is more sensitive to trinitrophenylation than is the decarboxylating activity. Therefore, modification of the single essential lysine did not greatly affect the ability of the enzyme to bind or to decarboxylate oxalacetate. This is consistent with the work of Jursinic and Robinson (1978) who were able to show that the decarboxylase activity of rabbit muscle pyruvate kinase did not significantly change till more than one site per subunit was modified.

The decrease in catalytic activity at high pH can be explained by an absolute dependence upon the protonated state of a particular residue with a pK of about 8.3 (Figure 18). The residue does not appear to be the trinitrophenylated lysine. The pH dependence of the trinitrophenylation reaction indicates a pK of approximately 9.6. The K_m of ADP drops slightly from 0.35 mM (Cardenas _et al_., 1973) at pH 7.0 to 0.25 mM at pH 7.8 indicating the loss in catalytic activity in this pH range is not due to a decreased affinity for ADP. Rose (1960) also found enhanced nucleotide binding with increasing pH. The K_m for ATP in the enolization of pyruvate dropped from 1.43 mM at pH 8.0 to 0.19 mM at pH 9. If the release of ATP and pyruvate is rate limiting, as previously suggested (Rao _et al_., 1979) than at least some of the decrease in catalytic activity at high pH could be explained by the tighter binding of ATP.

In view of the conformational change induced by PEP binding (James _et al_., 1973; Reuben and Kayne, 1971; Nowak, 1978b) the effect of saturating amounts of PEP on the pK of the reactive lysine was studied. As shown in Figure 17 the rate of inactivation tended to be higher in the presence of PEP but no significant change in the pK was seen. This is consistent with a independent random formation of the enzyme·substrate quaternary

complex. Since kinetic studies show that the presence of PEP does not affect the binding of ADP it would not be expected that PEP would affect the structure of the ADP binding site.

The pK of -amino groups vary widely depending on their local environment. Kixon and Webb (1964) reported that exposed protein -amino groups have pK's ranging from 9.4 to 10.6. Therefore, with the observed pK of 9.6, the reactive lysine does not appear to be located in a hydrophobic region of the molecule.

The question arises as to why only one lysine out of 37 per subunit (Cardenas et al., 1973) will react with TNBS under the conditions used. The specificity does not appear to be due to a uniquely low pK. The kinetics of inactivation do not show any evidence of "rate saturation" (Baker, 1967) that would indicate the presence of a specific enzyme·TNBS complex. Such a complex would be expected if the TNBS specificity was due to an affinity for the ADP binding site. However, if the 5-100μM concentrations of TNBS used in these studies were well below the dissociation constant of TNBS for the site, the saturation effect would not be seen. It is common to not see any kinetic evidence for an enzyme·inhibitor complex when using an affinity label, particularly if the amount of inhibitor is limited by low solubility or high enzyme inactivation rates. The binding of peptide chloromethyl ketones to the active site of chymotrypsin has been well studied using x-ray crystallography (Segal et al., 1971), however, no kinetic evidence was seen for the reversible enzyme·inhibitor complex when these compounds were used as affinity labels (Kurachi et al., 1973).

The Trinitrophenylated Peptide

The trinitrophenylation of pyruvate kinase leads to the modification of a unique lysine in or very near the ADP binding site, thereby providing the means by which this region of the protein could be isolated and its structure elucidated. The trinitrophenylated tryptic fragment shown in Figure 21 is a highly acidic peptide 34 amino acids long.

Since elucidation of the tryptic TNP-peptide from bovine muscle pyruvate kinase, Russell *et al*. (1979) reported the isolation and amino acid content of the CNBr - TNP-peptide from cat muscle pyruvate kinase. Except for the absence of leucyl residues in the peptide from cat muscle, the amino acid content of the cat muscle peptide can be arranged to fit the carboxyl region of the bovine muscle TNP-peptide (Table 5).

Table 5

TNP-Peptides from Bovine Muscle and Cat Muscle Pyruvate Kinase

Bovine Muscle: -M-L-S-G-E-T-A-TNPK-G-D-Y-P-L-E-A-V-R

Cat Muscle: S,G,E/Q,T,A,TNPK,G,D/N,Y,P, E/Q,A,V,R,V,Hse

Table 5. The amino acid content of the CNBr TNP-peptide from cat muscle pyruvate kinase (Russel et al., 1979.) can be arranged to very closely match the sequence of the carboxyl terminal region of the tryptic TNP-peptide from bovine muscle pyruvae kinase. A: alanine, D: aspartate, E: glutamate, G: glycine, Hse: homoserine, L: leucine, M: methionine, N: asparagine, P: proline, Q: glutamine, R: arginine, S: serine, T:threonine, TNPK: TNP-lysine, V: valine, Y: tyrosine.

With the vast majority of biological substrates being anionic, it would be expected that arginine and lysine would play an important role in binding substrates to enzymes. Riordan (1977) stressed the importance

of arginine in binding the phospho-intermediates of glycolysis. Inactivation of pyruvate kinase by phenylglyoxal (Berghauser, 1977) and 2,3-dibutanedione (Cardemil and Eyzaquirre, 1979) suggested the presence of an arginine involved in the binding of the phosphoryl group of PEP.

The role of lysine in binding phosphoryl groups has also been stressed (Milhausen and Levy, 1975). The importance of lysine is supported by the number and variety of enzymes that can be inactivated with pyridoxal 5'-phosphate. Pyridoxal 5'-phosphate will form a stable covalent modification. This method of active or allosteric site modification is not limited to those enzymes which normally use pyridoxal 5'-phosphate as a substrate or cofactor. It has been used to label the ATP allosteric site of nucleoside diphosphatase (Kawakita and Yamaziki, 1978), the 5-phosphoriboxyl-1-pyrophosphate site of anthranilate synthetase (Grove and Levy, 1979) and the 6-phosphogluconate site of 6-phosphogluconate dehydrogenase(Rippa et al., 1967). Milhausen and Levy (1975) proposed that the specificity of pyridoxal 5'-phosphate for phosphoryl binding sites was enhanced due to its own phosphate group.

Johnson and Deal (1970) inactivated rabbit muscle pyruvate kinase with pyridoxal 5'-phosphate but concluded that the lysine modified was not directly in the active site. They based this conclusion on their inability to detect any instantaneous inhibition by pyridoxal 5'phosphate. Here again, the rate constant for the schiff base formation may have been rapid compared to the dissociation constant for the enzyme·pyridoxal 5'-phosphate complex making it difficult to detect reversible inhibition. Pyridoxal did not inactivate pyruvate kinase, supporting the hypothesis that the phosphate group plays an important role in the binding of pyridoxal 5'-phosphate to the enzyme.

Several enzymes besides pyruvate kinase are susceptible to inactivation by TNBS. TNBS has recently been shown to modify the PEP binding site of PEP carboxylase (Naide et al., 1979) and a lysine which interacts with the C-1 carboxyl group of 3-phosphoglycerate in phosphoglycerate mutase (Sahaki et al., 1971; Russell et al., 1979).

The presence of a tyrosine only 3 residues away from the essential lysine in pyruvate kinase may also be significant. Recent work on 3-phosphoglycerate kinase (Roustan et al., 1979) has shown that a tyrosine and a lysine are located very close together within the phosphoryl binding site of this enzyme. When the enzyme was inactivated with 7-chloro-4-nitrobenzofurazan at pH 7.3 a tyrosine was modified. When excess inactivator was removed and the pH brought to 9 the modifying group migrated to a lysine. A similar migration has been shown to occur in mitrochondrial ATPase (Ferguson et al., 1975). The ATPase was inactivated by 7-chloro-4-nitrobenzofurazan modification of a single tyrosine. At pH 9 the group was transferred to a lysine residue. The rate of ransfer was reduced in the presence of either ATP or ADP. It would be very interesting to determine whether this reagent could be used to inactivate pyruvate kinase.

The exact chemical nature of the nucleotide binding site of pyruvate kinase is still not well understood. Hopefully the chemical modification and sequence analysis contributed by this work and others will provide the foundation on which the structural basis of the enzymatic catalysis of pyruvate kinase can be understood.

V. CONCLUSION

The results of this study are consistent with the location of a highly reactive lysyl residue in or near the nucleotide binding site of pyruvate kinase. Trinitrobenzenesulfonate will specifically modify this reactive lysyl group, preventing catalysis of those reactions which require substrate binding to the nucleotide site. The catalytic decarboxylation of oxalacetate, having no nucleotide requirement, is much less sensitive to trinitrophenylation than the kinase activity. The nucleotides ADP and ATP provide complete protection of the enzyme to modification, while saturating amounts of PEP and Mg^{++} have little or no effect on the inactivation rate. The specific role of the reactive lysyl group in the binding of the nucleotides is still unclear.

The finding of little or no effect of PEP on trinitrophenylation of pyruvate kinase is consistent with kinetic data showing independent, random binding of the substrates to the enzyme. Although magnetic resonance analysis has indicated a conformational change upon binding PEP to the enzyme (Nowak, 1978b), this change does not appear to affect either the affinity of the enzyme for ADP or the chemical nature of the neighboring nucleotide binding site.

The pH dependence of the trinitropheylation reaction is consistent with the involvement of an unprotonated lysyl residue with a pK of 9.6. This pK is not unusually low for ε-amino group, suggesting that the reactive lysyl group is not located in a hydrophobic region of the molecule. The primary structure of the tryptic peptide containing the reactive

lysyl group supports this conclusion: of the 34 residues in this peptide, six are either glutamic or aspartic acids and three are either serine or threonine. It should be noted that this highly acidic peptide comes from a rather basic protein, the isoelectric pH of bovine muscle pyruvate kinase being 8.9 (Cardenas *et al.*, 1973).

In addition to the reactive lysyl residue, chemical modification studies have implicated the presence of other amino acid residues within the active site of pyruvate kinase. Two of these, tyrosine and arginine, are also found in the TNP-peptide sequenced in this study. This work is one of the few studies on the primary structure in and around the active site of pyruvate kinase. Thus, the identification of these two residues as those which are required for catalysis or substrate binding has yet to be accomplished.

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