AN ABSTRACT OF THE THESIS OF

DAVID HAMILTON PORTER for the degree of DOCTOR OF PHILOSOPHY

in BIOCHEMISTRY AND BIOPHYSICS presented on 27 June, 1980

Title: THE RENATURATION OF BOVINE MUSCLE PYRUVATE KINASE

Abstract approved: *Redacted for Privacy*
Janet M. Cardenas, Ph.D.

Bovine muscle pyruvate kinase can be reversibly denatured
by solutions of guanidine hydrochloride. Subsequent dilution
of the enzyme into buffer containing 2-mercaptoethanol or
dithiothreitol results in recovery of enzymatic activity with
half-times that vary from 185 min at 0°C to 4 min at 45°C. In
the temperature range of 0-30°C, 90 percent of the enzymatic
activity is recovered. Above about 32°C the recovery drops
off sharply, with a yield of only 13 percent at 45°C. Removal
of inactive nonspecific aggregates and denatured monomer by gel
filtration yields enzyme with the same specific activity as
the original enzyme.

At enzyme concentrations below 3 μg/ml at 16°C or below
25 μg/ml at 7.8°C, the reactivation kinetics show a concentra-
tion dependence. In the presence of 5 mM L-phenylalanine at
16°C, the reactivation shows a concentration dependence at enzyme
concentrations below 20 μg/ml.

At higher concentrations (25.6 μg/ml) at 16°C or higher, no enzyme concentration dependence is seen and the reactivation kinetics are described by two first order relaxations, both in the absence and presence of L-phenylalanine. L-phenylalanine inhibits the two first order relaxations that are rate limiting and that have the following rate constants: 15.6×10^{-4} and 2.08×10^{-4} sec^{-1}, respectively, in the absence of phenylalanine and 5.07×10^{-4} and 1.27×10^{-4} sec^{-1}, respectively, in the presence of 8.0 mM phenylalanine. In the absence of L-phenylalanine, the fast and slow relaxations have rate constants with apparent activation energies of 10.6 and 11.9 kcal/mole, respectively.

At an enzyme concentration of 25.6 μg/ml and at 16°C, L-tyrosine and L-tryptophan also inhibit the reactivation but to a lesser extent than L-phenylalanine. Under these same conditions phenylpyruvate is a potent inhibitor of reactivation. The addition of 5 mM phenylpyruvate increases the half-time of recovery by 335 percent while 5 mM L-phenylalanine increases the half-time by 235 percent.

Neither L-alanine, L-valine, D-phenylalanine, D-tryptophan, phosphoenolpyruvate, nor fructose 1,6-bisphosphate have any appreciable effect on the reactivation kinetics either in the absence or presence of L-phenylalanine.

Combining the results presented here with earlier results from this laboratory (Cardenas and Dyson, 1973; Cardenas et al., 1977) indicates that a rapid major folding produces two species which undergo transconformational processes. This is followed by subunit association which produces the native tetramer. The results support

the hypothesis that an L-phenylalanine binding site that is probably

distinct from the catalytic site is formed early in the renaturation

process. L-phenylalanine binds to this site and inhibits both trans-

conformational and association processes involved in the renaturation

of bovine muscle pyruvate kinase.

Bovine muscle pyruvate kinase was immobilized onto cyanogen

bromide activated Sepharose 4B under conditions favoring the cross-

linking of only one subunit to the resin. Removal of the subunits

that are not covalently linked to the Sepharose leaves 28 percent of

the protein bound to the resin as determined by amino acid content.

The subunits that remain have 2.1 percent of the activity that was

originally bound to the Sepharose or about 8.4 percent of the activity

expected for a fully active monomer. Reconstitution of the quaternary

structure of the Sepharose bound subunits with active enzyme yields

61 percent of the original bound activity. Reconstitution with enzyme

that was inactivated with 2,4,6-trinitrobenzene sulfonate yields 18

percent of the original bound activity. This value agrees with that

predicted for a fully reactivated immobilized subunit when one takes

into consideration the reconstitution efficiency (61 percent) and

the fact that the trinitrophenylated enzyme retained about 6 percent

of its original activity.

Thus, isolated subunits of bovine muscle pyruvate kinase are

essentially inactive and activity appears on formation of the quaternary

structure.

The Renaturation of Bovine Muscle Pyruvate Kinase

by

David Hamilton Porter

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed 27 June 1980

Commencement June 1981

APPROVED:

Associate Professor of Biochemistry and Biophysics in charge of major

Chairperson of the Department of Biochemistry and Biophysics

Dean of the Graduate School

Date thesis is presented   27 June, 1980

Typed by Barbara Hannah for David Hamilton Porter

ACKNOWLEDGEMENT

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

The Renaturation of Bovine Muscle Pyruvate Kinase

I.   Introduction

Many of the most intriguing questions in molecular biology are
directed toward understanding the organization and function of macro-
molecular complexes. Often a complex has properties that are distinct
from those of its constituents.

To understand the functioning of biological complexes it is
necessary to determine how each component communicates with the other
components in the system and how this communication controls the
function of the complex as a whole. Studies with partially assembled
systems make it possible to correlate the state of assembly with
function. It follows that knowledge of the pathway by which the
complex is assembled is highly desirable.   In combination this in-
formation can potentially provide insight into the specific inter-
actions which give rise to particular functional properties.

Macromolecular assemblies range from the relatively simple dimeric
enzyme composed of identical subunits to the extremely complex, such
as chromatin of eukaryotic cells. An assembly containing only a few
components is not necessarily a system with a simple function for
which information is easily obtained.

<u>Pyruvate Kinase</u>

Among the relatively simple oligomeric systems is the enzyme pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40) which catalyses the transfer of a phosphate group from PEP to ADP with the production of pyruvate and ATP (see Boyer, 1962; Kayne, 1973). The reaction is essentially irreversible with an apparent ΔG of -4.7 kcal/mole at pH 7.4 (McQuate and Utter, 1959) and generally requires both a monovalent and a divalent cation. K^+ and Mg^{2+} are the apparent physiological activators.

<div align="center">
Pyruvate Kinase Isozymes:
A Brief Survey
</div>

There are three predominant isozymes of pyruvate kinase found in vertebrate tissues.

Type M (also called M_1) is the major isozyme of brain and cardiac muscle and the only isozyme found in skeletal muscle. It generally has hyperbolic kinetics with respect to PEP (Km values between 0.04–0.09 m$\underline{M}$) and is insensitive to fructose-1,6- bis phosphate(FDP) (Boyer, 1962; Tanaka <u>et al</u>., 1967; Flanders <u>et al</u>., 1971; Baranowska and Baranowski, 1975; Cardenas <u>et al</u>., 1975b). The muscle pyruvate kinases from many lower vertebrates (fish, amphibians, and reptiles) are activated by FDP and may have slightly sigmoidal kinetics with PEP (Story and Hochachka, 1974; Randall and Anderson, 1975; Zammit <u>et al</u>., 1978; Guderley and Cardenas, 1980). Typical Km values for ADP are about 0.03 m$\underline{M}$ (Boyer, 1962; Tanaka <u>et al</u>., 1967; Cardenas <u>et al</u>., 1973) and kinetics with ADP are hyperbolic.

Type L pyruvate kinase is the predominant isozyme of liver, where it is found only in parenchymal cells (Crisp and Pogson, 1972; Van Berkel et al., 1972). It is also found in kidney cell types thought to be capable of gluconeogenesis (Cardenas et al., 1978). Type L has sigmoidal kinetics with PEP ($K_{0.5}$ of 0.30–0.96 mM) and is activated to hyperbolic kinetics by FDP ($K_{0.5}$ of 0.03–0.15 mM). The apparent $K_{0.5}$ for FDP is 0.2–1.0×10^{-7} M (Kutzbach et al., 1973; Cardenas et al., 1975b; Ekman et al., 1976; Eigenbrodt and Schoner, 1977; Kohl and Cottam, 1977; Riou et al., 1978). The K_m for ADP (hyperbolic kinetics) has been reported to be 0.1–0.4 mM (Tanaka et al., 1967; Cardenas et al., 1975b; Ljungstrom et al., 1976) and is unaffected by the presence of FDP. Ljungstrom et al., (1974) have shown that type L could be inhibited by phosphorylation. The phosphorylation was later shown to occur in vivo (Ljungstrom and Ekman, 1977), and its activity was demonstrated to be under hormonal control.

Type K (also called M_2) is present in fetal tissues, many tumors and adult tissues, and in parenchymal cells of regenerating liver. It has slightly sigmoidal kinetics with PEP and the $K_{0.5}$ for PEP decreases slightly from 0.2–0.4 mM on the addition of FDP (Imamura et al., 1972; Corcoran et al., 1976; Eigenbrodt and Schoner, 1977). The K_m for ADP (kinetics are hyperbolic) is 0.2–0.4 mM (Corcoran et al., 1976; Eigenbrodt and Schoner, 1977).

Erythrocytes contain a form of pyruvate kinase (type R) which is kinetically similar to the L isozyme. Type R has sigmoidal kinetics with PEP and is activated by FDP. The $K_{0.5}$ for PEP drops from 0.48–0.63 mM to 0.06 mM in the presence of FDP (Blume et al.,

1971; Staal et al., 1971) with an apparent $K_{0.5}$ for FDP for 0.4–0.6 x 10^{-7} M (Blume et al., 1971; Garreau et al., 1977).

All three isozymes are inhibited by ATP, citrate, and certain amino acids, most notably phenylalanine (see section on the effects of amino acids).

Pyruvate kinases from higher plants typically show hyperbolic kinetics with both substrates. ATP and citrate inhibit but FDP has no effect, even in tissues that are known to be gluconeogenic (Tomlinson and Turner, 1973). Phenylalanine and alanine also have no effect. Kobr and Beevers (1971) have proposed separate metabolic compartments for gluconeogenesis and glycolysis. Peterson and Evans (1978) have concluded that control of soybean nodule cytosolic pyruvate kinase may be closely related to reactions involved in the assimilation of NH_4^+.

Pyruvate kinases from simple eukaryotes (yeast and E. gracilis) and bacteria tend to have diverse properties. They often show sigmoidal kinetics and are activated by FDP (Ohrmann, 1969; Barbalace et al, 1971; Johannes and Hess, 1973; Kapoor, 1975; Yoshizaki and Imahori, 1979). Some bacterial enzymes are not activated by FDP (Benziman, 1969; Liao and Atkinson, 1971). E. coli K12 (Malcovati and Kornberg, 1969; Waygood and Sanwal, 1974; Valentini et al., 1979), M. rouxii (Passeron and Roselino, 1971; Terenzi et al., 1971) and B. licheniformis (Tuominen and Bernlohr, 1971) each have two forms of pyruvate kinase. Generally, one form is activated by AMP and the other by FDP, the later forms often being activated by other glycolytic intermediates. Other bacteria appear to have only forms that are activated by AMP (Maeba and Sanwal, 1968; Ozaki and Shiio,

1969; Ide, 1970; Liao and Atkinson, 1971). A few bacterial and yeast enzymes show slightly cooperative binding of ADP (Haeckel et al., 1968; Ide, 1970; Tuominen and Bernlohr, 1971). Also unusual is the reported lack of a monovalent cation requirement in some gram negative bacteria (see section on cation cofactors for more detail). The yeast and Euglena enzymes are inhibited by alanine and phenylalanine (Haeckel et al., 1968; Ohrmann, 1969) as are the enzymes from some other micro-organisms (Ozaki and Shiio, 1969; Ng and Hamilton, 1975; Schedel et al., 1975). However, the enzyme from Thermus thermophilus is not in-hibited by phenylalanine (Yoshizaki and Imahori, 1979).

Structure

Each vertebrate isozyme (M, L, K) is composed of four subunits of equal molecular weight as determined by sodium dodecyl sulfate acrylamide gel electrophoresis or ultracentrifugation in guanidine hydrochloride or urea (Type M: Steinmetz and Deal, 1966; Peterson et al., 1974; Anderson and Randall, 1975; Baranowska and Baranowski, 1975; Cardenas et al., 1975a; Eigenbrodt and Schoner, 1977; Harkins et al., 1977a; Type L: Kutzbach and Hess, 1970; Cardenas and Dyson, 1973; Kutzbach et al., 1973; Ljungström et al., 1974; Kohl and Cottam, 1976; Marie et al., 1976; Eigenbrodt and Schoner, 1977; Riou et al., 1978; Type K: Imamura et al., 1972; Corcoran et al., 1976; Berglund et al., 1977; Eigenbrodt and Schoner, 1977; Harkins et al., 1977b). The subunits of each isozyme have very similar if not identical primary sequences as determined by tryptic or cyanogen bromide peptide maps. In each case the maps show a number of peptides equal to one

fourth of the number expected from the amino acid composition of the tetramer (Type M: Cottam et al., 1969; Peterson et al., 1974; Type L: Hall et al., 1978; Type K Corcoran et al., 1976). The amino terminal is blocked in each isozyme (Type M: Cottam et al., 1969; Parkinson and Easterby, 1977; Harkins et al., 1977a; Johnson, 1980; Type L: Hall et al., 1978; Type K: Corcoran et al., 1976) and the amino terminal of type M is N-acetylserine (Brummell et al., 1976; Johnson, 1980). The carboxyl terminal of type M is also blocked (Cottam et al., 1969; Brummell et al., 1976).

Type R is unusual in that two species are reported to exist (Garreau et al., 1977; Marie et al., 1977a). One is composed of four identical subunits and the other of two types of subunits. Peterson et al., (1974) have shown that the two types of subunits have similar molecular weights (approx. 60,000) and that the number of cyanogen bromide peptides is one half the number of methionine per molecule of enzyme. This suggests the presence of two types of subunits within the tetramer that have different primary structures.

The enzyme from yeast (Sossinka and Hess, 1974; Kapoor, 1975) and E. coli (Valentini et al., 1979) is also composed of four identical subunits as determined by gel electrophoresis, the number of tryptic peptides, and by amino terminal analysis in strains which have a free amino terminal. Valentini et al. (1979) have found two forms of pyruvate kinase in E. coli K12J53 that have free amino terminals, one with serine and the other with methionine as the amino terminal amino acid. E. coli K12 3000 has two forms with blocked amino termi-

nals. Bornmann et al. (1972) have found an acetylated amino terminal
and a free carboxy terminal valine in yeast.

High resolution X-ray diffraction studies (Stammers and Muirhead,
1975, 1977; Stammers et al., 1977; Levine et al., 1978) using cat
muscle pyruvate kinase indicate that each subunit consists of three
domains (see Fig. 1 of Levine et al., 1978). Domain A contains about
240 residues and consists of alternating β sheet and α helical regions.
The β sheet segments are parallel and form a cylinder while the eight
α helical segments form a cylinder that is coaxial with and outside of
the cylinder formed by the β sheet segments. This arrangement of α
helix and β sheet is also found in triose phosphate isomerase (TIM).
In TIM the chain terminates at the end of the final helix but in pyruvate
kinase it continues for another 120 residues and forms domain C. Trave-
ling along the peptide backbone, domain C begins with two anti-parallel
α helices, followed by alternating segments of β sheet and α helix.
The first three β segments and the interconnecting helices form a structure
characteristic of mononucleotide binding folds. The peptide ends with a
segment of β sheet which is antiparallel to the other β sheet segments of
domain C. Domain B consists largely of anti-parallel β sheet but flex-
ibility in this region has obscured some of the detail. The two initial
α helices of domain C pack against the corresponding helices in the adja-
cent subunit and the final β sheet segments of these two subunits forms
a continuous structure. This constitutes the major subunit contact between
subunits related by the dyad parallel to y. The subunits related by
the dyad parallel to z are in contact for most of their length with the

exception of domain B and adjacent areas of domain A. It appears that this contact results mostly in the packing of helix against helix. Subunits related by the dyad parallel to x have relatively few contacts.

The natural occurrence of K-L and K-M hybrids, the ability to form L-M hybrids in vitro (Cardenas and Dyson, 1973; Dyson and Cardenas, 1973), and the ability to form interspecies L-M hybrids (Cardenas et al., 1975b; Cardenas et al., 1975a) indicate that the regions of subunit contact are highly conserved. This is consistent with the idea that subunit interactions play an important role in the functioning of pyruvate kinase.

The muscle isozyme shows a high degree of homology for different vertebrates as indicated by amino acid compositions (see Hall and Cottam, 1978). A cyanogen bromide peptide containing a highly reactive sulfhydryl has been obtained from three animals (cat, rabbit, and sturgeon). Five out of the eight residues sequenced in each peptide are identical (Anderson and Randall, 1975; Harkins and Fothergill, 1977).

The immunological cross-reactivity within the pairs K, M and L, R (see Hall and Cottam, 1978) and interspecies cross-reactivity (Cardenas et al., 1975a; Eigenbrodt and Schoner, 1977; Lincoln et al., 1977) indicate further structural similarities between isozymes.

Saheki et al., (1978) have shown by peptide mapping of limited proteolytic digests that there is little homology between M, K, and L but considerable homology between L and R.

Binding studies with PEP indicate that there are four PEP sites per molecule of pyruvate kinase (Kayne, 1971; Cardenas et al., 1973). X-ray data on substrate binding (Stammers and Muirhead, 1977; Levine et al., 1978) indicate that PEP is bound at the carboxy end of the β sheet structure in domain A close to the barrel axis and near to domain B. Mg^{2+} binds very close to this. The electron density for Mn^{2+}-ATP lies radially perpendicular to the barrel axis so that the end of the density interpreted as the terminal phosphate overlaps the PEP binding site and the other end is between strands 3 and 4 of the barrel β sheet. Together these data are taken as identification of the active site of cat muscle pyruvate kinase. There is a second ADP binding site in domain A involving two α helices and a strand of β sheet.

Cation Cofactors

Generally pyruvate kinase requires both a monovalent and a divalent cation for activity. K^+ and Mg^{2+} are the apparent physiological activators. (see Boyer, 1962; Evans and Sorger, 1966; Kayne, 1973).

The order of effectiveness of various monovalent cations is

$$K^+ > NH_4^+ > Rb^+ > Tl^+ > Cs^+ > Na^+ > Li^+.$$
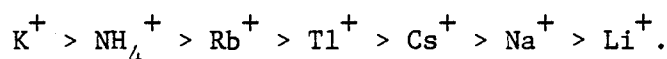
As the ionic radius (crystal) deviates (higher or lower) from that of K^+ the ability to activate is reduced, decreasing drastically when the difference in ionic radii exceeds about 0.3 $\overset{o}{A}$, as is the case with Cs^+, Na^+, and Li^+. Optimal concentrations are generally 100 m$\underline{M}$ with that for NH_4^+ being 50 m$\underline{M}$. Thallium (Tl^+) is unusual in that maximum activation occurs at a 3 m$\underline{M}$ concentration.

The absence of a monovalent requirement has been reported for enzyme from several sources (see Kayne, 1973; Yoshizaki and Imahori, 1979). Most often these reports are for enzyme obtained from gram negative bacteria. Two of these (Maeba and Sanwal, 1968; Waygood and Sanwal, 1974) have been attributed to the use of an undialyzed ammonium sulfate precipitate of lactate dehydrogenase in the coupled assay used to measure pyruvate kinase activity (Waygood et al., 1976).

Activating monovalent cations induce conformational changes in the enzyme as determined by ultraviolet difference sprectra and fluorescence emission and polarization. This and the high optimal concentrations led to the conclusion that the monovalent cations are involved in maintaining the conformation of the enzyme (Evans and Sorger, 1966).

The idea of Suelter (1970) that monovalent cations have a catalytic role in reactions involving enolate intermediates is supported by Kayne and Reuben (1970), who have shown that Tl^+ is located very near the active site Mn^{2+}. The high affinity of pyruvate kinase for Tl^+ allowed Kayne (1971) to determine that there were four Tl^+ binding sites on the enzyme, suggesting the presence of one Tl^+ per active site.

Atomic distances as determined by NMR and paramagnetic resonance measurements, as well as the effects of K^+ on the binding of PEP (higher affinity) and analogs in which the carbonyl group is lacking or blocked (lower affinity), led Nowak and Mildvan (1972) to suggest that the carbonyl group of PEP is coordinated to the enzyme-bound K^+. Today, this position of K^+ is generally proposed for substrate and cofactor geometries at the active site (Dunaway-Mariano et al., 1979).

The divalent cation requirement can be met by several metals, the order of effectiveness at the optimum pH being

$$Mg^{2+} > Mn^{2+} > Co^{2+} > Ni^+.$$
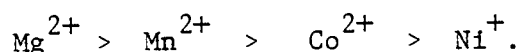
Early studies (Mildvan and Cohn, 1965, 1966; Sorger et al., 1965; Suelter, 1967) show that divalent metal binding causes a conformational change in rabbit muscle pyruvate kinase. NMR studies (Mildvan and Cohn, 1966; Cottam et al., 1972; Reuben and Cohn, 1970) demonstrate that Mn^{2+} binding is near the active site with the enzyme providing some liganding groups. This suggests that Mn^{2+} could be involved in an enzyme-metal-substrate bridge complex. The number of Mn^{2+} ions bound was determined by EPR (Reuben and Cohn, 1970; Cottam and Mildvan, 1971) to be four but the results are quite sensitive to the particular conditions used.

However, studies of the binding of mono- and divalent cations in the presence of Cr^{3+}-ATP indicate that two divalent cations are required per active site. One divalent cation is bound to the enzyme while the other binds directly to the phosphate chain of the nucleotide substrate. In the presence of mono- and divalent cations and Cr^{3+}-ATP, pyruvate kinase catalyzes the phosphorylation of glycolate (Dumaway-Mariano et al., 1979) and the enolization of pyruvate (Gupta et al., 1976; Gupta and Mildvan, 1977).

Chemical Modification

2,4,6-Trinitrobenzenesulfonate inactivates pyruvate kinase from rabbit muscle (Hollenberg et al., 1971), bovine muscle (Hubbard and Cardenas, 1975), and yeast (Roschlau and Hess, 1972) by modifying a single lysine per subunit. Inactivation is not complete. Up to 90-95% of the enzymatic activity of the muscle enzymes is eliminated, as is up to 60% of the activity of the yeast enzyme, but no experimental evidence has yet explained the incomplete inactivation. ATP, ADP, and AMP at high concentrations protect against inactivation. Mn^{2+} will provide some protection for the rabbit and yeast enzymes but not for the bovine enzyme (Johnson, 1980). PEP, FDP, and pyrophosphate will not protect against inactivation. The modification apparently blocks a portion of the nucleotide binding site. This is supported by NMR studies (Flashner et al., 1973) in which no evidence was found for the formation of an enzyme - Mn^{2+}- ATP complex with trinitrophenylated enzyme. These same authors could find no difference between native and modified enzyme in the formation of the enzyme -Mn^{2+}-PEP complex.

Trinitrophenylation affects only the adenine nucleotide mediated reactions of pyruvate kinase. Oxalacetate decarboxylase activity in the presence of inorganic phosphate is not appreciably affected (Jursinic and Robinson, 1978; Johnson, 1980). Neither is phosphate-mediated detritiation affected by trinitrophenylation of pyruvate kinase (Flashner et al., 1973).

After trinitrophenylation, the bovine muscle isozyme maintains its tetrameric structure with only a very slight decrease in sedimentation coefficient. It also maintains its ability to form hybrids with the bovine liver isozyme (Hubbard and Cardenas, 1975).

An active site peptide containing TNP-lysine has been isolated and sequenced (Johnson et al., 1979).

Effects of Alanine and Phenylalanine

L-phenylalanine inhibits the activity of type M pyruvate kinase (Jimenez de Asua et al., 1971; Carminatti et al., 1971; Kayne and Price, 1973; Ibsen and Trippet, 1973, 1974; Cardenas et al., 1975c; Ibsne and Marles, 1976). Most appear to retain their hyperbolic kinetics with PEP except bovine type M, which has sigmoidal kinetics in the presence of phenylalanine (Cardenas et al., 1975c). The phenylalanine inhibition is reversed by alanine (Carminatti et al., 1971; Cardenas et al., 1975c) and FDP (Weber, 1969; Ibsen and Trippet, 1973,1974; Cardenas et al., 1975b). For rabbit and rat type M the inhibition is reversed by serine (Carminatti et al., 1971; Kayne and Price, 1973; Ibsen and Trippet, 1974) with the inhibition of rabbit type M also being reversed by cysteine (Carminatti et al, 1971; Kayne and Price, 1973). Phenylalanine inhibition is also reversed by PEP (Carminatti et al., 1971; Cardenas, et al., 1975c). In contrast to the situation for mammalian pyruvate kinase, phenylalanine has much less effect on muscle pyruvate kinase from salmon and the inhibition is not reversed by alanine or FDP (Guderley and Cardenas, 1980).

Generally types K, L, and R are inhibited by alanine and pheny- lalanine and the effect on these isozymes is much more pronounced than for type M (Llorente et al., 1970; Stifel and Herman, 1971; Carbonell et al., 1973; Jimenez de Asua et al., 1971; Black and Henderson, 1972; Balinsky et al., 1973; Ibsen and Trippet, 1974; Imamura et al., 1972; Cardenas et al., 1975a). Bovine type L is unusual in that alanine is only slightly inhibitory (Cardenas et al.,

1975c). Alanine and phenylalanine inhibition can usually be reversed by FDP although there are reports of exceptions for some variants of type K (see Ibsen and Trippet, 1974). Alanine is only slightly inhibitory to salmon type K (Guderley and Cardenas, 1980). In studies on the specificity of amino acid inhibition, Ibsen and Trippet (1974) have shown that intact carboxyl and α-amino groups are necessary for inhibition by phenylalanine.

Phenylalanine binding enhances the fluorescence of rabbit muscle pyruvate kinase (Kayne and Price, 1972) but this is partially reversed by Mg^{2+} and PEP. The response of rabbit muscle pyruvate kinase to phenylalanine is cooperative (Carminatti et al., 1971; Kayne and Price, 1972) even though there is no evidence for sigmoidal kinetics with substrate. The cooperative response of type M is well illustrated in the case of the bovine enzyme, for which phenylalanine induces sigmoidal kinetics with PEP (Cardenas et al., 1975c).

Kayne and Price (1973) have found four alanine and four phenylalanine binding sites on the rabbit muscle enzyme (equilibrium dialysis) and conclude that the two amino acids compete for the same site. The allosteric effects of phenylalanine indicate that this site is not very near the active site. In contrast, Ibsen and Trippet (1974) and Ibsen and Marles (1976) propose two binding sites for amino acids on each subunit based on kinetic results. In this model small side chain amino acids bind at the PEP site while bulky side chain amino acids bind at another site. They suggest that phenylalanine binds to both sites. The effects of amino acids supposedly result from binding to one or both sites.

Yeast (Haeckel et al., 1968), Euglena (Ohrmann, 1969) and other microorganisms (Ozaki and Shiio, 1969; Schedel et al., 1975; Ng and Hamilton, 1975) have pyruvate kinases that are inhibited by alanine and phenylalanine. Plants (Tomlinson and Turner), 1973) and an extreme thermophile, Thermus thermophilus, (Yoshizaki and Imahori, 1979) have pyruvate kinases that are not inhibited by these amino acids.

Subunit Interactions

As mentioned above, pyruvate kinases generally show allosteric properties, indicating that subunit interactions play an important role in the function of the enzyme.

Kinetic studies have been made with hybrids formed between sub-units of the isozymes from liver and muscle (Dyson and Cardenas, 1973) or between liver and inactivated muscle subunits (Hubbard and Cardenas, 1975). The kinetic profiles of the hybrids with PEP are similar regard-of whether hybridization was performed with active or inactive muscle subunits. L_3M has sigmoidal kinetics and is activated by FDP, L_2M_2 has hyperbolic kinetics and is activated by FDP, while LM_3 has hyperbolic kinetics and is not activated by FDP. In each case the FDP activated enzyme has hyperbolic kinetics. Furthermore, the hybrids containing active muscle subunits have kinetics which are not just weighted sums of the properties of the parental isozymes. This indicates that the kinetic properties of a subunit are profound-ly affected by the nature of the other subunits present in the tetramer. The results are qualitatively consistent with a two-state (R-T) equilibrium in which a hybrid with more type M subunits are

more likely to be in the active conformation while hybrids with more L subunits are more likely to be in a less active conformation (sequential models could also explain the results).

The muscle and yeast enzymes dissociate into dimers in 2.4 $\underline{M}$ urea (Steinmetz and Deal, 1966; Boiteux et al., 1978). The dimers formed from muscle have been reported to be partially active (Cottam et al., 1969) while those from yeast are inactive (Boiteux et al., 1978).

Renaturation

The reversible denaturation of pyruvate kinase was first reported by Cottam et al. (1969) and Johnson et al. (1969) with recoveries of 35-50 percent and 70 percent, respectively, of the original enzymatic activity. The latter workers found that the native and renatured enzymes had the same sedimentation coefficients, K_m values, and heat stability profiles.

Bornmann et al. (1974) found the presence of L-valine to be necessary for the renaturation of the yeast enzyme. In the presence of L-valine, recovery of enzymatic activity followed pseudo first order kinetics with 50 percent recovery of the original enzymatic activity. O'Brien and Kapoor (1980) found that recovery of activity is enhanced in the presence of L-valine for the enzyme from Neurospora crassa. They also found that FDP was even more effective than L-valine in enhancing reactivation. The reactivation kinetics were sigmoidal. Bornmann et al. (1974) proposed that L-valine serves as a nucleation center for the folding of the monomer. O'Brien and Kapoor (1980) also concluded that FDP plays a role in the formation of structured monomer.

Previous work with the bovine muscle enzyme resulted in 50-60 percent recovery of activity at 15°C after denaturation in 3.5 $\underline{M}$ guanidine hydrochloride (Cardenas et al., 1977). The half-time of recovery of activity at 15°C was 37 min and was not affected by concentrations of guanidine hydrochloride in the range of 0.05-0.2 $\underline{M}$. Recovery of activity was sigmoidal. In the protein concentration range of 4 to 29 μg/ml, little or no effect of protein concentration

on the half-time of recovery at 15°C was seen. Reactivation was inhibited by phenylalanine and the inhibition was partially reversed by Mg^{2+}. Fluorescence properties and most of the circular dichroism characteristics were fully recovered within one minute of dilution of the guanidine hydrochloride, indicating rapid general folding of the polypeptide chains, but recovery of stable quaternary structure as demonstrated by hybridizability of type M subunits with those of type L was slow and roughly paralleled the regain of enzymatic activity.

General Considerations for Renaturation of
Multisubunit Proteins with Identical Subunits

The renaturation of multisubunit proteins from dissociated, unfolded monomers must involve both folding and association processes. For a complete understanding of the renaturation it is important to consider what is likely to be involved in the folding of subunits, including transconformational processes, the state of assembly and functionality of partially assembled species, and how these factors are interrelated. The probes used to follow renaturation have included the recovery of activity and spectral properties, light scattering, hybridization, and crosslinking.

Protein Folding

The classic experiments of Anfinsen and his colleagues (see Anfinsen, 1973) demonstrated that protein folding occurs spontaneously and indicated that the information required for folding was contained in the protein's primary sequence and its environment. They proposed that the three dimensional structure of native proteins corresponds to the kinetically accessible minimum of the potential energy (see Wetlaufer and Ristow, 1973).

The rapidity of folding and the high degree of cooperativity make a random search mechanism for folding unreasonable (Wetlaufer, 1973). This and kinetic evidence (Tsong et al., 1972b) lead to the idea of nucleation-initiated protein folding (see Wetlaufer, 1973). A nucleus is a localized three dimensional structure that is produced during the early stages of folding. This region grows rapidly by adding segments that are close to the nucleus in the amino acid sequence. The existence of distinct structural regions in globular proteins composed of single polypeptide chains supports this idea (Wetlaufer, 1973). Evidence for nucleus formation comes from the elegant experiments by Anfinsen and colleagues (see Anfinsen, 1973) involving specific antibodies to staphylococcal nuclease and its fragments.

An understanding of the pathway by which protein folding occurs is desirable and important. The usual method of determining a kinetic pathway is to identify and characterize intermediates and place them in the correct order in the pathway. For reasons of simplicity, most

folding studies are done with small globular proteins which have a single folding domain. Disulfide bridges are often left intact.

Early attempts to identify the presence of folding intermediates were unsuccessful. These experiments involved the monitoring of suitably chosen properties (ultraviolet or visible spectral properties, intrinsic viscosity, activity, etc.) as a function of denaturant concentration and determination of the enthalpy change that occurs during the folding process. (Brandt, 1964; Brandts and Hunt, 1967; Tanford, 1968, 1970; Privalov and Khechinashvili, 1974; Brandts, 1969; Baldwin, 1975).

Folding intermediates have been identified using proton magnetic resonance (staphylococcal nuclease: Epstein et al., 1971; ribonuclease A: Westmoreland and Matthews, 1973; Benz and Roberts, 1973; Roberts and Benz, 1973), fast reaction kinetics (ribonuclease: Tsong et al., 1972a; chymotrypsinogen: Tsong and Baldwin, 1972; metmyoglobin: Summers and McPhie, 1972; chytochrome C: Tsong, 1973; Ikai et al., 1973), rapid formation of a species which protects against exchange between tritiated amides and solvent hydrogen (ribonuclease A: Schmid and Baldwin, 1979), and by trapping of disulfide intermediates (bovine pancreatic trypsin inhibitor: see Creighton, 1978).

Cis-trans isomerization of proline residues produces heterogeneity in the unfolded state of RNAase A and other proteins (Brandts et al., 1975; Schmid and Baldwin, 1978a; Garel and Baldwin, 1973, 1975; Hagerman and Baldwin, 1976; Schmid and Baldwin, 1978b, 1979). Unfolded forms that contain non-native proline conformations result in the formation of folding intermediates.

The most detailed examination of folding intermediates has been with the disulfide-containing protein bovine pancreatic trypsin inhibitor (see Creighton, 1978). Quenching of renaturation mixtures at various times with iodoacetate followed by ion-exchange chromatography has permitted the preparative isolation of disulfide intermediates and the characterization of their disulfide bond structure (diagonal electrophoresis technique of Brown and Hartley, 1966).

The folding process determined for bovine pancreatic trypsin inhibitor has certain distinct features and these features appear to hold for other -SS- containing proteins (see Creighton, 1978). Only a small number of well defined intermediates are found, indicating that folding does not proceed by a random search mechanism. The intermediates are relatively compact, albeit unstable, and the slowest folding step is very late in the pathway.

There is no obvious reason why the features found for the refolding of bovine pancreatic trypsin inhibitor should not occur in the folding of proteins not involving disulfide bond formation (Creighton, 1978). While there is not direct evidence to support this statement, there is also no contradictory evidence. Since identical physical principles govern the chemistry of single and multisubunit proteins, these ideas should be applicable to the folding of subunits from oligomeric systems.

Multisubunit Proteins

Since the conditions required to disrupt the quaternary structure of multisubunit proteins often disrupt the secondary and tertiary structure as well (see Friedman and Beycock, 1979), it is often difficult if not impossible to obtain structured, partially assembled and stable systems for study. In these cases the presence of intermediates has been inferred from kinetic experiments or by finding a means of trapping assembly intermediates. Trapping of intermediates is usually accomplished by quenching a kinetic system with crosslinking agents (Hermann et al., 1979) or with an excess of another isozyme (Osborne and Hollaway, 1974; Tenenbaum-Bayer and Levitzki, 1976; Cardenas et al., 1977).

The renaturation of several dimeric enzymes has been studied fairly extensively. Malate dehydrogenase (Jaenicke et al., 1979) and triose phosphate isomerase (Jaenicke, 1978) reactivation experiments can be explained by an irreversible sequential unimolecular-bimolecular mechanism in which the monomer is inactive. Liver alcohol dehydrogenase (Rudolph et al., 1978; Gerschitz et al., 1978) appears to follow a similar scheme except that an intermediate on the main assembly pathway appears to be in equilibrium with a species that is not on the pathway. The renaturation of liver alcohol dehydrogenase has an absolute requirement for zinc (high concentrations will inhibit the renaturation) which is also required for catalytic activity. The renaturation of rabbit muscle creatine kinase (Bickerstaff et al., 1980) involves folding of the subunit, then rapid association to form

dimer, and finally, a slow rearrangement of the dimer to yield native enzyme. The first two processes are complete within 15 min and the initially produced dimer is 70% active. Bickerstaff et al.(1980) found the initial folding step to be slower than subunit reassociation, although the protein concentrations that they used were relatively high. The third step occurs over a period of several hours.

Tetrameric enzymes that have been studied with respect to enzyme renaturation include lactate dehydrogenase (LDH), aldolase (ALD), glyceraldehyde-3-phosphate dehydrogenase (GPD), and phosphofructokinase (PFK). Dimeric intermediates have been identified by hybridization (LDH: Tenenbaum-Bayer and Levitzki, 1976; GPD: Osborne and Hollaway, 1974), by crosslinking (LDH: Hermann et al., 1979), or from assembly kinetics (ALD: Chan et al., 1973; Rudolph et al., 1977d; PFK: Parr and Hammes, 1976). In the case of aldolase, differences in stability between the tetramer and smaller species in 2.3 $\underline{M}$ urea and of folded and unfolded species in the presence of trypsin have allowed the quantification of intermediates from the kinetics of reactivation. Aldolase is unusual among multisubunit enzymes in that the monomer is active as determined with immobilized subunits (Chan, 1976a) and from assembly kinetics (Rudolph et al., 1977d). However, the monomeric activity of aldolase is less stable than that of the tetramer.

Jaenicke and coworkers have found that the renaturation of ALD (Rudolph et al., 1977d), GPD (Rudolph et al., 1977b) and heart LDH (Rudolph et al., 1977c, 1977a) can be described by an irreversible sequential unimolecular-bimolecular reaction mechanism at all temperatures and protein concentrations tested. A more complicated mech-

anism, also involving first and second order processes has been proposed

for aldolase by Chan et al. (1973). Light scattering and sedimentation

results with high concentrations of aldolase indicate rapid formation of

structured monomer and dimer (Teipel, 1972) and that the renaturation

is limited by first order processes. The report of first order recovery

kinetics for heart LDH (Tenenbaum-Bayer and Levitzki, 1976) probably

results from the much larger protein concentrations used in this study.

In contrast, muscle LDH has been reported by some to follow second

order reactivation kinetics (Anderson and Weber, 1966; Levitzki and

Tenenbaum, 1974; Rudolph and Jaenicke, 1976) and by others to follow

first order kinetics (Tenenbaum-Bayer and Levitzki, 1976). These

differences probably reflect differences in protein concentration or in

the extent of denaturation.

Under optimal denaturation-renaturation conditions nearly 100

percent reactivation can be achieved for some enzymes and the product is

indistinguishable from the native enzyme (M_w, $S_{20,W}$, kinetic and spec-

tral properties). Incomplete reactivation is generally thought to be

the result of the formation of inactive aggregates. Removal of the

aggregates by gel filtration yields a reconstitution product that is

indistinguishable from the native enzyme (GPD: Rudolph et al., 1977b;

LDH: Rudolph and Jaenicke, 1976; Rudolph et al., 1977c; Zettlmeissl

et al., 1979; Rudolph et al., 1979).

Many early studies, mostly with dehydrogenases (Anderson and Weber,

1966; Chilson et al., 1966; Teipel and Koshland, 1971; Levi and Kaplan,

1971; Lindy, 1974; Tenenbaum-Bayer and Levitzki, 1976), have indicated

beneficial effects of nucleotide cofactors on renaturation based

on the extent of activity recovery, on recovery rates, and in some
cases on comparisons of structural properties with those of native
enzyme. However, when the percent recovery is defined as

$$\frac{\text{activity of renaturing enzyme at time t}}{\text{activity of control enzyme at time t}} \times 100, \text{ and } \frac{\text{\% recovery}}{\text{\% final recovery}}$$

is plotted as a function of time, the reactivation curves with and
without cofactor are identical (GDP: Rudolph et al., 1977b; LDH:
Rudolph et al., 1977a; liver alcohol dehydrogenase: Gerschitz et al.,
1978; malate dehydrogenase: Jaenicke et al., 1979). These results
indicate that the effect of cofactors is to stabilize the reactivation
product resulting in an increased yield but does not affect renaturation
rates per se. This stabilization of final product by a cofactor is
well-illustrated by the effects of zinc on the renaturation of alcohol
dehydrogenase (Rudolph et al., 1978; Jaenicke, 1978).

The results from the renaturation of multisubunit proteins with
identical subunits indicate that several features appear to be common
for these proteins. Generally, both transconformational and associa-
tion processes are significant. With the exception of aldolase, mono-
mers appear to be inactive. The major effect of cofactors appears to
be mainly the stabilization of the final renaturation product. It will
be interesting to see if this pattern is followed by other oligomeric
proteins.

II. Materials and Methods

Preparation of Bovine Muscle Pyruvate Kinase

Bovine neck muscle was obtained from a newly slaughtered animal and transported to the laboratory on ice. Excess fat and connective tissue were removed and the remaining muscle was used immediately or stored at -80°C. Bovine type M pyruvate kinase was prepared as described by Cardenas et al. (1973) with one modification. The initial extraction buffer was 0.02 $\underline{M}$ Tris-HCl, pH 7.5, 1 m$\underline{M}$ ethylenedinitri-lotetra acetic acid (EDTA), 10 m$\underline{M}$ 2-mercaptoethanol. Fractions from the carboxymethyl Sephadex column with specific activities of 200 units/mg protein or greater were pooled and precipitated by dialysis against saturated ammonium sulfate; pH 7.0, 4°C; containing 5 m$\underline{M}$ MgCl$_2$ and 10 m$\underline{M}$ 2-mercaptoethanol. At this stage the muscle pyruvate kinase is stable for several months. This material was chromatographed as needed (~ 15 mg at a time) on a 2.5 cm x 90 cm column of Biogel A 1.5 m. Elution was with 0.05 $\underline{M}$ potassium phosphate, pH 7.0, 0.1 $\underline{M}$ KCl, 10 m$\underline{M}$ 2-mercaptoethanol. The flow rate was 17 ml/hr and 2.5 ml fractions were collected. Fractions to be saved were pooled, dialyzed against ammonium sulfate (see above), and stored as an ammonium sulfate precipitate at 0-4°C.

Enzymatic Assay

Assays were performed on a Beckman Acta III spectrophotometer equipped with a sample changer and stirring accessory. A Lauda circulating water bath was used for temperature control.

Pyruvate kinase activity was measured by coupling the reaction to lactate dehydrogenase (Bücher and Pfleiderer, 1955) in a medium containing 0.05 $\underline{M}$ imidazole-HCl, pH 7.0, 0.1 $\underline{M}$ KCl, 10 $\underline{mM}$ MgCl$_2$, 2.0 $\underline{mM}$ ADP, 1.0 $\underline{mM}$ PEP, 0.16 $\underline{mM}$ NADH and the desired amount of lactate dehydrogenase.

Activity determinations in the reactivation studies were performed in 990 µl of assay medium plus 5 µl of 1100 units/ml lactate dehydrogenase. Samples of 10 µl were assayed at the same temperature as the corresponding reactivation unless otherwise indicated.

Activity determinations of immobilized enzyme were made in 3 ml of assay medium plus 25 µl of 1100 units/ml lactate dehydrogenase. The assay mixture was stirred using a spherical Teflon-coated stirring bead and the stirring accessory of the Beckman Acta III spectrophotometer. Samples of matrix bound enzyme were prepared as follows: Sufficient buffer was added to one volume of packed resin to make a total of two volumes. Aliquots, 25 µl, of the resulting slurry were transfered to the assay cuvette using an automatic pipette. The plastic tips used on the automatic pipette were cut with a scalpel to enlarge the opening. This allowed for the unhindered entry of the gel particles. All assays of immobilized enzyme were performed at 25°C.

Each mole of pyruvate produced in the reaction catalyzed by pyruvate kinase is converted to a mole of lactate by the lactate dehydrogenase with the concomitant conversion of one mole of NADH to NAD$^+$. The loss of NADH was followed by monitoring the decrease in absorbance at 340 nm in a 1 cm path length cell. Activities were

calculated using the equation

$$\frac{\Delta A}{min} = \frac{\mu moles}{6.22 \ ml} \ x \ \frac{Vol. \ of \ assay \ in \ ml.}{Vol. \ of \ sample \ in \ ml.} = \frac{\mu moles/min}{ml \ of \ sample} = \frac{units}{ml \ of \ sample}$$

The activity of matrix bound enzyme is expressed as units/ml of actual packed resin.

Protein Concentration

Protein concentrations of solutions of unmodified enzyme were determined from the absorbance at 280 nm using $E_{280}^{0.1\%} = 0.55$ (Cardenas et al., 1973). Protein concentrations of trinitrophenylated pyruvate kinase were determined by the Folin-Ciocalteau method as described by Clark (1964) using bovine muscle pyruvate kinase as a standard.

Denaturation

Enzyme that had been stored as an ammonium sulfate precipitate was centrifuged down and dissolved in 0.05 M Tris-HCl, pH 7.5, containing either 0.1 M 2-mercaptoethanol or 5 mM DTT or in standard renaturation buffer (see following section). For the studies on the effect of amino acids, analogs, etc. the standard renaturation buffer minus the magnesium chloride was used instead of the standard renaturation buffer. The resulting solution was diluted to give a protein concentration 52 times greater than the protein concentration desired during renaturation. An aliquot of this stock solution was mixed with an equal volume of 8 M guanidine hydrochloride and incubated at 0°C for 15 min unless otherwise stated. Each denaturation had a parallel con-

trol consisting of an aliquot of the stock enzyme which was diluted with an equal volume of distilled deionized water.

Reactivation

Reactivation was begun by diluting, with gentle swirling, an aliquot of enzyme in guanidine hydrochloride into 25 volumes of renaturation buffer at the desired temperature. Reactivation mixtures were incubated in a Blue M constant temperature bath. For the temperature and enzyme concentration studies the renaturation buffer was 0.05 $\underline{M}$ Tris-HCl, pH 7.5, 0.1 $\underline{M}$ KCl, 10 $\underline{mM}$ MgCl$_2$, 0.5 $\underline{M}$ sucrose, 5 $\underline{mM}$ DTT. This buffer will be referred to as standard renaturation buffer. For the studies with amino acids, amino acid analogs, and FDP, the standard renaturation buffer minus the MgCl$_2$ was used and the effector of interest was added. In the studies with tyrosine, tryptophan, and phenylalanine analogs the renaturation buffer contained 0.1 $\underline{mM}$ EDTA.

After reactivation was begun aliquots were removed at appropriate time intervals and were assayed for pyruvate kinase activity. The assay temperature was the same as the corresponding reactivation temperature with the following exceptions: (1) For the dependence of renaturation on enzyme concentration at 7.8°C, enzyme assays were performed at 15°C. (2) For other renaturations involving temperatures of less than 12.5°C, enzyme assays were performed at 12.5°C. Final activity yields were determined at least 24 hours after the beginning of reactivation and up to 3 days afterward for the lower temperatures.

Each reactivation had a parallel control consisting of enzyme that was not exposed to denaturing conditions but that was treated identically in all other respects. Sufficient solid guanidine hydrochloride was added to the control enzyme buffer so that control and renaturing enzyme were exposed to the same concentration of guanidine hydrochloride (0.15 $\underline{M}$).

Gel Filtration of Reactivated Enzyme

Gel filtration at 4°C was employed to detect protein aggregates formed during reactivation. A total of 2.4 mg of enzyme was subjected to the denaturation-renaturation procedure and was concentrated by ammonium sulfate precipitation. The precipitate was collected by centrifugation, dissolved in 0.05 $\underline{M}$ potassium phosphate, pH 7.0, 0.1 $\underline{M}$ KCl, 10 m$\underline{M}$ 2-mercaptoethanol and applied to a column (1.5 cm x 38 cm) of Biogel A 1.5 m, 100-200 mesh that had previously been equilibrated with the same buffer. Gravity flow from a Marriot flask was used to set the flow rate at 8.8 ml/hr and 0.88 ml fractions were collected. Fractions were assayed for pyruvate kinase activity as described above and protein concentrations were determined from the absorbance at 280 nm.

Data Analysis

For temperatures and enzyme concentration ranges where the reactivation did not show a dependence on enzyme concentration, the nonlinear first order plots of the reactivation data were analyzed by the method described by Ray and Koshland (1961) for the resolution of two exponentials.

Enzyme Immobilization

Sepharose 4B-200 obtained from Sigma Chemical Company was activated
with cyanogen bromide (CNBr) using the buffer method described by
Parikh et al. (1974) and the coupling of muscle pyruvate kinase was
performed as described by Chan (1976) for the immobilization of aldolase.

Sepharose was washed with 8 volumes of 1 $\underline{M}$ NaCl followed by 8 volumes
of distilled deionized water. Washing was by filtration under suction
through a sintered glass disc. Sepharose was centrifuged in a clinical
centrifuge at its lowest setting and the volume of packed resin was
used as a measure of the amount of resin.

A typical activation reaction involved adding a volume of 1:1
washed Sepharose to an equal volume of 2 $\underline{M}$ sodium carbonate. The slurry
was cooled to 5°C in an ice bath with gentle swirling. The desired
amount of CNBr dissolved in acetonitrile (1-2 mg/ml of packed resin)
was added and the rate of swirling was increased. After two minutes
the slurry was decanted into a sintered glass disc filtration apparatus
and the gel was washed with 20 volumes of 0.1 $\underline{M}$ carbonate buffer, pH 9.0.
The washed Sepharose was added to an equal volume of 5 mg/ml pyruvate
kinase that had previously been dialyzed against 0.1 $\underline{M}$ carbonate buffer,
pH 9.0, 1.0 m$\underline{M}$ EDTA, 10 m$\underline{M}$ 2-mercaptoethanol. This mixture was incu-
bated at 3°C with occasional gentle swirling. After 24 hours the gel
was washed by filtration with 10 volumes of 0.1 $\underline{M}$ ethanolamine-HCl,
pH 7.5. The Sepharose was suspended in 2.5 volumes of 0.1 $\underline{M}$ ethanola-
mine-HCl, pH 7.5, 10 m$\underline{M}$ 2-mercaptoethanol and incubated at 3°C with
occasional gentle swirling. After 24 hours the Sepharose was washed
with and stored in 0.05 $\underline{M}$ Tris-HCl, pH 7.5, 0.1 $\underline{M}$ KCl, 0.5 $\underline{M}$ sucrose,

5 m$\underline{M}$ MgCl$_2$, 0.1 m$\underline{M}$ EDTA, 5 m$\underline{M}$ DTT at 4°C.

Trinitrophenylation

Bovine muscle pyruvate kinase was inactivated with 2,4,6-trinitro-benzene sulfonic acid as described by Hubbard and Cardenas (1975). This procedure does not alter the quaternary structure of the enzyme or its ability to form hybrids with the liver isozyme. After the inactivation was stopped by addition 1/10 volume of 1 $\underline{M}$ Tris-HCl pH 7.5, the protein was precipitated by the addition of saturated ammonium sulfate and collected by centrifugation. The precipitate was dissolved in 1 ml of 0.05 $\underline{M}$ phosphate buffer, pH 7.0, 10 m$\underline{M}$ 2-mercapto-ethanol. This precipitation and centrifugation procedure was repeated two more times. The final precipitate was dissolved in 0.05 $\underline{M}$ Tris-HCl pH 7.5, 0.1 $\underline{M}$ KCl, 0.5 sucrose, 5 mM MgCl$_2$, 0.1 m$\underline{M}$ EDTA, 5 m$\underline{M}$ DTT and dialyzed against the same buffer for 3 hours at 4°C in the dark. Trinitrophenylated pyruvate kinase was stored at 4°C in the dark.

Removal of Subunits Not
Crosslinked to Sepharose

Sepharose immobilized enzyme was washed three times by centrifuga-tion and resuspension in 0.05 $\underline{M}$ Tris-HCl pH 7.5, 0.1 $\underline{M}$ KCl, 0.5 M sucrose, 5 m$\underline{M}$ MgCl$_2$, 0.1 m$\underline{M}$ EDTA, 5 mM DTT at 4°C. After the final centrifugation the supernatant was removed and a volume of 8 $\underline{M}$ guanidine hydrochloride equal to the volume of packed resin was added. The resulting slurry was incubated for 15 min on ice. The Sepharose containing immobilized

polypeptides was washed by filtration with 100 volumes of 4 $\underline{M}$

guanidine hydrochloride, .025 $\underline{M}$ Tris-HCl pH 7.5, 0.05 $\underline{M}$ KCl, 0.25

$\underline{M}$ sucrose, 2.5 m$\underline{M}$ MgCl$_2$, 0.05 m$\underline{M}$ EDTA, 2.5 m$\underline{M}$ DTT. The Sepharose was

transferred to 25 volumes of the same buffer used for the washing

by centrifugation and incubated at 16°C. After 24 hours the gel

was stored at 4°C. This preparation will be referred to as Sepharose

bound subunits.

Reconstitution of the Quaternary
Structure of Sepharose Bound Subunits

Sepharose bound subunits were washed by centrifugation and resus-

pension as described in the previous section. After the final centri-

fugation the supernatant was adjusted to give three volumes of packed

Sepharose plus seven volumes of supernatant. Soluble, non-Sepharose

bound, native or trinitrophenylated pyruvate kinase was denatured by

adding an aliquot of 8 $\underline{M}$ guanidine hyrochloride to an equal volume

of enzyme in 0.05 $\underline{M}$ Tris-HCl, pH 7.5, 0.1 $\underline{M}$ KCl, 0.5 $\underline{M}$ sucrose, 5 m$\underline{M}$

MgCl$_2$, 0.1 m$\underline{M}$ EDTA, 5 m$\underline{M}$ DTT. After 15 min on ice an aliquot was

diluted with gentle swirling into 25 volumes of the 3:7 slurry described

above. This mixture was incubated at 16°C for 24 hours. During the

first 1.5 hours, the renaturation vessel was gently swirled every 5-10

minutes in order to keep the Sepharose suspended.

Amino Acid Analysis

Sepharose bound protein, 0.5 ml packed Sepharose, was lyophilized in a hydrolysis vial. One ml of constant boiling HCl was added and the material was hydrolyzed at 110°C for 24 hours in the sealed evacuated vial. The solution was dried under vacuum and extracted with Durrum's Sample Diluent pH 2.0. Sample solutions were Millipore filtered and analyzed on a Dionex Amino Acid Analyzer (kit) using fluorescence detection. A blank containing Sepharose with no bound pyruvate kinase was treated identically to the samples containing bound enzyme. The amino acid content of the samples containing bound enzyme were corrected for the amino acid content of the blank.

III. Results

Part A: The Effect of Temperature and
Protein Concentration on the Renaturation
of Bovine Muscle Pyruvate Kinase.

Shown in Figure 1A are the reactivation curves of type M pyruvate kinase at temperatures from 0° to 20°C. Figure 1B shows the corresponding reactivation curves for temperatures from 35° to 45°C. For each data point, enzyme assays were performed on both the renaturing enzyme and a control solution of the same protein concentration (25 µg/ml) that had not been denatured. Each data point therefore represents a comparison of enzyme activity concentrations in renaturing and control samples. Control enzyme samples typically retained 85-100% of their original activity throughout the course of the experiment for all temperatures (see Table I). At all temperatures the recovery of activity as a function of time is sigmoidal. This indicates that activity recovery is not dependent on a single first or second order rate limiting step and therefore agrees with the earlier work by Tobes et al. (1972) with yeast pyruvate kinase and with the studies of Cardenas et al. (1977).

Half-times for recovery of enzymatic activity decreased with increasing renaturation temperature throughout the temperature range studied and varied from 185 min at 0°C to 4 min at 45°C. Decreases in half-times of renaturation as a function of increasing renaturation temperature were much less marked above 30°C (see Table I).

Figure 1. Reactivation as a function of temperature for bovine

type M pyruvate kinase after denaturation in 4 $\underline{M}$

guanidine hydrochloride. Each data point is the ratio

of the activity at time t to the activity of the

control at time t. The protein concentration was 25 $\mu g/ml$.

For reactivation at 0°, 5°, and 10°, enzymatic

activities were determined at 12.5^{o}. In each case no

reactivation in the assay cuvette was observed as

evidenced by a linear change in absorbance with time.

No enzymatic activity could be detected in enzyme

samples taken directly from solutions of 4 $\underline{M}$ guan-

idine hydrochloride. For Part A: 0°, (●); 5°, (□);

10°, (▲); 15°, (■); 20°, (○). For Part B: 35°, (+);

40°, (△); 45°, (✕).
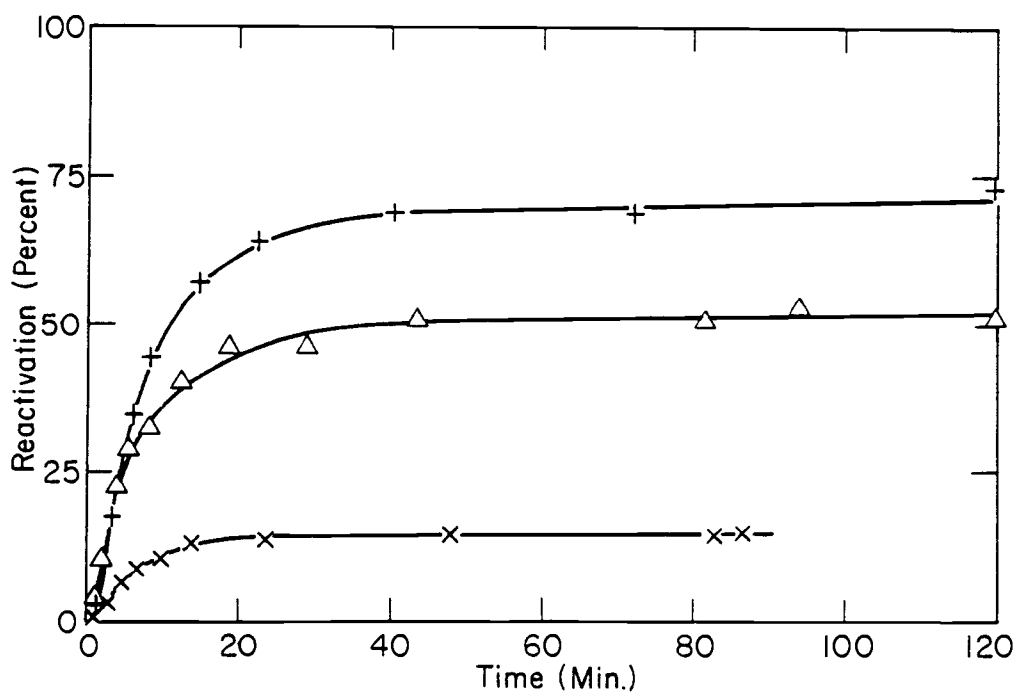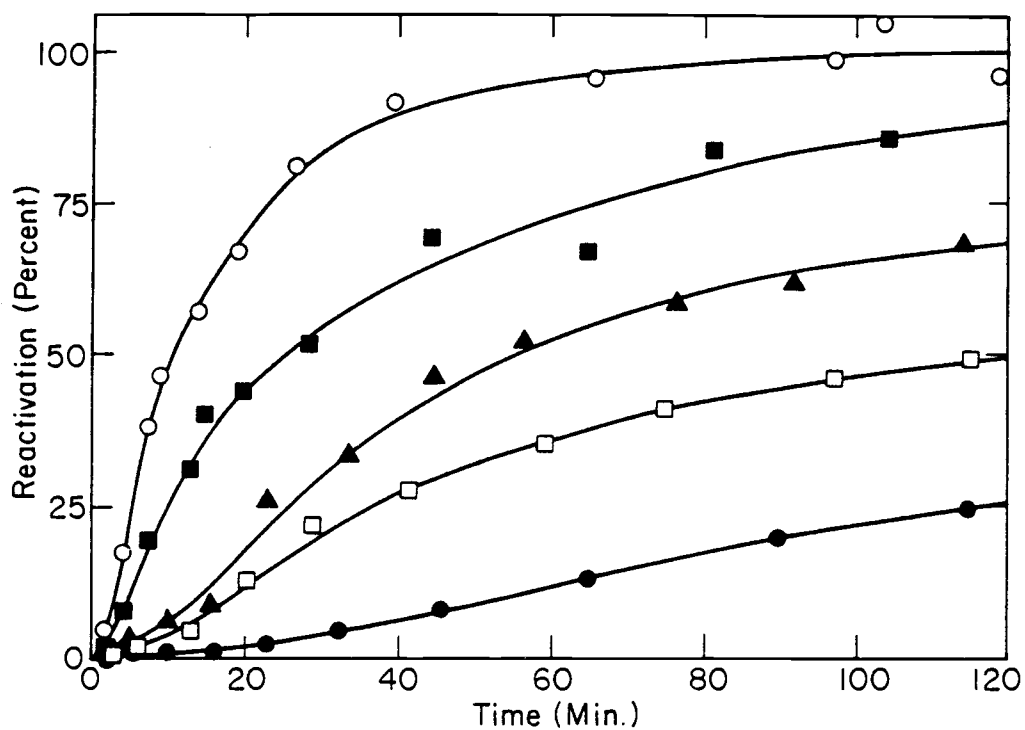
Figure 1. Part A: top. Part B: bottom.

TABLE I. Half-times and percent recovery for the enzymatic activity
of bovine type M pyruvate kinase as a function of temperature.

The enzyme concentration in each reactivation was 25.6 µg/ml.

Temperature	Half-time (min)	Percent Recovery	Control Enzyme Activity. Final percent compared to initial value.
0	185 ± 7.0	89 ± 3	88 ± 3
7.0	64.0 ± 4.0	93 ± 4	80 ± 3
11.2	29.3 ± 4.0	90 ± 6	90 ± 2
16.0	17.8 ± .60	89 ± 3	86 ± 3
20.3	10.5 ± .59	91 ± 3	85 ± 3
25.3	6.31 ± .20	88 ± 3	96 ± 4
30.3	5.85 ± .13	87 ± 3	94 ± 4
35.1	4.67 ± .29	73 ± 3	95 ± 4
40.0	5.12 ± .72	52 ± 3	100 ± 3
45.2	4.33 ± .32	13 ± 2	100 ± 3

Final activity yields, determined by assaying renaturing and control enzymes at least 24 h. after beginning renaturation for the higher temperatures and up to three days after beginning renaturation for the lower temperatures, are presented in Table I and Fig. 2. Final activity yields were constant at about 90% of control values for samples that were renaturated between 0° and 30°C. In the temperature range of 30-45°C, the percent recovery decreased with increasing renaturation temperature. Thus, while 91% of the activity, based on control samples of enzyme, was recovered when renaturation occurred at 20°C, only 73% of the activity could be recovered when renaturation occurred at 35°C, and a final activity yield of only 13% was found when renaturation took place at 45°C. The control enzyme retained 85-100% of its activity.

Kayne and Suelter (1968) found that an Arrhenius plot of catalytic activity of rabbit muscle pyruvate kinase consisted of two effectively linear segments of different slopes (larger slope at lower temperature) connected by a curved region. They proposed that the different slopes were due to a corresponding temperature dependent conformational change. The same type of Arrhenius plot is found for the catalytic activity of bovine muscle pyruvate kinase (Fig. 2). The plot is essentially linear at temperatures below about 31°C. Thus, there is therefore no kinetic evidence to suggest a critical conformational change in the temperature range of 0-31°C, although such a change may occur at 31° or above. It is interesting to note that the curvature in the Arrhenius curve for enzymatic activity occurs at about the same temperature where the recovery of enzymatic activity after renaturation begins to decrease.

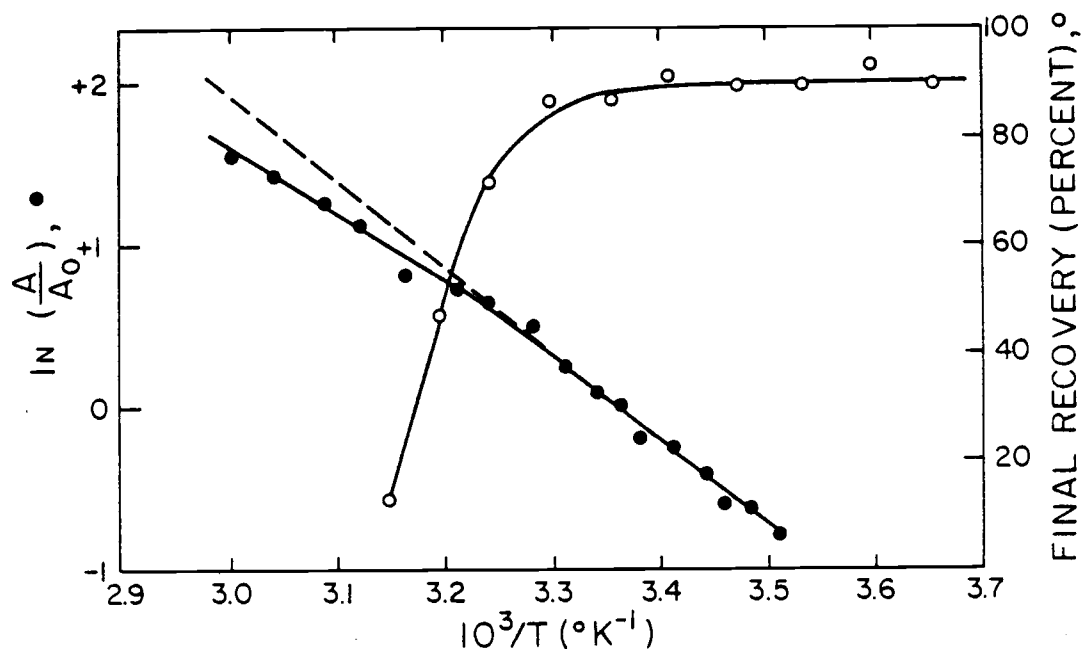Figure 2. Arrhenius plot of the catalytic activity of bovine

type M pyruvate kinase and a comparison with the final

recovery of enzymatic activity that is found upon

renaturation at various temperatures. A is the enzymatic

activity at temperature T and A_o is the enzymatic

activity at $25^{\circ}C$. The dashed line is an extrapolation

of the low temperature portion of the Arrhenius plot.

It is interesting to determine whether decreased recovery of enzymatic activity at the higher renaturation temperatures is due to the formation of enzyme of lower specific activity or whether some competing process, such as protein aggregation, might simply reduce the amount of enzyme available for renaturation. Gel filtration was employed in order to determine whether protein aggregates could be observed after renaturing the enzyme at any of the temperatures. Shown in Figure 3 is a gel filtration pattern of enzyme that had been re- natured at 25°C. The OD_{280}-absorbing peak that preceded the peak of enzymatic activity represents aggregated protein, while the peak trailing the activity peak probably represents denatured protein subunits. The specific activity of the active enzyme approximated the value for native enzyme, thus indicating that decreased yields of enzyme activity are caused by protein aggregation or by failure of protein subunits to reassociate to form a stable tetramer. These processes become more prevalent when renaturation is performed at higher temperatures. Renaturation at 45°C produced aggregates so large that they did not pass through the column but could be seen at the top of the column. A some- what larger quantity of inactive, monomeric protein was obtained when renaturation was performed at the higher temperature, but the active enzyme had a specific activity that was similar to that of native pyruvate kinase.

In order to determine whether aggregated or otherwise denatured protein affected the renaturation process of other enzyme subunits, a renaturation experiment was performed at 45°C for the purpose of pro-
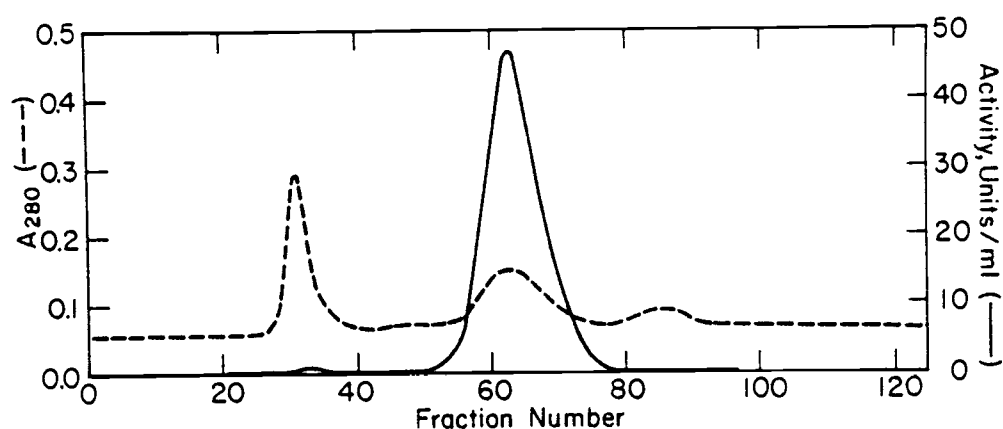
Figure 3. Gel filtration of enzyme renatured at 25°C. Absolute
absorbance at 280 nm (---) is uncorrected for buffer
absorbance. Pyruvate kinase activity is expressed as
units/ml (—). The enzyme concentration during the
renaturation was 25.6 μg/ml.

ducing large amounts of denatured protein. This solution of mainly

aggregated protein was then reequilibrated to 11.5°C. A second sample

of protein in guanidine HCl was added to the solution containing the

aggregates and was allowed to renature at the lower temperature. The

renaturation of the second aliquot of protein, added at 11.5°C, was

unaffected by the presence of aggregated protein from the renaturation

attempt at 45°C. Renaturation controls demonstrated that enzyme activity

recovered during the 11.5° incubation was due to the protein sample that

was added at that temperature and not due to additional activity regain

from the sample that had been previously incubated at 45°C.

The effect of protein concentration on the reactivation of pyruvate

kinase was also studied. Figure 4 shows the renaturation kinetics at

16°C over the concentration range of 6.4-25.6 μg/ml. No dependence on

protein concentration was seen under these conditions. Each data point

in Figure 4 represents the ratio of percent reactivation at time t to

the percent final reactivation. Within experimental error the percent

final reactivation was approximately the same for each protein concen-

tration (about 90%). Similar results were obtained for renaturation

at 55 μg protein /ml and 16°C (results not shown). Renaturation at

110 μg/ml resulted in only a 54% final reactivation, probably due to

increased nonspecific aggregation. At protein concentrations of 3.2

μg/ml or below and at the renaturation temperature of 16°C, renatura-

tion occurred with considerably reduced final yields (58% for 3.2 μg/ml).

Teipel and Koshland (1971) have also observed reduced renaturation

yields of other enzymes at low protein concentrations. Low concentrations
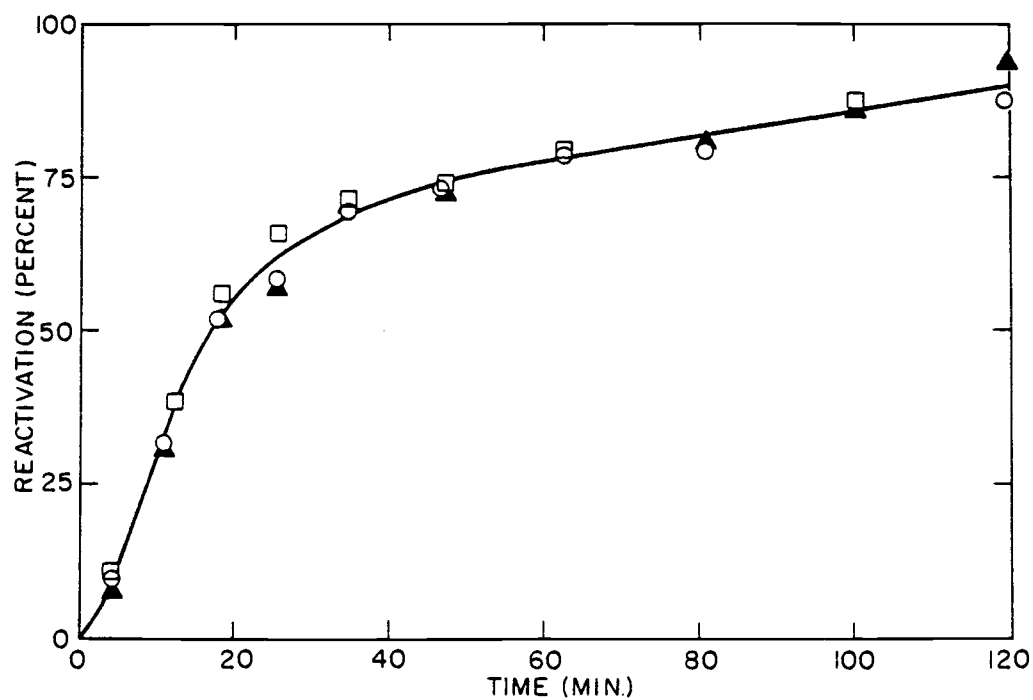
Figure 4.   Reactivation at 16° and the following protein concen-
trations (μg/ml): 25.6, (□); 12.8, (▲); 6.4, (○). Each
data point is the ratio of the percent reactivation at
time t to the percent final reactivation.

of aldolase (Chan et al., 1973) and of phosphofructokinase (Parr and Hammes, 1976) have been stabilized by the addition of bovine serum albumin. The addition of bovine serum albumin (1.0 mg/ml) to the pyruvate kinase renaturation mixture eliminates the problem of reduced final activity yields at the lower concentrations of enzyme.

Other than increasing the final recovery of enzymatic activity at the lower concentrations of pyruvate kinase, bovine serum albumin had no significant effect on the renaturation kinetics of pyruvate kinase at any temperatures or concentrations of enzyme tested. BSA had no effect on the renaturation kinetics of pyruvate kinase at the higher enzyme concentrations used. Cook and Koshland (1969) have shown that the renaturation of a given enzyme is generally independent of the renaturation of other added purified enzymes. Thus, bovine serum albumin in the renaturation medium appears to function in a relatively nonspecific manner, neutralizing factors such as vessel wall effects that tend to decrease the amount of enzyme available for renaturation.

Figure 5 shows the renaturation kinetics in the presence of 1 mg/ml bovine serum albumin at 16°C. No concentration dependence is seen in the enzyme concentration range of 6.4-25.6 µg/ml and the renaturation results are similar to those obtained in the absence of bovine serum albumin. However, the renaturation rate is slower at enzyme concentrations of 2.5 µg/ml or less than at higher concentrations of enzyme (see Fig. 5 and Table II). In the presence of bovine serum albumin, the final recovery of enzymatic activity is approximately 90% at all enzyme concentrations tested.

Figure 5. Reactivation at 16° in the presence of 1 mg/ml bovine
serum albumin. Enzyme concentrations (μg/ml) are:
25.6, (□); 12.8, (▲); 6.4, (○); 2.5, (■); 1.0, (△).
Each data point is the ratio of the percent reactivation
at time t to the percent final reactivation.

Table II. Half-times and percent recovery of enzymatic activity as a
function of enzyme concentration at two temperatures. Bovine serum
albumin, 1 mg/ml, was present in each case.

Temperature	Enzyme Concentration (µg/ml)	Half-time (min)	Percent Recovery
16°C	25.6	17.8	90
	12.8	17.8	90
	6.4	17.8	90
	2.5	38	90
	1.0	91	90
7.8°C	25.6	45	90
	12.8	59	90
	6.4	80	90
	2.5	118	90
	1.0	210	90

Figure 6 shows the reactivation curves for various concentrations of enzyme at 7.8°C in the presence of 1 mg/ml bovine serum albumin. Table II shows the half-time and percent recoveries. Here we see a concentration dependence in the range of 6.0-25.6 µg/ml as well as at lower concentrations. Thus, manipulation of enzyme concentration and/or renaturation temperature can produce conditions in which the association processes involved in the assembly of active pyruvate kinase are slow enough that they contribute to the rate of recovery of enzymatic activity. This is shown by the enzyme concentration effects at low renaturation temperatures and/or concentrations. Also seen in the reactivation curves of Figure 6 is a lag time which is highly dependent on enzyme concentration.

By choosing the appropriate temperature and enzyme concentrations for renaturation so that concentration effects are not seen, we can examine the transconformational processes involved in the reactivation of pyruvate kinase. Figure 7 shows a first order plot of reactivation data for temperatures between 16° and 30.3°C. The solid lines were calculated as described below. The enzyme concentration in each case was 25.6 µg/ml. In this temperature range, no concentration dependence on the renaturation kinetics could be seen. The reactivation rates clearly do not follow simple first order kinetics. However, the data can be satisfactorily represented as the sum of two exponentials, according to the equation

$$1 - v/V_f = ae^{-\lambda_1 t} + be^{-\lambda_2 t} \tag{1}$$
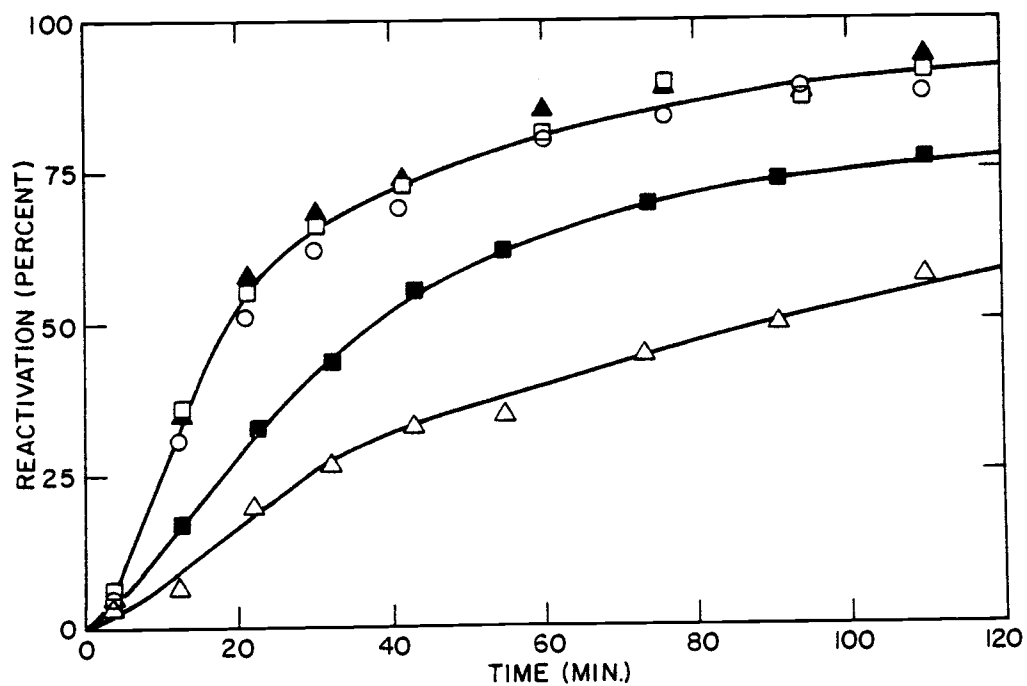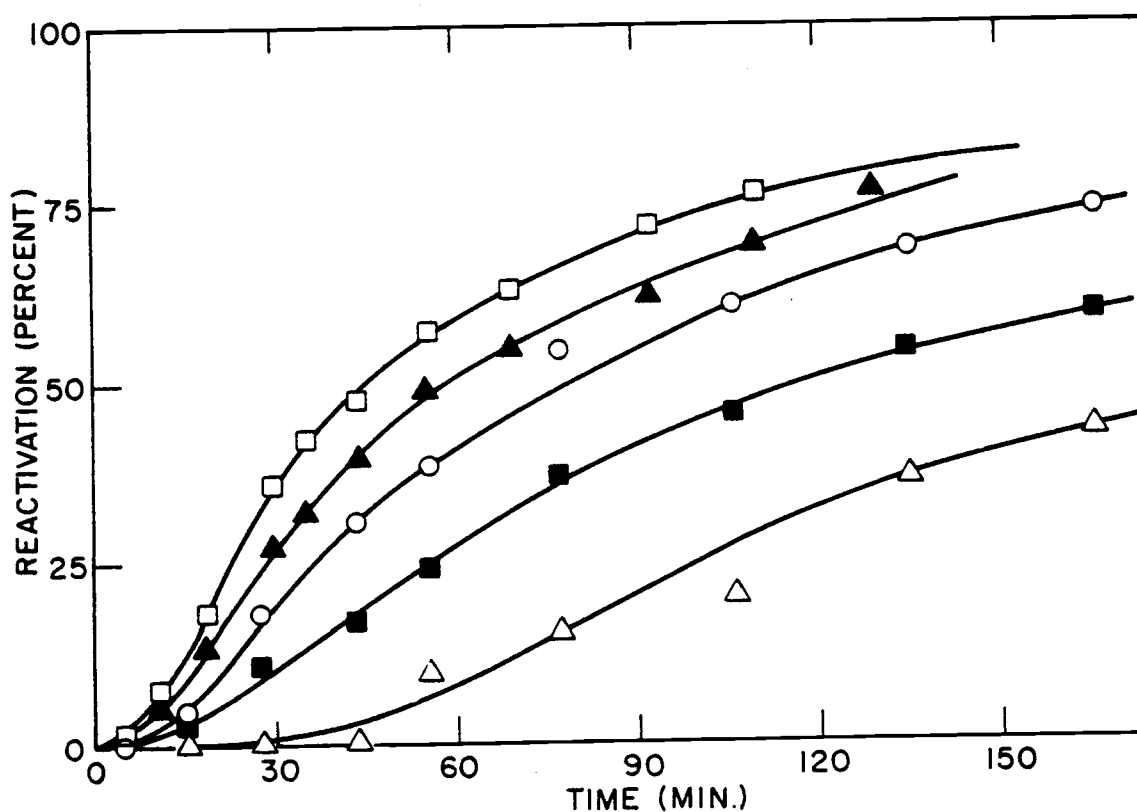
Figure 6.   Reactivation at 7.8° in the presence of 1 mg/ml bovine
serum albumin. Enzyme concentrations (μg/ml) are:
25.6, (□); 12.8, (▲); 6.0, (○); 2.5, (■); 1.0, (△).
Each data point is the ratio of the percent reactivation
at time t to the percent final reactivation. Enzymatic
activities were measured at 15°C.
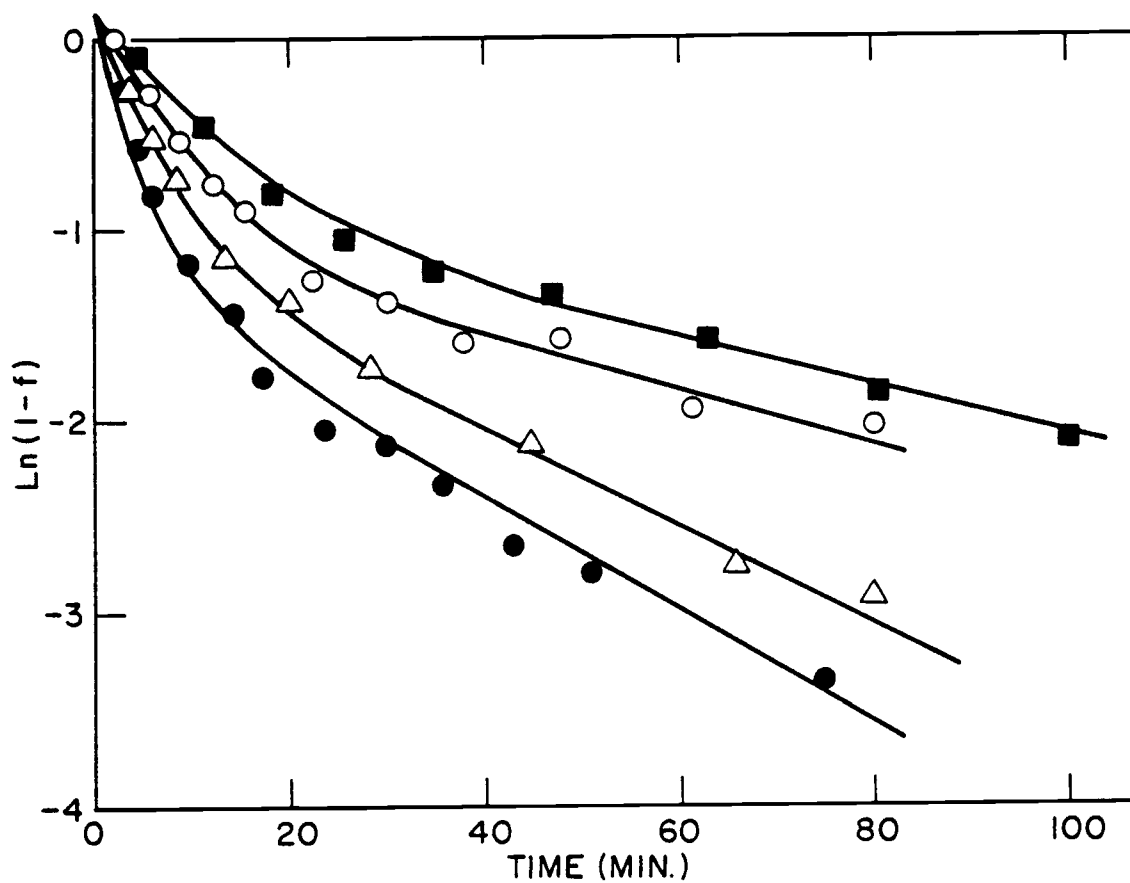
Figure 7. First order plot of reactivation data at 25.6 μg/ml for the following temperatures (°C): 16.0, (■); 20.3, (O); 25.3, (△); 30.3, (●). The solid lines were drawn based on equation 1 using the parameters given in Table III.

Where a and b are constants, v is the % recovery at time t, V_f is the final % recovery, and λ_1 and λ_2 are the macroscopic rate constants.

The data were analyzed by the method described by Ray and Koshland (1961) and yielded the values given in Table III. The value for a increases with increasing temperature while b decreases. There is a short induction period as indicated by the failure of the reactivation curves to extrapolate to $\ln (1-v/V_f) = 0$ at t = 0 and by the fact that the sum of a and b is 1.14 rather than 1.0. From equation 1 and taking v = 0 at t = 0 we would expect the sum of a and b to be 1.0. The solid lines of Figure 7 were calculated from equation 1, using the parameters given in Table III. As seen in Figure 7 , the calculated lines provide a good fit for the actual experimental data points.

Figure 8 is an Arrhenius plot of the macroscopic rate constants λ_1 and λ_2. The data are highly linear and yield apparent activation energies of 10.6 and 11.9 kcal/mole, respectively, for λ_1 and λ_2.

The reactivation kinetics of pyruvate kinase at 16°C and 25.6 μg/ml are found to be identical (a) whether denaturation is performed in 4.0 or 6.0 M guanidine HCl; (b) whether 2.5 mM or 5.0 mM dithiothreitol or 0.1 M mercaptoethanol is used in the denaturation buffer; (c) at any dithiothreitol concentration in the renaturation buffer from 5 mM up to at least 15 mM; (d) whether the enzyme remains in the denaturation medium 8, 15, or 30 min prior to beginning renaturation; (e) regardless of whether or not the solutions are flushed with N_2 prior to use; (f) whether enzyme denaturation is performed in a solution containing Tris-HCl and 2-mercaptoethanol or in the renaturation buffer described in the Materials and Methods section.

Table III: Values of the parameters in Eq. 1 determined for temperatures
of 16°C to 30.3°C. Each value is the mean $\pm$ the standard deviation
of three determinations. The units of λ_1 and λ_2 are sec^{-1}. The protein
concentration was 25.6 µg/ml.

Temp	a	λ_1 x 10^3	b	λ_2 x 10^4
16°C	.662 $\pm$ .032	1.65 $\pm$ 0.30	.487 $\pm$ .040	2.08 $\pm$ 0.38
20.3°C	.766 $\pm$ .044	2.22 $\pm$ 0.02	.380 $\pm$ .024	2.40 $\pm$ 0.45
25.3°C	.800 $\pm$ .050	3.00 $\pm$ 0.42	.342 $\pm$ .057	4.13 $\pm$ 1.08
30.3°C	.850 $\pm$ .089	3.88 $\pm$ 0.55	.317 $\pm$ .105	5.03 $\pm$ 1.70
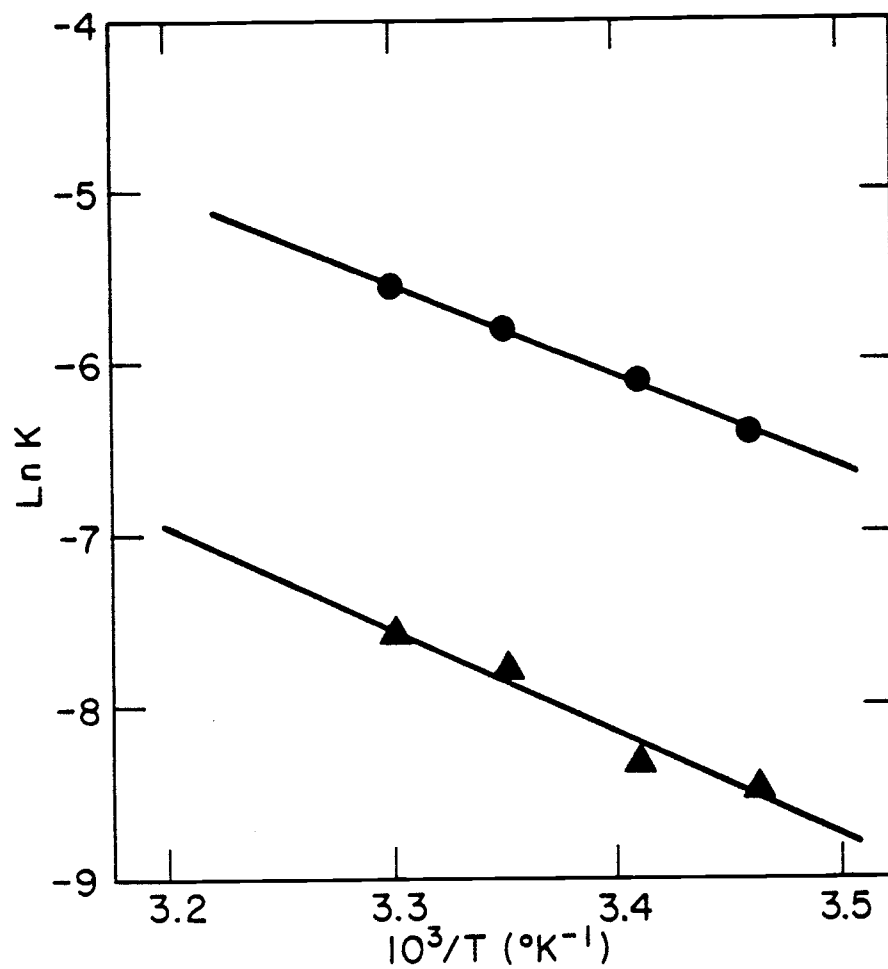
Figure 8. Arrhenius plot for the macroscopic rate constants λ_1 ($\bullet$) and λ_2 ($\blacktriangle$).

Part B: Effects of Amino Acids, Amino

Acid Analogs, Fructose-1,6-bisphosphate

and Phosphoenolpyruvate on renaturation

of pyruvate kinase.

Shown in Figure 9 are the renaturation curves for bovine type M

pyruvate kinase at various concentrations of L-phenylalanine. Since

Mg^{2+} reverses the phenylalanine inhibition of renaturation (Cardenas

et al., 1977), the reactivation experiments described in this section

were done in the absence of Mg^{2+}. All reactivations were performed

at 16°C. Half-times for activity recovery and percents of final re-

covery are listed in Table IV, Part I. Final recoveries were determined

by comparing activities in control and renaturing enzyme solutions

approximately 24 h after beginning the renaturation. All other pro-

cedures are as described in the methods section. Some variation in

percent and half-times of recovery was seen from one set of experiments

to another, (for example, compare the values for 5 mM L-phenylalanine

in Part I with the data presented in the first line of Part II, and

compare the values given in the first lines of Parts I and III). How-

ever, the ratios of kinetic parameters were the same from one experi-

mental set to another, and each experimental set included its own

control performed as close as possible in time and procedure to the

experimental samples. Control enzyme solutions typically retained

60-80% their original activity. Even 1.0 mM L-phenylalanine, the

lowest concentration tested, produced an approximately 50 percent

increase in the half time for recovery of enzymatic activity, while
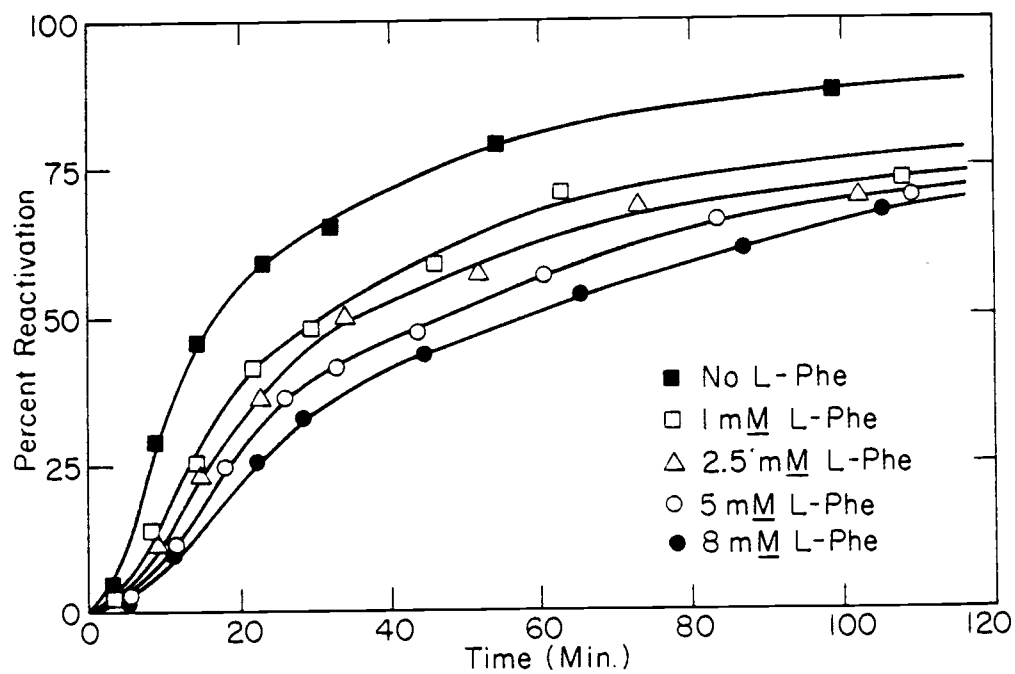
Figure 9.   Reactivation kinetics of bovine type M pyruvate kinase

as a function of L-phenylalanine concentration.

Reactivation was performed at 16° and at an enzyme

concentration of 25.6 µg/ml.

Table IV. Effect of Amino acids on the renaturation rate and final recovery of enzymatic activity.

Amino Acid	Final Recovery (Percent of Control)	Half time of Renaturation (min)
PART I Concentration and stereospecific effects of phenylalanine		
none	97	17.1
1.0 mM L-Phenylalanine	94	25.8
2.5 mM L-Phenylalanine	92	30.5
5.0 mM L-Phenylalanine	93	39.8
8.0 mM L-Phenylalanine	90	45.5
5.0 mM D-Phenylalanine	100	19.5
PART II Alanine effects in the presence of 5 mM L-Phenylalanine		
no Alanine	90	46.5
5 mM L-Alanine	93	43.8
15 mM L-Alanine	95	38.8
PART III Alanine effects – no Phenylalanine present		
no Alanine	88	21.5
5 mM L-Alanine	97	22
15 mM L-Alanine	94	22.5
PART IV Tyrosine and Tryptophan effects		
no amino acid	80	17.1
2 mM L-Tyrosine	80	19.3
2 mM L-Phenylalanine	80	26
5 mM L-Tryptophan	80	27
5 mM L-Phenylalanine	80	40.5

8.0 m$\underline{M}$ L-phenylalanine, the highest concentration tested, produced a 166 percent increase in the half time. A small but consistent decrease in overall recovery of enzymatic activity was seen for increasing L-phenylalanine concentrations. This decrease in overall recovery was <u>not</u> caused by direct inhibition by these concentrations of L-phenylalanine on enzymatic activity, as was demonstrated by the following two determinations: (a) control (nondenatured) enzyme was not inhibited when incubated with the same concentration of phenylalanine; (b) the small aliquots of phenylalanine-containing enzyme solutions that were added to each assay cuvette resulted in a sufficient dilution (at least one hundredfold) to produce final phenylalanine concentrations that were so far below the apparent K_i of 22 m$\underline{M}$ (see Cardenas <u>et al</u>., 1975c) that no detectable inhibition by the amino acid resulted. Addition of phenylalanine two minutes after the start of renaturation gave the same results as when phenylalanine was present from the beginning of the renaturation.

Fig. 10 shows the reactivation of pyruvate kinase as a function of enzyme concentration in the absence of Mg^{2+} and phenylalanine. Over the concentration range of 8-32 μg/ml no dependence on enzyme concentration is seen. In the presence of 5 m$\underline{M}$ L-phenylalanine there is no dependence on enzyme concentration in the range of 21-32 μg/ml, but a concentration dependence is seen at enzyme concentrations below 20 μg/ml. (see Fig. 11).

Thus, in the presence of L-phenylalanine the reactivation has a concentration dependence at enzyme concentrations 6-7 times greater than is seen in the absence of L-phenylalanine. This indicates that
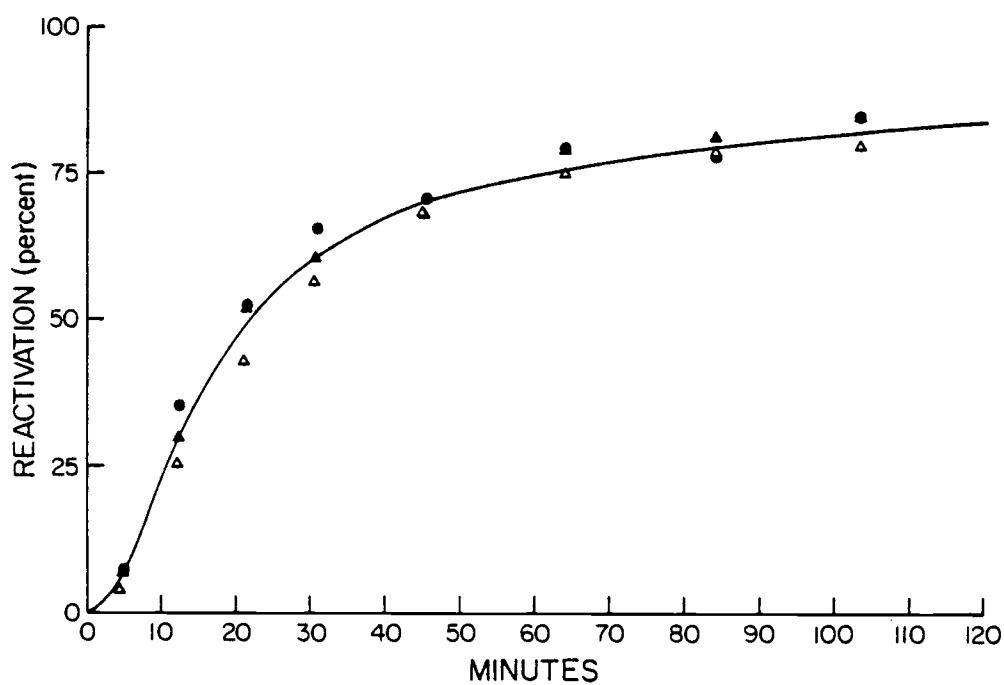
Figure 10.   Reactivation at 16° in the absence of Mg^{2+} and

phenylalanine. Enzyme concentrations (μg/ml) are:

32, (●); 16, (▲); 8, (△). Each data point is the ratio

of the percent reactivation at time t to the percent

final reactivation.
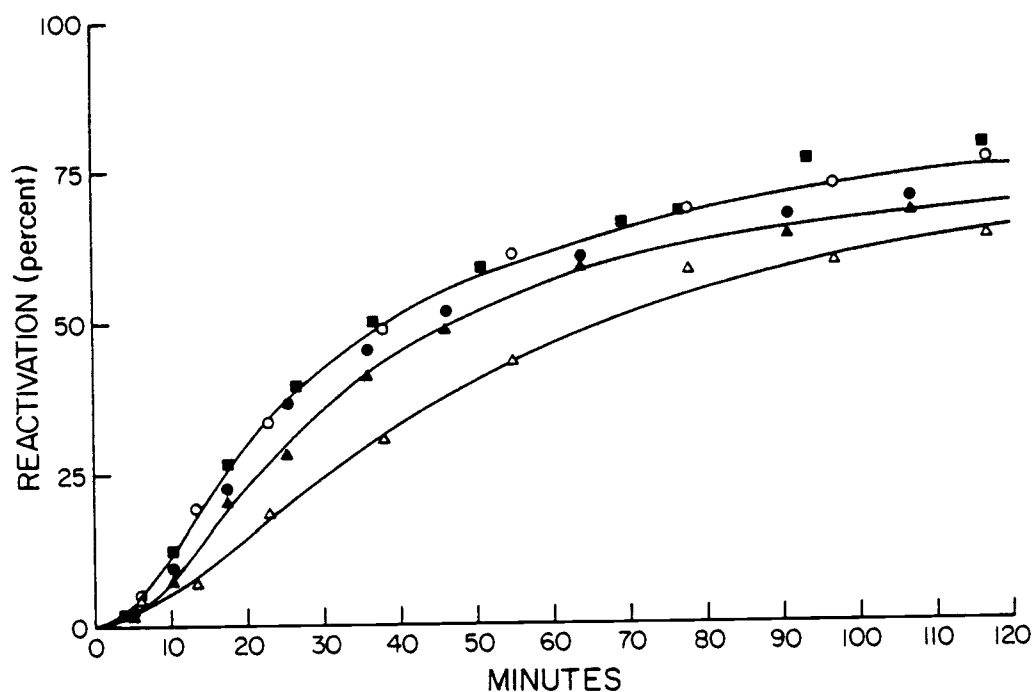
Figure 11. Reactivation at 16° in the presence of 5 m$\underline{M}$ L-phenyl-
alanine. Enzyme concentrations (µg/ml) are: 32, (○);
25, (■); 20.8, (●); 16, (▲); 8, (△). Each data
point is the ratio of the percent reactivation at time t
to the percent final reactivation.

L-phenylalanine interferes with the association processes involved in the reactivation of pyruvate kinase.

Fig. 12 shows first order plots of reactivation curves obtained with various concentrations of L-phenylalanine. The enzyme concentration used (25.6 μg/ml) was in a range where no effect of protein concentration on the renaturation rate could be seen either in the absence or the presence of 5 mM L-phenylalanine. Again the first order plots are satisfactorily represented by the sum of two exponentials as given by equation 1. Analysis of the data by the method described by Ray and Koshland (1961) yield the values given in Table V.

Phenylalanine inhibits the faster relaxation to a greater extent than it inhibits the slower relaxation. The addition of 2.5 mM and 8.0 mM L-phenylalanine reduce λ_1 to 47% and 35%, respectively, of its value in the absence of phenylalanine. The same concentrations of L-phenylalanine reduce λ_2 to 73% and 62%, respectively, of its value in the absence of phenylalanine. The amplitudes a and b are also dependent on the concentration of phenylalanine; a decreases with increasing phenylalanine concentrations while b increases. The sum of a and b is constant at 1.10.

Again, there is a short induction period as indicated by the failure of the reactivation curves to extrapolate to ln $(1-v/V_f) = 0$ at t = 0 and by the fact that the sum of a and b is not 1.0.

The experiments corresponding to the absence of phenylalanine in Table V and to 16°C in Table III differ only in that the former was performed in the absence of Mg^{2+} and the latter was performed in the presence of 5 mM Mg^{2+}. Comparison of the parameters (Tables III and V)
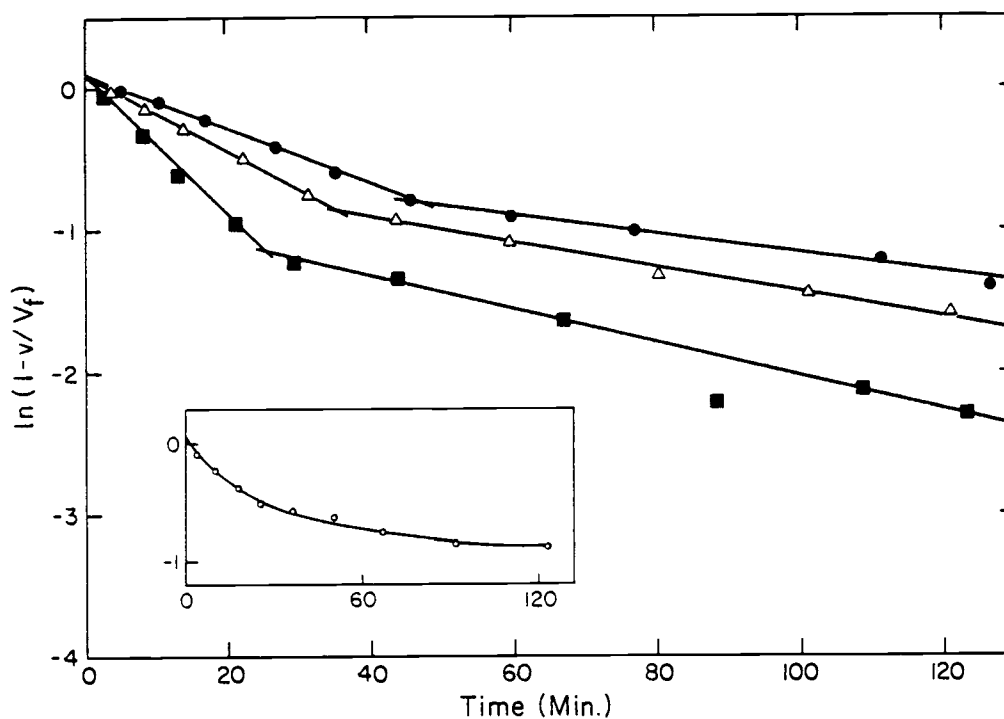
Figure 12. First order plots of reactivation curves obtained in
the absence of L-phenylalanine (■) or in the presence
of 2.5 mM L-phenylalanine (△) or 8.0 mM L-phenyl-
alanine (●). The insert is a first order plot of the
reactivation data in the presence of 5 mM phenyl-
pyruvate. The units of the coordinates are the same
for the insert as for the larger plot.

Table V. Values of the Parameters for Equation 1 as Determined by the Method of Ray and Koshland (1961). The units of λ_1 and λ_2 are sec^{-1}. All reactivations were performed at 16° and at an enzyme concentration of 25.6 μg/ml.

L-phenylalanine concentration (m$\underline{M}$)	a	$\lambda_1 \times 10^4$	b	$\lambda_2 \times 10^4$
0.0	0.658 ± 0.021	14.6 ± 0.10	0.442 ± 0.021	2.07 ± 0.01
2.5	0.511 ± 0.021	6.88 ± 0.02	0.591 ± 0.019	1.51 ± 0.02
8.0	0.404 ± 0.094	5.07 ± 0.27	0.698 ± 0.092	1.27 ± 0.16

for these two cases indicates that the reactivation is the same either in the presence or absence of Mg^{2+}. Mg^{2+} does, however, increase the stability of the control enzyme. With Mg^{2+} present the control enzyme retained 86% of its original activity after 24 hours whereas in the absence of Mg^{2+} the control enzyme retained about 70% of its original activity.

In contrast to the effect of L-phenylalanine described above, D-phenylalanine does not have a significant effect on either the rate or extent of recovery of enzymatic activity and does not alter the effect of L-phenylalanine on the reactivation kinetics (see Figure 13 and Table III, Part I.). In agreement with results reported earlier by Weber (1969) with brain pyruvate kinase, I find that the inhibition of native enzyme activity by phenylalanine concentrations much higher than those used in the renaturation experiments is also stereospecific. At 0.5 mM PEP, concentrations of L-phenylalanine greater than 9 mM are significantly inhibitory, but the same concentrations of D-phenylalanine have no effect.

L-alanine is known to reverse the L-phenylalanine inhibition of enzymatic activity but to have no effect by itself on the activity of mammalian type M pyruvate kinase (Carminatti et al., 1971; Cardenas et al., 1975a). Thus, it is of interest to determine whether L-alanine can reverse the inhibitory effect of L-phenylalanine on the renaturation of pyruvate kinase. As shown in Figure 14 and Table III, Parts II and III, the addition of 5 mM or 15 mM L-alanine to the solution of renaturing enzyme does not alter the
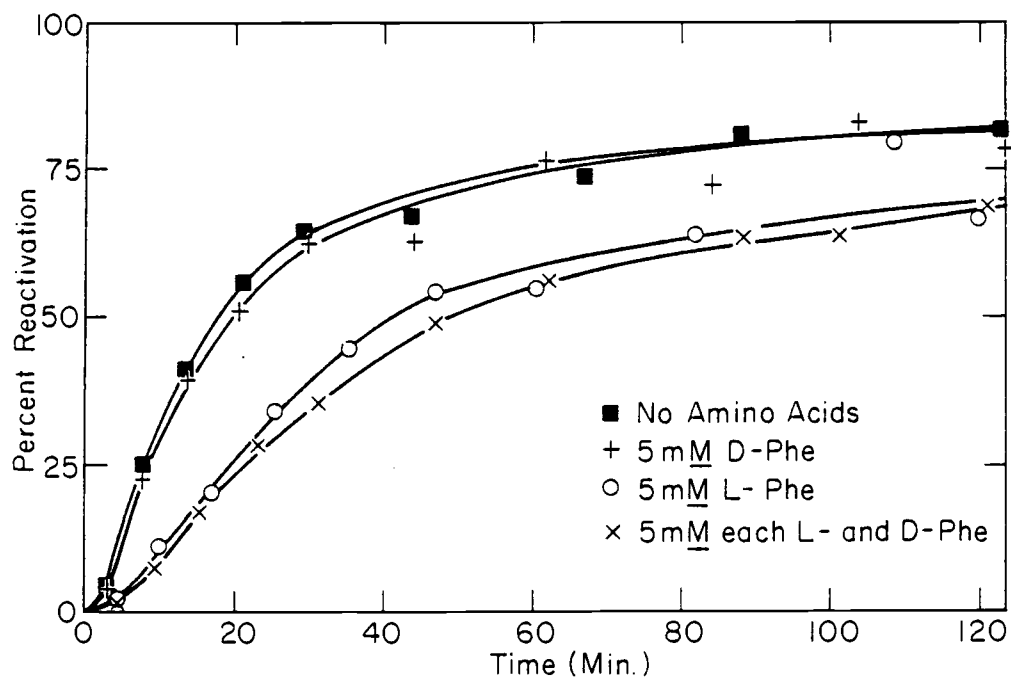
Figure 13.   Reactivation kinetics of bovine type M pyruvate kinase

as influenced by the absence or presence of D- and/or

L-phenylalanine. Reactivation was at 16° and at an

enzyme concentration of 25.6 ug/ml.

68

Figure 14.   Reactivation kinetics of bovine type M pyruvate kinase

as influenced by the absence or presence of L-phenyl-

alanine and/or L-alanine. Reactivation was at 16° and

at an enzyme concentration of 25.6 µg/ml.

reactivation kinetics in the absence of L-phenylalanine, and 5 mM L-alanine has no significant effect when phenylalanine is present. However, 15 mM L-alanine does slightly and reproducibly increase the reactivation rate in the presence of 5 mM L-phenylalanine. This very weak reversal by high concentrations of L-alanine contrasts sharply with the dramatic reversal by this amino acid of the L-phenylalanine induced inhibition of native enzymatic activity (Carminatti et al., 1971; Ibsen and Trippet, 1974; Cardenas et al., 1975c).

Bornmann et al. (1974) found evidence for the presence of non-covalently bound L-valine in yeast pyruvate kinase and concluded that the presence of L-valine was required for renaturation of this enzyme. L-valine also enhances the renaturation of pyruvate kinase from Neurospora crassa (O'Brien and Kapoor, 1980). The possibility that L-phenylalanine inhibits renaturation of bovine type M pyruvate kinase by interfering with the binding of L-valine was considered. However, no effect of L-valine on the reactivation kinetics of the bovine enzyme could be detected, either in the absence or presence of L-phenylalanine.

L-tyrosine and L-tryptophan inhibit the reactivation of pyruvate kinase but are much less inhibitory than is L-phenylalanine (see Table IV, Part IV). The experiments with tyrosine and tryptophan were performed in the presence of 0.1 mM EDTA. This addition resulted in complete stability of the control enzyme and a final recovery of 80%. The reactivation kinetics were not affected by the presence of EDTA.

The low solubility of L-tyrosine precluded the use of concentrations

higher than 2 mM. Like D-phenylalanine, D-tryptophan has no effect

on the reactivation kinetics. The reactivation curves for these

amino acids (not shown) have the same basic sigmoidal shape as the

curves with and without L-phenylalanine.

Table VI shows the effect of some phenylalanine analogs on the

renaturation of pyruvate kinase. Phenyllactate and phenylacetate are

only slightly inhibitory, increasing the half-time by about 2 minutes.

L-phenylalanine amide is a slightly stronger inhibitor, increasing

the half-time by 3.5 minutes. Ibsen and Trippet (1974) found L-phenyl-

alanine amide to have only a slight inhibitory effect on the activity

of native rat muscle pyruvate kinase. Thus, considerable specificity

is exhibited in the L-phenylalanine inhibition of the renaturation of

bovine muscle pyruvate kinase.

Of all the compounds tested, phenylpyruvate is by far the most

effective inhibitor of reactivation (Table VI and Figure 15), being

more effective than phenylalanine. Furthermore, the reactivation curve

has an entirely different shape in the presence of phenylpyruvate,

suggesting that its mechanism of action may be different from that of

phenylalanine. Phenylpyruvate does not affect the final recovery of

activity when EDTA is present in the renaturation medium.

PEP reverses the phenylalanine inhibition of native bovine

muscle pyruvate kinase (Cardenas et al., 1975c) and the phenylalanine

induced enhancement of rabbit muscle pyruvate kinase fluorescence

(Kayne and Price, 1972). FDP also reverses the phenylalanine inhib-

Table VI. The effect of phenylalanine analogs on the reactivation

of bovine type M pyruvate kinase. EDTA, 0.1 m$\underline{M}$, was

present in each case.

Analog	Half-time of Reactivation(minutes)
no addition	17.4
10 m$\underline{M}$ DL-Phenyllactate	19.3
5 m$\underline{M}$ Phenylacetate	19.3
5 m$\underline{M}$ L-Phenylalanine Amide	21.0
5 m$\underline{M}$ L-Phenylalanine	36.0
5 m$\underline{M}$ Phenylpyruvate	57.0
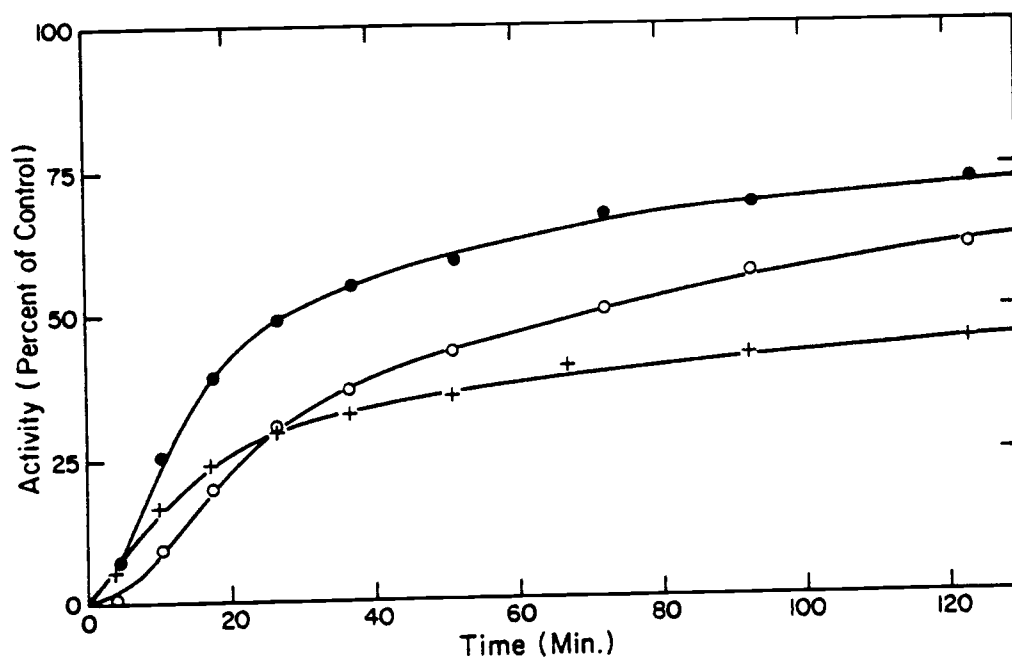
Figure 15. Reactivation kinetics of bovine type M pyruvate kinase in the presence of 5 mM phenylpyruvate and comparison with reactivation kinetics in the absence and presence of 5 mM L-phenylalanine. EDTA, 0.1 mM, was present in each case. No additions (●), 5 mM L-phenylalanine (○), 5 mM phenylpyruvate (+).

ition of the native bovine muscle enzyme (Cardenas et al., 1975b).

However, neither PEP nor FDP reverses the inhibitory effect of

L-phenylalanine on the renaturation rate of pyruvate kinase (see

Table VII for the results with PEP). As stated above, L-phenylalanine

tends to decrease the final yield as well as the renaturation rate.

The presence of either 1.0 m$\underline{M}$ PEP or 1.0 m$\underline{M}$ FDP leads to slightly

increased yields but does not speed up the renaturation rate, either

in the absence or presence of 5 m$\underline{M}$ L-phenylalanine.

Table VII. The effect of PEP on the reactivation rate of
bovine type M pyruvate kinase.

Additions	Final Yield (percent of control)	Half-time of Reactivation (minutes)
none	72.5	23.5
5 m$\underline{M}$ L-Phenylalanine	68.5	65.7
1 m$\underline{M}$ PEP	83.8	27.3
5 m$\underline{M}$ L-Phenylalanine plus 1 m$\underline{M}$ PEP	72.5	69.8

Part C: Immobilized Enzyme and

Subunit Interactions

Figure 16 and Table VIII shows the properties of the stirred assay.
In the assay of Sepharose bound activity, the Δ A/min is a linear
function of the amount of packed Sepharose assayed. Also, when soluble
enzyme is assayed in the presence of Sepharose containing no bound
enzyme, the soluble activity shows only a very slight decrease as the
amount of Sepharose is increased (Fig. 16). When a 25 μl aliquot of
Sepharose (1:1 slurry) containing no bound enzyme is added to an assay
of a sample containing 2.28 units/ml of soluble enzyme, the activity is
not changed by the presence of the Sepharose. When a 25 μl aliquot of
Sepharose containing 0.93 units/ml of bound enzyme activity is added
to an assay of a sample containing 2.28 units/ml of soluble enzyme,
the total activity is 3.25 units/ml (Table VIII). Thus, soluble and
bound activities as assayed are additive. There is also no detectable
solubilization of Sepharose bound activity as a result of stirring
during the course of the assay. This was shown by centrifuging assay
mixtures containing immobilized enzyme to remove it before the sub-
strates had been consumed completely. The supernatant was then re-
turned to the spectrophotometer to determine whether the assay con-
tained soluble enzyme. In no case was any enzymatic activity found
after removal of the Sepharose-bound enzyme. Thus, the stirred assay
system is able to reliably and accurately measure bound enzymatic
activity.

The immobilized monomer and tetramer have protein contents of 5.53
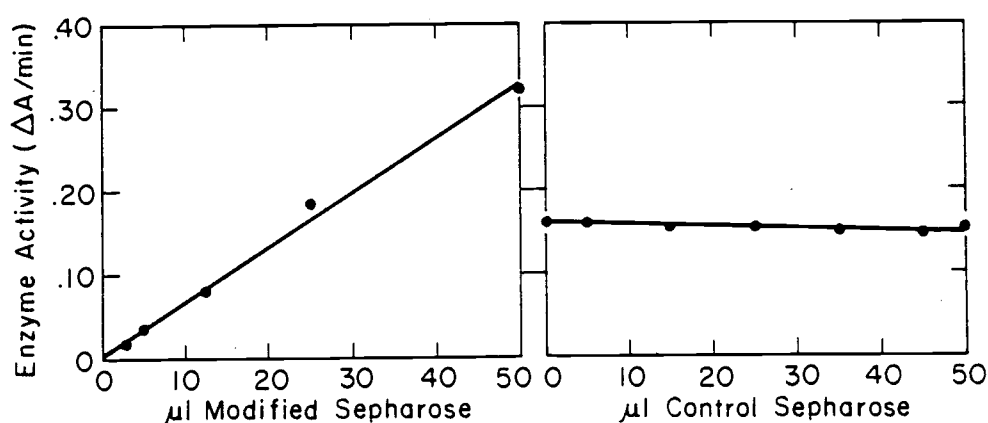
Figure 16.   Properties of the stirred assay.

Left: Enzymatic activity (ΔA_{340}/min) of immobilized

pyruvate kinase as a function of the amount of

Sepharose containing bound enzyme.

Right: Enzymatic activity of soluble pyruvate kinase

in the presence of Sepharose containing no bound

enzyme.

In each case, the units of the abscissa are µl of

packed Sepharose.

Table VIII. Properties of the Stirred Assay

Used to Determine Pyruvate Kinase Activity.

Activities are Expressed as Units/ml of Sample

	Without Sepharose	With Sepharose 25 μl(1:1 slurry)	Difference
Soluble Enzyme +Sepharose	2.28	2.26	0.02
Sepharose Bound Enzyme	-	0.93	-
Soluble Enzyme + Sepharose Bound Enzyme	2.28	3.25	0.97

and 1.56 µg/ml,respectively,as determined by amino acid analysis (Table IX). The bound monomer contains 28% of the protein that was present for the bound tetramer which is close to that expected if only one subunit per tetramer had been covalently linked to the resin.

Table X shows the activity of the bound monomer and a comparison with the activity expected for a fully active monomer. In each case the monomer is essentially inactive. Thus, it may be that subunit interactions are required for activity of the pyruvate kinase monomer. However, two other possibilities could account for the lack of enzymatic activity of the immobilized monomer and these possibilities must be considered. One possibility is that the immobilized subunit did not refold correctly. The other possibility is that the immobilization procedure modified an amino acid residue that is required for enzymatic activity. It is particularly important to consider this latter possibility since CNBr activated Sepharose immobilizes proteins by reacting with the ε-amino group of lysine residues, and bovine muscle pyruvate kinase is known to have an essential lysine residue at or near the active site (Hubbard and Cardenas, 1975; Johnson, 1980).

The reason for the existence of inactive monomers can be checked by attempting to reconstitute the quaternary structure of the immobilized subunit using active and inactive subunits. If the immobilized subunit is capable of refolding correctly, it should be able to interact with other subunits. Thus, reconstitution with active subunits should result in the appearance of Sepharose bound enzymatic activity. If reconstitution is performed with inactivated subunits, then appearance

Table IX Amino acid content of Sepharose bound tetramer and monomer
Values are corrected for the amino acid content of the Sepharose. Units
are nmoles/ml of packed resin.

Amino acid	Tetramer	Monomer	Monomer as fraction of tetramer
Asp	3.61	1.09	0.30
Glu	5.25	1.24	0.24
Gly	4.61	1.30	0.28
Ala	5.00	1.58	0.32

Based on the known amino acid content and averaging the values for the
four amino acids yields (µg/ml of packed Sepharose)

Tetramer	Monomer
5.53 ± 0.98	1.56 ± 0.25

Table X.   Activities of Sepharose Bound Tetramer and Monomer

Activities are Expressed as units/ml Sepharose

	Bound Tetramer	Bound 'Monomer'	Expected for Fully Active Monomer	Bound 'Monomer' / Expected for Fully Active Monomer
Exp. 1	1.07	0.030	0.268	0.110
Exp. 2	3.06	0.049	0.765	0.064
Exp. 3	2.52	0.052	0.63	0.082

The Sepharose activation was performed with 1.2 mg/ml CNBr in Exp. 1 and with 2.0 mg/ml in Exp. 2 and 3.

of Sepharose bound activity would result from reactivation of the immobilized subunit via its interaction with inactivated, soluble sub-units. Providing that essential active site residues have not been modified, reconstitution with inactive subunits should yield a bound activity equal to one fourth of the activity of a bound native tetramer. If critical active site residues have been modified during immobilization, then very little immobilized activity would result from reconstitution of the tetramer with inactivated subunits.

Two inactivations of bovine muscle pyruvate kinase with 2,4,6-tri-nitrobenzene sulfonate left 2.1 and 9.7% of the original enzymatic activity. These preparations of inactive enzyme and a preparation of active enzyme were used for the reconstitution experiments described above. Reconstitution of immobilized tetramers via addition of active subunits to immobilized monomer yields 61 percent recovery of bound activity (Table XI). Over the enzyme concentration used (32-44 µg/ml), the recovery is essentially independent of the initial concentration of soluble enzyme. The addition of a second aliquot of soluble monomers after the first reconstitution was complete (24 h) produced no further increase in the yield of bound activity. Reconstitution with trinitro-phenylated subunits yields about 13 percent recovery. The recovered ac-tivities agree well with the values that are expected for complete acti-vation of the bound subunit when one takes into account the reconstitution efficiency (61 percent) and the fact that three of the subunits in the reconstituted system would be expected to retain small amounts of activity, due to incomplete inactivation (see Table XI for results and calculations).

Table XI. Reconstitution of the Quaternary Structure

of the Sepharose Bound Subunits

Activity of the final product is expressed as units/ml Sepharose.

Reconstitution of Immobilized Monomer with Active Subunits

	Bound Tetramer	Reconstituted	Percent Recovery	Initial Concentration of Soluble Enzyme in Reconstitution Mixture (μg/ml)
Exp. 1	1.07	.676	63%	44
Exp. 2	3.01	1.81	60%	42
Exp. 3	2.87	1.70	60%	32

Reconstitution of Immobilized Monomer with Inactive Subunits

	Bound Tetramer	Reconstituted	Percent Recovery	Initial Concentration of Soluble Enzyme in Reconstitution Mixture (μg/ml)
Exp. 1	1.07	0.169	15.9%	44
Exp. 2	3.01	0.585	19.4%	42

Expected recovery if the covalently linked subunit becomes fully active:

Exp. 1 $1.07 \ (.63)[1/4 + 0.021(3/4)] = .179$

Exp. 2 $3.01 \ (.60)[1/4 + 0.097(3/4)] = 0.584$

Thus, isolated subunits of bovine muscle pyruvate kinase are essentially inactive, but activity appears when the quaternary structure of the enzyme is established.

DISCUSSION

This work describes both transconformational and association processes in the renaturation of bovine muscle pyruvate kinase and represents the first report of a concentration dependence in the renaturation kinetics of pyruvate kinase. Also described is a stereospecific and structurally specific effect of L-phenylalanine in inhibiting the renaturation of muscle pyruvate kinase.

Earlier work in this laboratory (Cardenas et al., 1977) indicates that the fluorescence properties and most of the circular dichroism characteristics of bovine muscle pyruvate kinase are recovered within one minute after beginning renaturation. However, the formation of stable tetramer and the recovery of enzymatic activity are much slower under their conditions. These earlier studies indicate that major folding of the peptide chain occurs on a much shorter time scale than does the regain of either native tetrameric structure or enzymatic activity. The addition of L-phenylalanine two minutes after the start of renaturation gives the same results as when the phenylalanine is present at the beginning of renaturation (this thesis) indicating that L-phenylalanine inhibits processes that are distinct from the fast folding that occurs within the first minute of renaturation. Thus, this work describes transconformational and association processes that occur after the major folding of the peptide chain and the effect of phenylalanine on these later processes.

Final activity recoveries are essentially constant at 90 percent in the temperature range of 0-30°C but drop off sharply as the temperature increases above 32°C. The incomplete recovery of enzymatic activity at 25° and 45°C results from the formation of inactive aggregates and denatured monomer. It is interesting to note that protein folding under the renaturation conditions described here would result in only about 70 percent yields at in vivo temperatures of 37°C. Thus, either cells have different folding conditions at in vivo temperatures, or some additional factor augments the yield of active enzyme, or the cell operates with only 70 percent of the synthesized monomers being converted to active tetramer.

The sharp decrease in recovery of enzyme activity with increasing temperature begins at about the same temperature where curvature appears in an Arrhenius plot of the enzymatic activity of bovine muscle pyruvate kinase. Similar Arrhenius plots are obtained for the activity of rabbit muscle pyruvate kinase and are thought to be indicative of a temperature dependent conformational change (Kayne and Suelter, 1968). Thus, enzyme conformations that both enhance the aggregation of bovine muscle pyruvate kinase and decrease the ability to form active tetramer may be favored at temperatures above 32°C.

Under appropriate conditions of temperature and enzyme concentration, subunit association is rapid enough that it is not rate limiting. Under these conditions the reactivation kinetics are satisfactorily represented by the sum of two exponentials, both in the absence and presence of L-phenylalanine. The temperature dependence of the two macroscopic

rate constants has been determined in the absence of phenylalanine

and was found to obey normal Arrhenius behavior for the temperature

range of 16-30°C. These rate constants have apparent activation ener-

gies of 10.6 and 11.9 kcal/mole. The results are consistent with the

presence of two populations at zero time that may renature either

      (a)   via a single pathway

$$A \longrightarrow B \longrightarrow N$$
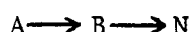
where $[B] \neq 0$ at $t = 0$

      (b)   or via parallel pathways

$$\begin{array}{c} A \searrow \\ \quad\;\; \searrow N \\ B \nearrow \end{array}$$
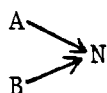
A and B are intermediates, $[B]$ is the concentration of B, and N is the

native enzyme. Since the reactivation conditions that gave rise to

these results strongly favor the formation of active enzyme, the in-

dividual steps are assumed to be essentially kinetically irreversible.

For the single pathway mechanism, the data require that species B is

populated at $t = 0$ and that the conversion of B to N is the faster of

the two steps (see appendix).

Isomerization of proline residues while a protein is unfolded is

known to produce heterogeneity in the unfolded state and this hetero-

geneity is thought to lead to fast and slow phases in the folding process

(Brandts et al., 1975; Schmid and Baldwin, 1978a). For example, carp

parvalbumins that contain no proline have only a fast folding phase

while a parvalbumin containing one proline has both fast and slow

folding phases (Lin and Brandts, 1978). The presence of a slow and a

fast folding species of ribonuclease A is thought to result from proline isomerization in the unfolded state (Brandts et al., 1975; Schmid and Baldwin, 1978a). However, non-native isomers of proline in the slow folding species allow some folding to occur (Schmid and Baldwin, 1978a; 1979) and the final folding to yield the native form of ribonuclease A apparently is not limited by proline isomerization (Schmid and Baldwin, 1978b).

The question arises whether the rate limiting conformational processes in the renaturation of bovine muscle pyruvate kinase represent proline isomerization. The apparent activation energy of proline isomerization is 20 ± 3 kcal/mole (determined with model compounds) but may depend somewhat on neighboring amino acid residues (Brandts et al., 1975; Schmid and Baldwin, 1978a). The apparent activation energies for the rate limiting conformational processes in the renaturation of bovine muscle pyruvate kinase are 10.6 and 11.9 kcal/mole. These values appear to be too low to be attributed to proline isomerization, even when possible effects of neighboring residues are taken into consideration. Thus, it seems unlikely that the transconformational steps described above in the renaturation of bovine muscle pyruvate kinase represent proline isomerization.

This work represents the first report of a protein concentration dependence in the renaturation of pyruvate kinase. At 16°C, the renaturation rate is dependent on the enzyme concentration at concentrations below 3.0 µg/ml. If the renaturation temperature is lowered to 7.8°C,

a concentration dependence is seen at protein concentrations as high as 25.6 μg/ml.

If the change in concentration dependence with temperature is only the result of a temperature effect on rate constants, then the activation energy for the association processes is much larger than the activation energies for the limiting transconformational processes. Rudolph et al. (1977b) obtained an apparent activation energy of 58 kcal/mole for the association process that is rate limiting in the renaturation of porcine muscle lactate dehydrogenase. A concentration dependence based solely on diffusion rates is unlikely, since diffusion limited reactions have low activation energies (Gardiner, 1969).

An alternate explanation for the change in concentration dependence with temperature could be destabilization of quaternary structure at decreased temperatures. However, equilibrium ultracentrifugation at 4°C of native bovine muscle pyruvate kinase yields only one species of molecular weight 230,000, which is the tetramer (Cardenas et al., 1973). Furthermore, hybridization experiments performed at 15°C and at enzyme concentrations of approximately 20 μg/ml did not reveal any subunit dissociation (Cardenas et al., 1977). Thus, while tetramer dissociation has not been rigorously looked for, no evidence exists for a destabilization of quaternary structure by low temperatures.

Addition of phenylalanine increases the minimum concentration at which an enzyme concentration dependence appears in the renaturation rate. In the presence of 5 mM phenylalanine at 16°C, there is an enzyme concentration dependence at concentrations below 20 μg/ml

whereas in the absence of phenylalanine at 16°C the concentration

dependence in the renaturation rate appears only at concentrations

below 3 μg/ml. Phenylalanine therefore inhibits both the association

and the transconformational processes involved in the renaturation of

bovine muscle pyruvate kinase. The phenylalanine inhibition of the

association processes could result either from direct steric inter-

ference with the establishment of subunit contacts or from a confor-

mational effect that alters the structure of the regions where subunit

contacts occur. In support of the latter alternative, phenylalanine

is known to cause a cooperative conformational change in native rabbit

muscle pyruvate kinase (Kayne and Price, 1972).

It is interesting to note that L-alanine, which inhibits the

enzyme from Ehrlich Ascites tumor cells, causes a dissociation of

that enzyme from the tetrameric to the dimeric state (Hofman et al.,

1975). The dissociation is reversed by FDP. In contrast, gel filtra-

tion in the presence of L-phenylalanine reveals no dissociation of

bovine muscle pyruvate kinase (Cardenas et al., 1977).

It should be noted that as the renaturation proceeds at any tem-

perature or enzyme concentration the concentration of unassociated

monomers will decrease. Thus, even under conditions where no concentra-

tion dependence has been detected, it should be possible to see a

concentration dependence at very long times after the beginning of

renaturation. However, over the time span during which I followed the

renaturation, I have not seen this effect. There are two possible

reasons for this. (1) I have not looked at long enough times after

the beginning of renaturation. (2) At very long times one is attempting to measure very small changes in a relatively large amount of enzymatic activity. At these long times the change in recovered activity with time is so slow that the expected concentration dependence would be difficult to distinguish from normal experimental error.

A short induction period occurs at the beginning of renaturation, as indicated by the initial lag in activity seen in Figure 6 and by the failure of the lines in Figures 7 and 12 to converge to $\ln(1-f) = 0$. This lag is seen under conditions of temperature and enzyme concentration high enough that no concentration dependence is observed in the overall renaturation process. However, it is more pronounced at the lower renaturation temperatures and enzyme concentrations where a concentration dependence does occur. The lag could not result from an inaccurate assessment of time zero, as mixing of solutions is complete within 5-10 seconds and the lag lasts for several minutes. Part of the induction period could result from the fast folding process described above. Under conditions where a concentration dependence is seen, part of the lag is no doubt due to the kinetic contribution of the association process. However, the fact that the lag is so long and is seen under conditions where no protein concentration is evident suggests that the renaturation process could involve additional kinetically significant steps that have not yet been described.

The question arises whether the transconformational processes precede or follow the association processes. The results of previous work (Cardenas and Dyson, 1973; Cardenas et al., 1977) with the

formation of hybrids of muscle and liver pyruvate kinases make it unlikely that significant amounts of stable dimer are formed early during renaturation. Furthermore, the formation of stable tetramers occurs relatively late during renaturation and at a rate that approximates the recovery of enzymatic activity. Combining this information with the mathematical necessity that species A and B are populated at $t = 0$ leads to the conclusion that the rate limiting transconformational processes precede the association processes.

Also of interest is the effect of substrates, cofactors, etc., on the renaturation. Reactivation kinetics are not altered by the substrate PEP, the cofactor Mg^{2+} or the effector FDP. However, PEP and FDP slightly increase the final yield. The addition of either 0.1 m$\underline{M}$ EDTA or 5 m$\underline{M}$ Mg^{2+} increases the stability of the renaturation controls, most likely by eliminating or by counteracting detrimental effects of trace metal ions.

The general features of the renaturation of bovine muscle pyruvate kinase are consistent with the features found for the renaturation of other oligomeric proteins with identical subunits. The kinetic significance of both transconformational and association processes has been examined for muscle pyruvate kinase (this thesis), malate dehydrogenase (Jaenicke et al., 1979), triose phosphate isomerase (Jaenicke, 1978), liver alcohol dehydrogenase (Rudolph et al., 1978; Gerschitz et al., 1978), creatine kinase (Bickerstaff et al., 1980), aldolase (Chan et al., 1973; Rudolph et al., 1977d), glyceraldehyde-3-phosphate dehydrogenase (Rudolph et al., 1977c),

and heart lactate dehydrogenase (Rudolph et al., 1977a; 1977b). Under conditions where activity recovery is incomplete, removal of inactive aggregates yields a final renaturation product that is indistinguishable from the native enzyme (pyruvate kinase: this thesis; also see Johnson et al., 1969; LDH: Rudolph and Jaenicke, 1976; Rudolph et al., 1977a; Zettlmeissl et al., 1979; Rudolph et al., 1979; GPD: Rudolph et al., 1977c). With the exception of the effect of phenylalanine on the renaturation of bovine pyruvate kinase, the major effect of most substrates, cofactors, and effectors appears to be the stabilization of the final renaturation product (pyruvate kinase: this thesis; GPD: Rudolph et al., 1977c; LDH: Rudolph et al., 1977b; liver alcohol dehydrogenase: Gerschitz et al., 1978; malate dehydrogenase: Jaenicke et al., 1979).

The folding of the bovine muscle pyruvate kinase subunit involves a fast folding (Cardenas et al., 1977) followed by slower transconformational processes (this thesis; Cardenas et al., 1977). This same sequence of events is found with disulfide bond formation in bovine pancreatic trypsin inhibitor (Creighton, 1978) and with the slow folding species of ribonuclease A (Schmid and Baldwin, 1979). Thus, the occurrence of the slowest step late in the folding pathway may be a general feature of protein folding.

The stability of the final form requires the simultaneous acquisition of precise and specific interactions and a very close packing of atoms. This process might be expected to be relatively slow because it would be improbable. Thus, the rate limiting step seems to result from entropic factors (see Creighton, 1978).

While phenylalanine and phenylpyruvate are known to inhibit the activity of many enzymes (Kaufman, 1977), effects of phenylalanine on the renaturation of enzymes have not been previously examined. The L-phenylalanine inhibition of the renaturation of bovine muscle pyruvate kinase shows considerable stereoisomeric and structural specificity. The data are consistent with the binding of phenylalanine to the enzyme early during the renaturation, thereby inhibiting each of the two rate limiting first order relaxations. Since the two relaxations are inhibited to different extents by phenylalanine, species A and B may have different affinities for phenylalanine. The lack of an effect of D-phenylalanine, the very small inhibition by phenyllactate and phenylalanine amide, and the reduced inhibitory effect of tyrosine and tryptophan indicate that the stereoisomeric form, the α-amino and carboxyl groups, and the exact nature of the side chain affects binding. This specificity rules out nonspecific and simple nonpolar binding.

Other publications have described a stereospecific inhibition by L-phenylalanine of the activity of native pyruvate kinase; this inhibition can be dramatically reversed by alanine, PEP, or FDP (Weber, 1969; Ibsen and Trippet, 1973; Cardenas et al., 1975a; 1975b). However, none of these substances significantly relieves the L-phenylalanine induced inhibition of the renaturation of bovine muscle pyruvate kinase. This implies some differences in phenylalanine effect on the renaturation as compared to its effect on native enzyme activity or could indicate that the phenylalanine binding site is formed earlier during the renaturation than are the other ligand binding sites.

I now turn to the question of the number of phenylalanine binding sites on each subunit of pyruvate kinase. Kayne and Price (1973) find four phenylalanine and four alanine binding sites per tetramer of rabbit muscle pyruvate kinase, suggesting the presence of one site per subunit. They conclude that the two amino acids compete for the same site and that this site is far from the active site. In contrast, Ibsen and Trippet (1974) and Ibsen and Marles (1976) propose two amino acid binding sites on each subunit based on kinetic results. They suggest that phenylalanine binds at both the PEP site and at another site removed from the PEP site. In their model, the second site binds amino acids with bulky side chains while alanine and other small side chain amino acids bind at the PEP site. It is unclear whether the phenylalanine binding site that effects renaturation is the same as the proposed binding site for bulky side chain amino acids that affects native enzymatic activity. The observed K_i for phenylalanine in the presence of 10 m$\underline{M}$ MgCl$_2$ and 0.5 m$\underline{M}$ PEP is 22 m$\underline{M}$ (Cardenas $\underline{et}$ $\underline{al}$., 1975a). Phenylalanine enhances the fluorescence of rabbit muscle pyruvate kinase, the half point for the enhancement occurring at 90 $\mu\underline{M}$ phenylalanine in the absence of divalent cations or PEP and at 6.5 m$\underline{M}$ in the presence of 5 m$\underline{M}$ Mg^{2+} and 0.1 m$\underline{M}$ PEP (Kayne and Price, 1972). In the present work, 1 m$\underline{M}$ L-phenylalanine causes a 50 percent increase in the renaturation half time. It is interesting to note that alanine reverses the phenylalanine inhibition of the native enzyme but has no effect on the phenylalanine inhibition of renaturation. This suggests that alanine does not bind to the phenylalanine binding site that affects the rate of renaturation.

Whether there are one or two phenylalanine binding sites per subunit on the native enzyme, the phenylalanine inhibition of renaturation can be adequately explained on the basis of a single phenylalanine binding site that is formed early in the renaturation process. Since PEP, FDP, and alanine all reverse the phenylalanine inhibition of native bovine muscle pyruvate kinase but do not reverse the phenylalanine inhibition of renaturation, the phenylalanine binding site that affects renaturation is probably formed before the substrate and effector binding sites.

Phenylpyruvate appears to inhibit enzyme reactivation in a manner which differs from that of phenylalanine, as suggested by the very different shapes of the reactivation curves (Figure 15). It is unclear whether phenylpyruvate binds to the same site that binds phenylalanine or whether the two compounds inhibit renaturation via entirely different mechanisms.

<div align="center">

Subunit Structure

and

Pyruvate Kinase Activity

</div>

Since conditions required to disrupt the quaternary structure of miltisubunit proteins often disrupt the secondary and tertiary structure as well, it is often difficult if not impossible to obtain structured, partially assembled, and stable systems for study. It is important to know whether the tetrameric structure is required for catalytic activity or whether smaller structural units are also active. Rabbit muscle and

yeast pyruvate kinase dissociate into dimers in 2.4 $\underline{M}$ urea. The dimers

formed from the rabbit muscle enzyme are reported to be partially active

(Cottam et al., 1969) while those from yeast are inactive (Boiteux et

al., 1978).

While the present work provides no information as to the enzymatic

activity of the dimer, the existence of a concentration dependence in

the renaturation kinetics of bovine muscle pyruvate kinase indicates that

the monomer can not be fully active. Immobilization of subunits to

Sepharose followed by removal of noncovalently bound enzyme subunits and

renaturation of matrix-bound subunits gave results demonstrating 2.1

percent retention of the original catalytic activity on the resin.

Most of the enzyme molecules covalently bound to the Sepharose under

these conditions were bound via a single subunit, and removal of the

noncovalently bound subunits results in mostly monomers bound to the

Sepharose. If one assumes a 61 percent yield for renaturation of

matrix bound enzyme, then the maximum activity possible for the matrix

bound monomer would be about 3 percent that of the matrix-bound tetra-

mer.

One must, however, consider that some enzyme molecules would

probably be covalently bound via more than one subunit. Hence, it

is quite likely that the true monomer has no enzymatic activity.

That the immobilized subunit is capable of enzymatic activity is

shown by the reappearance of enzymatic activity when soluble renaturing

subunits, either native or inactivated by trinitrophenylation, are

added to the immobilized subunits. This regain of enzymatic activity

presumably occurs via the reformation of matrix bound tetramer. Hence,
the tetramer, either soluble or matrix-bound, is active while the monomer
at the very most possesses 13 percent of the activity it possesses when
it is part of a tetramer. While this is the first study of the sub-
unit activity of immobilized pyruvate kinase, subunit immobilization
has been used to study other partially assembled systems. Isolated
monomers of aldolase and transaldolase are partially active but less
stable than the native enzyme. Monomers of lactate dehydrogenase are
inactive (see Chan, 1976a; 1976b).

Conclusion

The renaturation of bovine muscle pyruvate kinase involves both
transconformational and association processes. An initial rapid folding
produces two partially folded species. These species undergo transcon-
formational steps followed by association processes that produce the
native tetramer. Other than the inhibitory effect on the renaturation
kinetics by L-phenylalanine, the major effect of substrates, cofactors,
and other ligands appears to be the stabilization of the final renatura-
tion product. The general features of the renaturation are consistent
with those found for the renaturation of other oligomeric systems with
identical subunits.

L-phenylalanine inhibits both the transconformational and associa-
tion processes but does not inhibit the fast folding. The results are
consistent with the formation of a single phenylalanine binding site
early during the renaturation. This site is probably distinct from

the catalytic site. PEP, FDP, and alanine reverse the phenylalanine inhibition of native pyruvate kinase but have no significant effect on the renaturation either in the presence or absence of phenylalanine. This suggests that the binding sites for these ligands are formed after the phenylalanine binding site that affects renaturation is formed. The question of the number of phenylalanine binding sites on the native enzyme has yet to be resolved.

The presence of a concentration dependence in the renaturation kinetics indicates that the monomer cannot be fully active. The studies with immobilized subunits demonstrate that the isolated monomer of bovine muscle pyruvate kinase is essentially inactive and that activity appears on formation of the quaternary structure.

REFERENCES

Anderson, P. J. and Randall, R. F. 1975. Comparison of the subunit
    and primary structures of the pyruvate kinases from rabbit and
    sturgeon muscles. Biochem. J. 145: 575-579.

Anderson, S. and Weber, G. 1966. The reversible acid dissociation and
    hybridization of lactic dehydrogenase. Arch. Biochem. Biophys.
    116: 207-223.

Anfinsen, C. B. 1973. Principles that govern the folding of protein
    chains. Science 181: 223-230.

Baldwin, R. L. 1975. Intermediates in protein folding reactions and
    the mechanism of protein folding. Ann. Rev. Biochem. 44: 453-
    475.

Balinsky, D., Cayanis, E., and Bersohn, I. 1973. The effects of various
    modulators on the activities of human pyruvate kinases isolated
    from normal adult and foetal liver and hepatoma tissue. Int. J.
    Biochem. 4: 489-501.

Baranowska, B. and Baranowski, T. 1975. Pyruvate kinase from human
    skeletal muscle. Molec. Cell. Biochem. 6: 197-201.

Barbalace, D. S., Chambliss, G. H., and Brady, R. J. 1971. Some
    differences in pyruvate kinase regulation in selected yeasts.
    Biochem. Biophys. Res. Comm. 42: 287-291.

Benz, F. W. and Roberts, G. C. K. 1973. NMR studies of the unbolding
    of ribonuclease by guanidine hydrochloride evidence for inter-
    mediate states. FEBS Lett. 29: 263-266.

Benziman, M. 1969. Factors affecting the activity of pyruvate kinase
    of Acetobacter xylinum. Biochem. Jour. 112: 631-636.

Berglund, L., Ljungström, O., and Engström, L. 1977. Purification and
    characterization of pig kidney pyruvate kinase (Type A). J. Biol.
    Chem. 252: 6108-6111.

Bickerstaff, G. F., Paterson, C., and Price, N. C. 1980. The refolding
    of denatured rabbit muscle creatine kinase. Biochim. Biophys.
    Acta. 621: 305-314.

Black, J. A. and Henderson, M. H. 1972. Activation and inhibition of
    human pyruvate kinase by organic phosphates, amino acids,
    dipeptides and anions. Biochim. Biophys. Acta. 284: 115-127.

Blume, K. G., Hoffbauer, R. W., Busch, D., Arnold, H. and Löhr, G. W. 1971. Purification and properties of pyruvate kinase in normal and in pyruvate kinase deficient human red blood cells. Biochim. Biophys. Acta. 227: 364-372.

Boiteux, A., Doster, W., Hess, B., Markus, M., Plesser, Th., and Wieker, H.-J. 1978. The function of pyruvate kinase. Proc. 12th FEBS Meeting. Dresden (Hofmann, E., ed.) 52: 35-43.

Bornmann, L., Hess, B., and Zimmermann-Telschone, H. 1974. Mechanism of renaturation of pyruvate kinase of Saccharomyces carlsbergenis: Activation by L-valine and magnesium and manganese ions. Proc. Natl. Acad. Sci. U.S.A. 71: 1525-1529.

Bornmann, L., Röschlau, P., and Hess, B. 1972. Essentielle aminosäurereste von hefe-pyruvatkinase. Hoppe-Seyler's Z. Physiol. Chem. 353: 696.

Boyer, P. D. 1962. Pyruvate kinase. in the Enzymes, 2nd ed. 6: 95-113.

Brandts, J. F. 1964. The thermodynamics of protein denaturation. II. A model of reversible denaturation and interpretations regarding the stability of chymotrypsinogen. J. Am. Chem. Soc. 86: 4302-4314.

Brandts, J. F. 1969. The thermodynamics of protein denaturation. Biol. Macromol. 2: 213-219.

Brandts, J. F., Halvorson, H. R., and Brennan, M. 1975. Consideration of the possibility that the slow step in protein denaturation reactions is due to cis-trans isomerization of proline residues. Biochemistry 14: 4953-4963.

Brandts, J. F. and Hunt, L. 1967. The thermodynamics of protein denaturation. III. The denaturation of ribonuclease in water and in aqueous urea and aqueous ethanol mixtures. J. Am. Chem. Soc. 89: 4826-4838.

Brown, J. R. and Hartley, B. S. 1966. Location of disulphide bridges by diagonal paper electrophoresis: The disulphide bridges of bovine chymotrypsinogen A. Biochem. J. 101: 214-228.

Brummell, M. C., Boaz, D. P., Flashner, M., and Stegink, L. D. 1976. Rabbit muscle pyruvate kinase amino- and carboxyl-terminal studies. Biochim. Biophys. Acta. 438: 102-107.

Bucher, T. and Pfleiderer, G. 1955. Pyruvate kinase from muscle. Methods in Enzymology. 1: 435-440.

Carbonell, J., Feliu, J. E., Marco, R., and Sols, A. 1973. Pyruvate kinase: Classes of regulatory isoenzymes in mammalian tissues. Eur. J. Biochem. 37: 148-156.

Cardenas, J. M., Blachly, E. G., Cecootti, P. L., and Dyson, R. D. 1975a. Properties of chicken skeletal muscle pyruvate kinase and a proposal for its evolutionary relationship to the other avian and mammalian isozymes. Biochemistry 14: 2247-2252.

Cardenas, J. M. and Dyson, R. D. 1973. Bovine pyruvate kinases. II. Purification of the liver isozyme and its hybridization with skeletal muscle pyruvate kinase. J. Biol. Chem. 248: 6938-6944.

Cardenas, J. M., Dyson, R. D., and Strandholm, J. J. 1973. Bovine pyruvate kinases. I. Purification and characterization of the skeletal muscle isozyme. J. Biol. Chem. 248: 6931-6937.

Cardenas, J. M., Dyson, R. C., and Strandholm, J. J. 1975b. Bovine and chicken pyruvate kinase isozymes intraspecies and inter-species hybrids. in Isozymes: Molecular Structure. Markert, C., Ed., Academic Press, New York, pp. 523-541.

Cardenas, J. M., Hubbard, D. R., and Anderson, S. 1977. Subunit structure and hybrid formation of bovine pyruvate kinases. Biochemistry. 16: 191-197.

Cardenas, J. M., Richards, T. C., and Gabourel, L. 1978. Localization of pyruvate kinase isozymes in bovine kidney and comparison of these patterns with those of lactate dehydrogenases and aldolases. J. Cell. Physiol. 96: 189-198.

Cardenas, J. M., Strandholm, J. J., and Miller, J. M. 1975c. Effects of phenylalanine and alanine on the kinetics of bovine pyruvate kinase isozymes. Biochemistry. 14: 4041-4045.

Carminatti, H., Jimenez de Asau, L., Leiderman, B., and Rozengurt, E. 1971. Allosteric Properties of skeletal muscle pyruvate kinase. J. Biol. Chem. 246: 7284-7288.

Chan, W. W. C. 1976a. Some experimental approaches for studying subunit interactions in enzymes. Can. J. Biochem. 54: 521-528.

Chan. W. W. C. 1976b. Immobilized subunits. Methods in Enzymology. 44: 491-503.

Chan, W. W. C., Mort, J. S., Chong, D. K. K., and MacDonald, P. D. W. 1973. Studies on Protein Subunits. III. Kinetic evidence for the presence of active subunits during the renaturation of muscle aldolase. J. Biol. Chem. 248: 2778-2784.

Chilson, O. P., Kitto, G. B., Pudles, J., and Kaplan, N. O. 1966. Reversible inactivation of dehydrogenases. J. Biol. Chem. 241: 2431-2445.

Clark, J. M., Jr. 1964. Experimental Biochemistry. pp. 109. W. H. Freeman and Co. San Francisco.

Cook, R. A. and Koshland, D. E. 1969. Specificity in the Assembly of multisubunit proteins. Proc. Natl. Acad. Sci. U.S.A. 64: 247-254.

Corcoran, E., Phelan, J. J., and Fottrell, P. F. 1976. Purification and properties of pyruvate kinase from human lung. Biochim. Biophys. Acta. 446: 96-104.

Cottam, G. L., Hollenberg, P. F., and Coon, M. J. 1969. Subunit structure of rabbit muscle pyruvate kinase, J. Biol. Chem. 244: 1481-1486.

Cottam, G. L. and Mildvan, A. S. 1971. Effect of urea on the divalent cation binding site of pyruvate kinase. J. Biol. Chem. 246: 4363-4365.

Cottam, G. L., Mildvan, A. S., Hunsley, J. R., and Suelter, C. H. 1972. Proton relaxation and kinetic studies of ternary complexes of an allosteric pyruvate kinase from yeast. J. Biol. Chem. 247: 3802-3809.

Creighton, T. E. 1978. Experimental studies of protein folding and unfolding. Prog. Biophys. Mol. Biol. 33: 231-297.

Crisp, D. M. and Pogson, C. I. 1972. Glycolytic and gluconeogenic enzyme activities in parenchymal and non-parenchymal cells from mouse liver. Biochem. J. 126: 1009-1023.

Dunaway-Mariano, D., Benovic, J. L., Cleland, W. W., Gupta, R. K., and Mildvan, A. S. 1979. Stereospecificity of the metal-adenosine 5$'$-triphosphate complex in reactions of muscle pyruvate kinase. Biochemistry 18: 4347-4354.

Dyson, R. D. and Cardenas, J. M. 1973. Bovine pyruvate kinases. III. Hybrids of the liver and skeletal muscle isozymes. J. Biol. Chem. 248: 8482-8488.

Eigenbrodt, E. and Schoner, W. 1977. Purification and properties of the pyruvate kinase isoenzymes type land M_2 from chicken liver. Hoppe-Seyler's Z. Physiol. Chem. 358: 1033-1046.

Ekman, P., Dahlqvist, U., Humble, E., and Engström, L. 1976. Comparative kinetics studies on the L-type pyruvate kinase from rat liver and the enzyme phosphorylated by cyclic 3$'$,5$'$-AMP-stimulated protein kinase. Biochim. Biophys. Acta. 429: 374-382.

Epstein, H. F., Schechter, A. N., and Cohen, J. S. 1971. Folding of staphylococcal nuclease: magnetic resonance and fluorescence studies of individual residues. Proc. Nat. Acad. Sci. U.S.A. 68: 2042-2046.

Evans, H. J. and Sorger, G. J. 1966. Role of mineral elements with emphasis on the univalent cations. Ann. Rev. Plant Physiol. 17: 47-76.

Flanders, L. E., Bamburg, J. R., and Sallach, H. J. 1971. Pyruvate kinase isozymes in adult tissue and eggs of Rana pipens. II. Physical and kinetic studies of purified skeletal and heart muscle pyruvate kinases. Biochim. Biophys. Acta. 242: 566-579.

Flashner, M., Tamir, I., Mildvan, A. S., Meloche, H. P., and Coon, M. J. 1973. Magnetic resonance and catalytic studies of pyruvate kinase with essential sulfhydryl and ϵ-amino groups chemically modified. J. Biol. Chem. 248: 3419-3425.

Friedman, F. K. and Beychok, S. 1979. Probes of subunit assembly and reconstitution pathways in multisubunit proteins. Ann. Rev. Biochem. 48: 217-250.

Gardiner, W. C., Jr. 1969. Rates and mechanisms of chemical reactions. W. A. Benjamin, Inc. p. 167.

Garel, J. R. and Baldwin, R. L. 1973. Both the fast and slow refolding reactions of ribonuclease A yield native enzyme. Proc. Nat. Acad. Sci. U.S.A. 70: 3347-3351.

Garel, J. R. and Baldwin, R. L. 1975. The heat-unfolded state of ribonuclease A is an equilibrium mixture of fast and slow refolding species. J. Mol. Biol. 94: 611-620.

Garreau, H., Columelli, S., Marie, J., and Kahn, A. 1977. Fructose-1,6-diphosphate binding by two forms of pyruvate kinase purified from human erythrocytes. FEBS Lett. 78: 95-97.

Gerschitz, J., Rudolph, R., and Jaenicke, R. 1978. Refolding and reactivation of liver alcohol dehydrogenase after dissociation and denaturation in 6 M guanidine hydrochloride. Eur. J. Biochem. 87: 591-599.

Guderley, H. and Cardenas, J. M. 1980. A study of the catalytic properties of pyruvate kinase from salmon and an examination of their functional relationships. J. Exp. Zool., in press.

Gupta, R. K., Fung, C. H., and Mildvan, A. S. 1976. Chromium (III)-adenosine triphosphate as a paramagnetic probe to determine intersubunit distances on pyruvate kinase. J. Biol. Chem. 251: 2421-2430.

Gupta, R. K. and Mildvan, A. S. 1977. Structures of enzyme-bound metal-nucleotide complexes in the phosphoryl transfer reaction of muscle pyruvate kinase ^{31}P NMR studies with magnesium and kinetic studies with chromium nucleotides. J. Biol. Chem. 252: 5967-5976.

Haeckel, R., Hess, B., Lauterborn, W., and Wüster, K-H. 1968. Purification and allosteric properties of yeast pyruvate kinase. Hoppe-Seyler's Z. Physiol. Chem. 349: 699-714.

Hagerman, P. J. and Baldwin, R. L. 1976. A quantitative treatment of the kinetics of the folding of ribonuclease A. Biochemistry 15: 1462-1473.

Hall, E. R. and Cottam, G. L. 1978. Isozymes of pyruvate kinase in vertebrates: Their physical, chemical, kinetic and immunological properties. Int. J. Biochem. 9: 785-793.

Hall, E. R., Kohl, E. A., and Cottam, G. L. 1978. The subunit structure of rat liver pyruvate kinase. Biochem. Biophys. Res. Commun. 80: 586-592.

Harkins, R. N., Black, J. A., and Rittenberg, M. B. 1977a. Purification and characterization of human muscle pyruvate kinase. Can. J. Biochem. 55: 301-307.

Harkins, R. N., Black, J. A., and Rittenberg, M. B. 1977b. M_2 isozyme of pyruvate kinase from human kidney as the product of a separate gene: Its purification and characterization. Biochemistry 16: 3831-3837.

Harkins, R. N. and Fothergill, L. A. 1977. Cat muscle pyruvate kinase: Isolation of a reactive thiol-group-containing peptide. Biochem. Soc. Trans. 5: 772-774.

Hermann, R., Rudolph, R., and Jaenicke, R. 1979. Kinetics of In Vitro reconstitution of oligomeric enzymes by cross-linking. Nature 277: 243-245.

Hofman, E., Kurganov, B. I., Schellenberger, W., Schulz, J., Sparmann, G., Wenzel, K.-W., and Zimmerman, G. 1975. Association-dissociation behavior of erythrocyte phosphofructokinase and tumor pyruvate kinase. Adv. Enzymatic Regul. 13: 247-267.

Hollenberg, P. F., Flashner, M., and Coon, M. J. 1971. Role of lysyl E-amino groups in adenosine diphosphate binding and catalytic activity of pyruvate kinase. J. Biol. Chem. 246: 946-953.

Hubbard, D. R. and Cardenas, J. M. 1975. Kinetic properties of pyruvate kinase hybrids formed with native type L and inactivated type M subunits. J. Biol. Chem. 250: 4931-4936.

Ibsen, K. H. and Marles, S. W. 1976. Inhibition of chicken pyruvate kinases by amino acids. Biochemistry. 15: 1073-1079.

Ibsen, K. H. and Trippet, P. 1973. A comparison of kinetic parameters obtained with three major noninterconvertible isozymes of rat pyruvate kinase. Arch. Biochem. Biophys. 156: 730-744.

Ibsen, K. H. and Trippet, P. 1974. Effects of amino acids on the kinetic properties of three noninterconvertible rat pyruvate kinases. Arch. Biochem. Biophys. 163: 570-580.

Ide, M. 1970. Pyruvate kinase from Brevibacterium liquefaciens. Arch. Biochim. Biophys. 140: 408-414.

Ikai, A., Fish, W., and Tanford, C. 1973. Kinetics of unfolding and refolding of proteins. II. Results for cytochrome c. J. Mol. Biol. 73: 165-184.

Imamura, K., Taniuchi, K., and Tanaka, T. 1972. Multimolecular forms of pyruvate kinase. II. Purification of M_2-type pyruvate kinase from yoshida ascites hepatoma 130 cells and comparative studies on the enzymological and immunological properties of the three types of pyruvate kinases L, M_1, and M_2. J. Biochem. 72: 1001-1015.

Jaenicke, R. 1978. Subunit refolding and reassociation of oligomeric enzymes. Proc. 12th FEBS Meeting. Dresden (Hofmann, E., ed.) 52: 187-198.

Jaenicke, R., Rudolf, R., and Heider, I. 1979. Quaternary structure, subunit activity, and in vitro association of porcine mitochondrial malic dehydrogenase. Biochemistry. 18: 1217-1223.

Jimenez de Asua, L., Rosengurt, E., DeValle, J. J., and Carminatti, H. 1971. Some kinetic differences between the M isozymes of pyruvate kinase from liver and muscle. Biochim. Biophys. Acta. 235: 326-334.

Johannes, K.-J., and Hess, B. 1973. Allosteric kinetics of pyruvate kinase of Saccharomyces carlsbergensis. J. Mol. Biol. 76: 181-205.

Johnson, S. C. 1980. Trinitrophenylation of bovine muscle pyruvate kinase. Ph.D. Thesis Oregon State University.

Johnson, S. C., Bailey, T., Becker, R. R., and Cardenas, J. M. 1979. Isolation and sequencedetermination of a peptide located in or near the active site of bovine muscle pyruvate kinase. Biochem. Biophys. Res. Comm. 90: 525-530.

Johnson, G. S., Kayne, M. S., and Deal, W. C., Jr. 1969. Metabolic control and structure of glycolytic enzymes. VIII. Reversal of the dissociation of rabbit muscle pyruvate kinase into unfolded subunits. Biochemistry 8: 2455-2462.

Jursinic, S. B. and Robinson, J. L. 1978. The active site of rabbit muscle pyruvate kinase. Evidence for a site common to the oxalacetate decarboxylase and pyruvate kinase reactions. Biochim. Biophys. Acta. 523: 358-367.

Kapoor, M. 1975. Subunit structure and some properties of pyruvate kinase of Neurospora. Can. J. Biochem. 53: 109-119.

Kaufman, S. 1977. Phenylketonuria: Biochemical Mechanisms. in Advances in Neurochemistry. Agranoff, B. and Aprison, M., Eds., Plenum Press, New York, 2: 1-109.

Kayne, F. J. 1971. Thallium (I) activation of pyruvate kinase. Archs. Biochem. Biophys. 143: 232-239.

Kayne, F. J. 1973. Pyruvate kinase. in Enzymes, 3rd ed. 8: 353-382.

Kayne, F. J. and Price, N. C. 1972. Conformational changes in the allosteric inhibition of muscle pyruvate kinase by phenylalanine. Biochemistry 11: 4415-4420.

Kayne, F. J. and Price, N. C. 1973. Amino acid effector binding to rabbit muscle pyruvate kinase. Arch. Biochem. Biophys. 159: 292-296.

Kayne, F. J. and Reuben, J. 1970. Thallium-205 nuclear magnetic resonance as a probe for studying metal ion binding to biological macromolecules. Estimate of the distance between the monovalent and divalent activators of pyruvate kinase. J. Am. Chem. Soc. 92: 220-222.

Kayne, F. J. and Suelter, C. H. 1968. The temperature-dependent conformational transitions of pyruvate kinase. Biochemistry 7: 1678-1684.

Kobr, M. L. and Beevers, H. 1971. Gluconeogenesis in the castor bean endosperm I changes in glycolytic intermediates. Plant Physiol. 47: 48-52.

Kohl, E. A. and Cottam, G. L. 1976. Alteration in liver pyruvate kinase protein and catalytic activity upon starvation and refeeding. Archs. Biochem. Biophys. 176: 671-682.

Kohl, E. A. and Cottam, G. L. 1977. Kinetic studies with L-type pyruvate kinase from rats either fed a high carbohydrate, low protein diet or starved. Biochim. Biophys. Acta. 484: 49-58.

Kutzbach, C., Bischofberger, H., Hess, B., and Zimmerman-Telschow, H. 1973. Pyruvate kinase from pig liver. Hoppe-Seyler's Z. Physiol. Chem. 351: 272-273.

Kutzbach, C. and Hess, B. 1970. Reinigung und einige eigenschaften von pyruvatkinase aus schweineleber. Hoppe-Seyler's Z. Physiol. Chem. 351: 272-273.

Levi, A. S. and Kaplan, N. O. 1971. Physical and Chemical properties of reversibly inactivated lactate dehydrogenases. J. Biol. Chem. 246: 6409-6417.

Levine, M., Muirhead, H., Stammers, D. K. and Stuart, D. I. 1978. Structure of pyruvate kinase and similarities with other enzymes: possible implications for protein taxonomy and evolution. Nature 271: 626-630.

Levitzki, A. and Tenenbaum, H. 1974. Dimers as intermediates in the assembly of tetrameric proteins: A study on lactate dehydrogenase isozymes. Isr. J. Chem. 12: 327-337.

Liao, C.-L. and Atkinson, D. E. 1971. Regulation at the phosphoenolpyruvate branchpoint in Azotobacter vinelandii: Pyruvate kinase. J. Bacteriol. 106: 37-44.

Lin, L.-N. and Brandts, J. F. 1978. Further evidence suggesting that the slow phase in protein unfolding and refolding is due to proline isomerization: A kinetic study of carp parvalbumins. Biochemistry 17: 4102-4110.

Lincoln, D. R., Rittenberg, M. B., and Black, J. A. 1977. Immunological cross-reaction between eukaryote and prokaryote pyruvate kinase. FEBS Lett. 80: 145-147.

Lindy, S. 1974. Synthesis and degradation of rat liver lactate dehydrogenase M_4. J. Biol. Chem. 249: 4961-4968.

Ljungström, O., Hjelmquist, G., and Engström, L. 1974. Phosphorylation of purified rat liver pyruvate kinase by cyclic $3',5'$-AMP-stimulated protein kinase. Biochim. Biophys. Acta. 358: 289-298.

Ljungström, O., Berglund, L., and Engström, L. 1976. Studies on the kinetic effects of adenosine-$3',5'$-monophasphate-dependent phosphorylation of purified pig liver pyruvate kinase type L. Eur. J. Biochem. 68: 497-506.

Ljungström, O. and Ekman, P. 1977. Glucagon-induced phosphorylation of pyruvate kinase (Type L) in rat liver slices. Biochem. Biophys. Res. Commun. 78: 1147-1155.

Llorente, P., Marco, R., and Sols, A. 1970. Regulation of liver pyruvate kinase and the phosphoenol-pyruvate crossroads. Eur. J. Biochem. 13: 45-54.

Maeba, P. and Sanwal, B. D. 1968. The regulation of pyruvate kinase of Escherichia coli by fructose diphosphate and adenylic acid. J. Biol. Chem. 243: 448-450.

Malcovati, M. and Kornberg, H. L. 1969. Two types of pyruvate kinase in Escherichia coli K12. Biochim. Biophys. Acta. 178: 420-423.

Marie, J., Kahn, A., and Boivin, P. 1976a. L-type pyruvate kinase from human liver. Purification by double affinity elution, electrofocusing, and immunological studies. Biochim. Biophys. Acta. 438, 393-406.

Marie, J., Garreau, H, and Kahn, A. 1977a. Evidence for a postsynthetic proteolytic transformation of human erythrocyte pyruvate kinase into L-type enzyme. FEBS Lett. 78: 91-94.

McQuate, J. T. and Utter, M. F. 1959. Equilibrium and kinetic studies of the pyruvic kinase reaction. J. Biol. Chem. 234: 2151-2157.

Mildvan, A. S. and Cohn, M. 1965. Kinetic and magnetic resonance studies of the pyruvate kinase reaction. J. Biol. Chem. 240: 238-246.

Mildvan, A. S. and Cohn, M. 1966. Kinetic and magnetic resonance studies of the pyruvate kinase reaction. J. Biol. Chem. 241: 1178-1193.

Ng, S. K. C. and Hamilton, I. R. 1975. Purification and regulatory properties of pyruvate kinase from Veillonella parvula. J. Bacteriol. 122: 1274- 1282.

Nowak, T. and Mildvan, A. S. 1972. Nuclear magnetic resonance studies of the function of potassium in the mechanism of pyruvate kinase. Biochemistry 11: 2819-2828.

O'Brien, M. D. and Kapoor, M. 1980. Studies of the structure-function relations of Neurospora pyruvate kinase: Denaturation by urea and the effect of ligands on the refolding and renaturation process. Int. J. Biochem. 11: 107-116.

Ohrmann, E. 1969. Die regulation der pyruvat-kinase in Euglena gracilis. Arch. Mikrobiol. 67: 273-292.

Osborne, H. H. and Hollaway, M. R. 1974. The hybridization of Glyceraldehyde-3-phosphate dehydrogenases from rabbit muscle and yeast. Biochem. J. 143: 651-662.

Ozaki, H. and Shiio, I. 1969. Regulation of the TCA and glyoxylate-cycles in Brevibacterium flavum. J. Biochem (Tokyo) 66: 297-311.

Parikh, I., March, S., and Cuatrecasas, P. 1974. Topics in the methodology of substitution reactions with agarose. Methods in Enzymology. 34: 77-81.

Parkinson, J. and Easterby, J. S. 1977. Purification and molecular properties of bovine heart pyruvate kinase. Biochim. Biophys. Acta. 481: 471-480.

Parr, G. R. and Hammes, G. G. 1976. A kinetic study of the subunit dissociation and reassembly of rabbit muscle phosphofructokinase. Biochemistry 15: 857-862.

Passeron, S. and Roselino, E. 1971. A new form of pyruvate kinase in mycelium of Mucor rouxii. FEBS Lett. 18: 9-12.

Peterson, J. S., Chern, C. J., Harkins, R. N., and Black, J. A. 1974. The subunit structure of human muscle and human erythrocyte pyruvate kinase isozymes. FEBS Lett. 49: 73-77.

Peterson, J. B. and Evans, H. J. 1978. Properties of pyruvate kinase from soybean nodule cytosol. Plant Physiol. 61: 909-914.

Privalov, P. L. and Khechinashvili. 1974. A thermodynamic approach to the problem of stabilization of globular protein structure: A calorimetric study. J. Mol. Biol. 86: 665-684.

Randall, R. F. and Anderson, P. J. 1975. Purification and properties of the pyruvate kinase of sturgeon muscle. Biochem. J. 145: 569-573.

Ray, W. J. and Koshland, D. E., Jr. 1961. A method for characterizing the type and numbers of groups involved in enzyme action. J. Biol. Chem. 236: 1973-1979.

Reuben, J. and Cohn, M. 1970. Magnetic resonance studies of manganese (II) binding sites of pyruvate kinase. J. Biol. Chem. 245: 6539-6546.

Riou, J. P., Claus, T. H., and Pilkis, S. J. 1978. Stimulation by glucagon of In Vivo phosphorylation of rat hepatic pyruvate kinase. J. Biol. Chem. 253: 656-659.

Roberts, G. C. K. and Benz, F. W. 1973. Proton fourier transform NMR studies of the unfolding of ribonuclease. Ann. N. Y. Acad. Sci. 222: 130-148.

Roschlau, P. and Hess, B. 1972. Modification of yeast pyruvate kinase by 2, 4, 6-trinitrobenzene-sulphonic acid. Hoppe-Seyler's Z. Physiol. Chem. 353: 994-998.

Rudolph, R., Gerschitz, J., and Jaenicke, R. 1978. Effect of zinc (II) on the refolding and reactivation of liver alcohol dehydrogenase. Eur. J. Biochem. 87: 601-606.

Rudolph, R., Heider, I., and Jaenicke, R. 1977a. Effect of coenzymes and temperature on the process of in vitro refolding and reassociation of lactic dehydrogenase isoenzymes. Biochemistry 16: 5527-5531.

Rudolph, R., Heider, I., and Jaenicke, R. 1977b. Mechanism of reactivation and refolding of glyceraldehyde-3-phosphate dehydrogenase from yeast after denaturation and dissociation. Eur. J. Biochem. 81: 563-570.

Rudolph, R., Heider, I., Westhof, E., and Jaenicke, R. 1977c. Mechanism of refolding and reactivation of lactic dehydrogenase from pig heart after dissociation in various solvent media. Biochemistry 16: 3384-3390.

Rudolph, R. and Jaenicke, R. 1976. Kinetics of reassociation and reactivation of pig-muscle lactic dehydrogenase after acid dissociation. Eur. J. Biochem. 63: 409-417.

Rudolph, R., Westhof, E., and Jaenicke, R. 1977d. Kinetic analysis of the reactivation of rabbit muscle aldolase after denaturation with guanidine hyrochloride. FEBS Lett. 73: 204-206.

Rudolph, R., Zettlmeissl, G., and Jaenicke, R. 1979. Reconstitution of lactic dehydrogenase. Noncovalent aggregation versus reactivation. 2. Reactivation of irreversibly denatured aggregates. Biochemistry 18: 5572-5575.

Saheki, S., Saheki, K., and Tanaka, T. 1978. Peptide mapping by limited proteolysis of four pyruvate kinase isozymes. FEBS Lett. 93: 25-28.

Schedel, M., Klemme, J.-H., and Schlegel, H. G. 1975. Regulation of C_3-enzymes in facultative phototrophic bacteria: The cold-labile pyruvate kinase of Rhodopseudomonas sphaeroides. Arch. Microbiol. 103: 237-245.

Schmid, F. X. and Baldwin, R. L. 1978a. Acid catalysis of the formation of the slow-folding species of RNase A: Evidence that the reaction is proline isomerization. Proc. Natl. Acad. Sci. U.S.A. 75: 4764-4768.

Schmid, F. X. and Baldwin, R. L. 1978b. The nature of the slow-folding species of ribonuclease A. Proc. 12th FEBS Meeting. Dresden (Hofmann, E., ed.) 52: 173-185.

Schmid, F. X. and Baldwin, R. L. 1979. Detection of an early intermediate in the folding of ribonuclease A by protection of amide protons against exchange. J. Mol. Biol. 135: 199-215.

Sorger, G. J., Ford, R. E., and Evans, H. J. 1965. Effects of univalent cations on the immunoelectrophoretic behavior of pyruvic kinase. Proc. Natl. Acad. Sci. U.S.A. 54: 1614-1621.

Sossinka, J. and Hess, B. 1974. Electron microscopy of pyruvate kinase crystals from Saccharomyces carlsbergensis. J. Mol. Biol. 83: 285-287.

Staal, G. E. J., Koster, J. F., Kamp, H., Van Milligen-Boersma, L., and Veeger, C. 1971. Human erythrocyte pyruvate kinase its purification and some properties. Biochim. Biophys. Acta. 227: 86-96.

Stammers, D. K. and Muirhead, H. 1975. Three dimensional structure of cat muscle pyruvate kinase at 6 Å resolution. J. Mol. Biol. 95: 213-225.

Stammers, D. K., Levine, M., Stuart, D. I., and Muirhead, H. 1977. Structure of cat muscle pyruvate kinase at 0.26 nm resolution. Biochem. Soc. Trans. 5: 654-657.

Stammers, D. K. and Muirhead, H. 1977. Three-dimensional structure of cat muscle pyruvate kinase at 3.1 Å resolution. J. Mol. Biol. 112: 309-316.

Steinmetz, M. A. and Deal, W. C., Jr. 1966. Metabolic control and structure of glycolytic enzymes. III. Dissociation and subunit structure of rabbit muscle pyruvate kinase. Biochem. 5: 1399–1405.

Stifel, F. B. and Herman, R. H. 1971. Effect of L-histidine on human and rat jejunal pyruvate kinase activity. Can. J. Biochem. 49: 1105–1116.

Story, R. B. and Hochachka, P. W. 1974. Enzymes of energy metabolism in a vertebrate facultative anaerobe, Pseudemys scripta. J. Biol. Chem. 249: 1423–1427.

Suelter, C. H. 1967. Effects of temperature and activating cations on the fluorescence of pyruvate kinase. Biochemistry 6: 418–423.

Suelter, C. H. 1970. Enzymes activated by monovalent cations. Science 169: 789–795.

Summers, M. R. and McPhie, P. 1972. The mechanism of unfolding of globular proteins. Biochem. Biophys. Res. Commun. 47: 831–837.

Tanaka, T., Harano, Y., Sue, F., and Morimura, H. 1967. Crystallization, characterization and metabolic regulation of two types of pyruvate kinase isolated from rat tissues. J. Biochem. 62: 71–91.

Tanford, C. 1968. Protein denaturation. Adv. Prot. Chem. 23: 121–182.

Tanford, C. 1970. Protein denaturation. Theoretical models for the mechanism of denaturation. Adv. Prot. Chem. 24: 1–95.

Teipel, J. W. 1972. In vitro assembly of aldolase. Kinetics of refolding, subunit reassociation, and reactivation. Biochemistry 11: 4100–4107.

Teipel, J. W. and Koshland, D. E., Jr. 1971. Kinetic aspects of conformational changes in proteins. I. Rate of regain of enzyme activity from denatured proteins. Biochem. 10: 792–805.

Tenenbaum-Bayer, H. and Levitzki, A. 1976. The refolding of lactate dehydrogenase subunits and their assembly to the functional tetramer. Biochim. Biophys. Acta. 445: 261–279.

Terenzi, H., Roselino, E., and Passeron, S. 1971. Two types of pyruvate kinase in Mucor rouxii: Changes in the enzymatic pattern related to aerobic development. Eur. J. Biochem. 18: 342–350.

Tobes, M., Kuczenski, R. T., and Suelter, C. H. 1972. Yeast pyruvate kinase renaturation after dissociation by guanidine hyrochloride. Arch. Biochem. Biophys. 151: 56–61.

Tomlinson, J. D. and Turner, J. F. 1973. Pyruvate kinases from higher plants. Biochim. Biophys. Acta. 329: 128-139.

Tsong, T. Y. 1973. Detection of three kinetic phases in the thermal unfolding of ferricytochrome c. Biochemistry 12: 2209-2214.

Tsong, T. Y. and Baldwin, R. L. 1972. Kinetic evidence for intermediate states in the unfolding of chymotrypsinogen A. J. Mol. Biol. 69: 145-148.

Tsong, T. Y., Baldwin, R. L. and Elson, E. L. 1972a. Properties of the refolding and unfolding reactions of ribonuclease A. Proc. Natl. Acad. Sci. U.S.A. 69: 1809-1812.

Tsong, T. Y., Baldwin, R. L., and McPhie, P. 1972b. A sequential model of nucleation-dependent protein folding: Kinetic studies of ribonuclease A. J. Mol. Biol. 63: 453-475.

Tuominen, F. W. and Bernlohr, R. W. 1971. Pyruvate kinase of the spore-forming bacterium, Bacillus licheniformis. J. Biol. Chem. 246: 1733-1745.

Valentini, G., Iadarola, P., Somani, B. L., and Malcovati, M. 1979. Two forms of pyruvate kinase in Escherichia Coli. A comparison of chemical and molecular properties. Biochim. Biophys. Acta. 570: 248-258.

Van Berkel, J. C., Koster, J. F., and Hülsman, W. C. 1972. Distribution of L- and M-type pyruvate kinase between parenchymal and kupffer cells of rat liver. Biochim. Biophys. Acta. 276: 425-429.

Waygood, E. B., Mort, J. S., and Sanwal, B. D. 1976. The Control of pyruvate kinase of Escherichia Coli. Binding of substrate and allosteric effectors to the enzyme activated by fructose 1,6-bisphosphate. Biochemistry 15: 277-282.

Waygood, E. B. and Sanwal, B. D. 1974. The control of pyruvate kinase of Escherichia Coli I. Physico-chemical and regulatory properties of the enzyme activated by fructose 1,6-diphosphate. J. Biol. Chem. 249: 265-274.

Weber, G. 1969. Inhibition of human brain pyruvate kinase and hexokinase by phenylalanine and phenylpyruvate: Possible relevance to phenylketonuria brain damage. Proc. Natl. Acad. Sci. U.S.A. 63: 1365-1369.

Westmoreland, D. G. and Matthews, C. R. 1973. Nuclear magnetic resonance study of the thermal denaturation of ribonuclease A: Implications for multistate behavior at low pH. Proc. Nat. Acad. Sci. U.S.A. 70: 914-918.

Wetlaufer, D. B. 1973. Nucleation, rapid folding, and globular intrachain regions in proteins. Proc. Nat. Acad. Sci. U.S.A. 70: 697-701.

Wetlaufer, D. B. and Ristow, S. S. 1973. Acquisition of three-dimensional structure of proteins. Ann. Rev. Biochem. 42: 135-158.

Yoshizaki, F. and Imahori, K. 1979. Regulatory properties of pyruvate kinase from an extreme thermophile Thermus thermophilus HB8. Agric. Biol. Chem. 43: 527-536.

Zammit, V. A., Beis, I., Newsholme, E. A. 1978. Maximal activities and effects of fructose bisphosphate on pyruvate kinase from muscles of vertebrates and invertebrates in relation to the control of glycolysis. Biochem. J. 174: 989-998.

Zettlmeissl, G., Rudolph, R., and Jaenicke, R. 1979. Reconstitution of lactic dehydrogenase. Noncovalent aggregation versus reactivation. 1. Physical properties and kinetics of aggregation. Biochemistry 18: 5567-5571.

APPENDIX

Appendix

Let

[A] = concentration of A

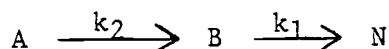[B] = concentration of B

[N] = concentration of N

A_o = [A] at t = 0

B_o = [B] at t = 0

The kinetic system

$$A \xrightarrow{k_2} B \xrightarrow{k_1} N$$

with the initial conditions [A] = A_o and [B] = B_o at t = 0 is described

by the rate equations

$$\frac{d[A]}{dt} = -k_2[A] \qquad\qquad A1$$

$$\frac{d[B]}{dt} = -k_1[B] + k_2[A] \qquad\qquad A2$$

$$\frac{d[N]}{dt} = k_1[B] \qquad\qquad A3$$

A1 is easily solved and substituting into A2 gives

$$\frac{d[B]}{dt} + k_1[B] = k_2 A_o e^{-k_2 t} \qquad\qquad A4$$

A4 is also easily solved subject to the initial condition on [B] and

yields

$$[B] = \frac{k_2 A_o}{k_1 - k_2} e^{-k_2 t} + \frac{k_1 B_o - k_2(A_o + B_o)}{k_1 - k_2} e^{-k_1 t} \qquad\qquad A5$$

Substituting A5 into A3 and solving for [N] subject to the conditions

[N] = 0 at t = 0 and [N] = $A_o + B_o$ at t = ∞ yields

$$[N] = A_o + B_o - \frac{k_1 A_o}{k_1 - k_2} e^{-k_2 t} - \frac{k_1 B_o - k_2 (A_o + B_o)}{k_1 - k_2} e^{-k_1 t}$$

which can be rearranged to give

$$1 - f = \frac{k_1 (1 - F)}{k_1 - k_2} e^{-k_2 t} + \frac{k_1 F - k_2}{k_1 - k_2} e^{-k_1 t} \qquad A6$$

where

$$f = \frac{[N]}{A_o + B_o}$$

$$F = \frac{B_o}{A_o + B_o}$$

From equation A6 it is easy to show that the initial slope

$$\frac{d \ln(1 - f)}{dt} \Big|_{t = 0} = -k_1 F \qquad A7$$

and that the final slope

$$\lim_{t \to \infty} \frac{d \ln(1 - f)}{dt} = -k_i \quad \text{where } k_i \text{ is the smaller of } k_1 \text{ and } k_2.$$

To fit the data, the initial slope of the $\ln(1 - f)$ vs time plot must

be less than the final slope. Thus, from A7 we must have F ≠ 0, that

is B_o ≠ 0 and then 0 < F < 1. If $k_1 < k_2$, then the final slope is $-k_1$

and, since, 0 < F < 1, $-k_1 F$ is not less than $-k_1$. Thus, we can not

fit the data if $k_1 < k_2$. If $k_1 > k_2$, then from A6 and equation 1

$$\frac{k_1 F - k_2}{k_1 - k_2} = a$$

Thus $-k_1 F < -k_2$, which does meet the requirement that the initial slope be less than the final slope. So to fit the data, with a sequential mechanism requires $B_o \neq 0$ and $k_1 > k_2$.

The kinetic mechanism

is described by the equations

$$\frac{d[A]}{dt} = -k_1 [A]$$

$$\frac{d[B]}{dt} = -k_2 [B]$$

$$[N] = A_o - [A] + B_o - [B]$$

which are easily solved to yield

$$1 - f = (1-F)e^{-k_1 t} + Fe^{-k_2 t}$$

where f and F are defined by the same expressions as are given above.