Conformational Changes in the Allosteric Inhibition of Muscle Pyruvate Kinase by Phenylalanine†

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ABSTRACT: Evidence has been obtained for a conformational change in muscle pyruvate kinase on binding the allosteric inhibitor, L-phenylalanine. Phenylalanine enhances the fluorescence of the enzyme by about 22%. The saturation curve is slightly sigmoidal (Hill coefficient, $n = 1.5$) and the half-point occurs at a phenylalanine concentration of 90 μM. In the presence of the divalent metal activators, Mg^{2+} or Mn^{2+}, or Mg^{2+} plus substrate (phosphoenolpyruvate), the saturation curves are much more sigmoidal (n values up to 2.6) and are shifted to higher phenylalanine concentrations. These fluorescence changes are reversed by L-alanine (which relieves the phenylalanine inhibition of the enzyme) in the presence of the divalent cation and/or substrate. A striking difference is observed between the ternary complexes pyruvate kinase–Mn^{2+}–phosphoenolpyruvate and pyruvate kinase–Mg^{2+}–phosphoenolpyruvate; 20 mM phenylalanine only slightly increases the fluorescence of the former, but almost completely titrates the fluorescence increase of the latter. The distinction between Mn^{2+} and Mg^{2+} as divalent cation activators of the enzyme is also observed in kinetic studies. Phenylalanine is almost without effect on the Mn^{2+}-activated reaction, whereas 50% inhibition of the Mg^{2+}-activated reaction occurs at phenylalanine concentrations of about 14 mM. Ultraviolet difference spectra measurements are in good accord with the fluorescence studies and lend support to the postulate that conformational changes in the enzyme are involved in the inhibition by phenylalanine. Phenylalanine weakens the binding of Mn^{2+} to the enzyme by a factor of 3.5; this effect being substantially reversed by addition of alanine. However, little change in the proton relaxation rate of water by the Mn^{2+} bound to the enzyme is seen on addition of phenylalanine, suggesting that the environment of the bound Mn^{2+} is only slightly changed. Preliminary nuclear magnetic resonance studies indicate that the phenylalanine and Mn^{2+} sites are at least 12 Å apart on the enzyme, and imply that the heterotropic interactions between these ligands are of an indirect, allosteric nature.

Rabbit muscle pyruvate kinase (EC 2.7.1.40) has long been recognized as a multisubunit enzyme with specific requirements for a monovalent and a divalent cation (for reviews, see Boyer, 1962, and Kayne, 1972). Potassium and Mg^{2+} are presumed to be the natural activators of the enzyme, but other combinations do show appreciable activating ability (Boyer, 1962; Kayne, 1971). The fact that the paramagnetic Mn^{2+} ion will substitute for Mg^{2+} provides a powerful tool for the investigation of the catalytic and structural properties of the enzyme (Cohn and Reuben, 1971; Reed and Cohn, 1972a). Until recently, no firm evidence had been presented for interactions between the subunits from either kinetic or substrate binding studies. Carminatti *et al.* (1971) have now reported that pyruvate kinase can be inhibited in an allosteric fashion by L-phenylalanine.[1] In particular, the inhibition displays sigmoidal saturation, kinetic behavior of a "mixed" type, and can be relieved by Ala. Since the optical properties of muscle pyruvate kinase (ultraviolet absorption and fluorescence spectroscopy) have been used to detect changes in conformation induced by various ligands (Suelter, 1967; Kayne and Suelter, 1968) it was felt that these properties might be useful in determining the effect of Phe on the enzyme. In conjunction with these studies, magnetic resonance and kinetic investigations were able to provide further insight into the various heterotropic and homotropic interactions involved in this inhibition.

Materials and Methods

Pyruvate kinase was isolated from frozen rabbit muscle (Pel-Freeze, Rogers, Ark.) according to the method of Tietz and Ochoa (1958). The preparation had a specific activity of 250 μmoles of product formed per min per mg of protein at optimal substrate concentrations and at pH 7.5 ($T = 24.5°$). Enzyme activity was assayed using the coupled lactate dehydrogenase assay (Kayne, 1971). Pyruvate kinase concentrations were determined spectrophotometrically using published values of the extinction coefficient at 280 nm and molecular weight (Boyer, 1962).

L-Phenylalanine, Ala, NADH, and the tricyclohexylammonium salt of P-enolpyruvate were purchased from Sigma, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid from Calbiochem, and ADP and lactate dehydrogenase from Boehringer. Other reagent were of the highest purity commercially available.

Fluorescence measurements were made on a Perkin-Elmer MPF2A fluorescence spectrophotometer equipped with a thermostated cell compartment. Protein fluorescence was excited at 295 nm and observed at 350 nm. No correction was necessary for any inner filter effects since Phe does not absorb radiation of these wavelengths. Pyruvate kinase concentrations were typically about 0.5 mg/ml for these experiments.

Difference spectra measurements were made on a Cary 15 spectrophotometer using 1-cm cells and the 0.1-*A* slide-wire. The tandem cell method was used to compensate the sample

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‡ Harkness Fellow of the Commonwealth Fund of New York, 1971–1972.

[1] Abbreviations used are: Phe, L-phenylalanine; Ala, L-alanine.

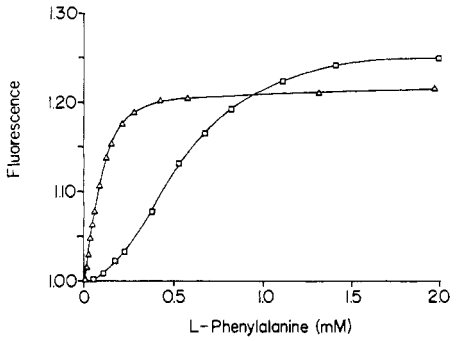

FIGURE 1: Titrations of pyruvate kinase with Phe, monitored by changes in protein fluorescence. (Δ) Pyruvate kinase and (□) pyruvate kinase + 2.5 mM $MnCl_2$. Enzyme was at a concentration of 0.5 mg/ml in 50 mM potassium ion–*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (75 mM KCl added) at pH 7.5. The temperature was 23.5°.

or reference beams for the added Phe absorption and for dilution of the enzyme.

Electron paramagnetic resonance (epr) measurements were made on an X-band Varian E3 spectrometer, as described by Reuben and Cohn (1970). A standard variable-temperature accessory was used for thermostating; the temperature in the cavity was measured at intervals during the experiment.

Proton relaxation rate measurements were made at 24.3 MHz by the 180–90° null method as described by Reuben and Cohn (1970). During the experiment the temperature was constant at 22 ± 0.5°. The enhancement parameter of the bound Mn^{2+} was calculated as described previously (Mildvan and Cohn, 1965).

Nuclear magnetic resonance (nmr) measurements were made on a Varian HR220 instrument operating at 220 MHz, equipped with a Fourier transform accessory. Measurements of the longitudinal relaxation times (T_1) were made using a special program written in this laboratory (McDonald and Leigh, 1972). For these experiments, the pyruvate kinase solutions were lyophilized and redissolved in D_2O (Thomson Packard, Inc; 99.8% isotopic purity). This procedure did not affect the specific activity of the enzyme.

Results

Fluorescence Studies. The addition of Phe to pyruvate kinase resulted in an increase in protein fluorescence as shown in Figure 1. Little change was observed in the wavelength of maximum emission. In the limit, the fluorescence was increased by approximately 22%, and the half-point for this change ($S_{0.5}$) is observed at a Phe concentration of 90 μM. This is considerably below the $S_{0.5}$ obtained in the kinetic study reported by Carminatti *et al.* (1971). Figure 1 also shows the titration curve in the presence of 2.5 mM Mn^{2+} (saturating concentrations). In this case, the saturation curve is much more sigmoidal, as described by the increased Hill coefficient, *n* (Table I), and the $S_{0.5}$ is shifted to higher Phe concentrations. In the presence of the "natural" activator Mg^{2+}, the titration curves are similar to those seen with Mn^{2+} (see Table I). For ease of comparison, the titration curves shown in Figures 1 and 2 are normalized to the same initial fluorescence. In fact, the addition of saturating amounts of divalent metal ion and/or substrate caused a small (*ca.* 5%) decrease in the protein fluorescence.

In the presence of P-enolpyruvate, the saturation curves for Phe are shifted to higher Phe concentrations although the value of the Hill coefficient is not markedly changed from that observed in the absence of P-enolpyruvate (Table I and Figure 2). However, in the presence of both Mg^{2+} and P-enolpyruvate, the n value is substantially increased and the $S_{0.5}$ for Phe is now approximately 7 mM, in the range of that observed in the kinetic experiments (Carminatti *et al.*, 1971). A striking difference is seen in this case, however, when Mn^{2+} is used as the divalent cation. Only a very slight increase in protein fluorescence was observed even after addition of 20 mM Phe (Figure 2).

L-Alanine has been shown to relieve the Phe inhibition of pyruvate kinase (Carminatti *et al.*, 1971). The fluorescence enhancement caused by Phe is completely reversed by subsequent addition of Ala, provided that either P-enolpyruvate, or divalent metal ion, or both are present. However, in the absence of these, Ala only partially reverses the Phe-induced enhancement of protein fluorescence. Further investigation

TABLE I: Titrations of Pyruvate Kinase with Phenylalanine under Various Conditions, as Monitored by Protein Fluorescence Changes.[a]

Sample	$S_{0.5}$ (mM)	*n* Value	Ala Reversal Obsd	$S_{0.5}$ for Reversal (mM)
Pyruvate kinase	0.09	1.5	Partial	≃5
Pyruvate kinase + 50 μM Mg^{2+}	0.11	1.65	Partial	≃6
Pyruvate kinase + 2.5 mM Mg^{2+}	0.44	2.25	Complete	1.9
Pyruvate kinase + 5 mM Mg^{2+}	0.60	2.2	Complete	1.0
Pyruvate kinase + 50 μM Mn^{2+}	0.23	1.9	Complete	3.2
Pyruvate kinase + 100 μM Mn^{2+}	0.30	2.0	Complete	2.8
Pyruvate kinase + 2.5 mM Mn^{2+}	0.52	2.3	Complete	1.1
Pyruvate kinase + 100 μM P-enolpyruvate	1.5	1.6	Complete	1.5
Pyruvate kinase + 200 μM P-enolpyruvate	3.9	1.7	Not determined	
Pyruvate kinase + 2.5 mM Mg^{2+} + 100 μM P-enolpyruvate	7.0	2.5	Complete	0.45
Pyruvate kinase + 5 mM Mg^{2+} + 100 μM P-enolpyruvate	6.5	2.6	Complete	0.35
Pyruvate kinase + 50 μM Mn^{2+} + 100 μM P-enolpyruvate	Too weak to determine			
Pyruvate kinase + 2.5 mM Mn^{2+} + 100 μM P-enolpyruvate	Too weak to determine			

[a] Pyruvate kinase was at a concentration of 0.5 mg/ml in 50 mM potassium ion–*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (75 mM KCl added) at pH 7.5. The temperature was 23.5°. $S_{0.5}$ is the ligand concentration required for half-maximal effect.

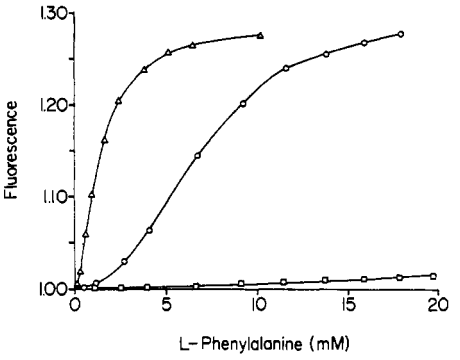

FIGURE 2: Titrations of pyruvate kinase with Phe. (Δ) Pyruvate kinase + 100 μM P-enolpyruvate, (○) pyruvate kinase + 2.5 mM $MgCl_2$ + 100 μM P-enolpyruvate, and (□) pyruvate kinase + 2.5 mM $MnCl_2$ + 100 μM P-enolpyruvate. Conditions were as in Figure 1.

showed that Ala itself, in the absence of substrate and divalent cations, causes an enhancement in the protein fluorescence, some 75% that of the Phe effect. In the presence of substrate and/or divalent cations Ala has no effect on the protein fluorescence. The $S_{0.5}$ values for the reversal of the Phe effect by Ala are included in Table I. It is seen that in the presence of Mg^{2+} and P-enolpyruvate, the $S_{0.5}$ for the Ala reversal is much lower than in their absence. Thus, it appears that the effects of magnesium ions and substrate on the Phe and Ala affinities of the enzyme are reciprocal.

Difference Spectra. Protein ultraviolet difference spectra were recorded under conditions similar to those used in fluorescence measurements. Addition of 170 μM Phe gives rise to a positive difference spectrum (Figure 3, curve A) which suggests a change in environment of tryptophan residues. The addition of Mg^{2+} (5 mM) would almost completely reverse the fluorescence enhancement brought about by this concentration of Phe (Figure 1 and Table I), and in accord with this prediction, 5 mM Mg^{2+} almost completely abolished the difference spectrum at 295 nm (Figure 3, curve B). Addition of Mg^{2+} to the reference protein solution abolishes the residual (negative) difference (Figure 3, curve C). Raising the Phe concentration to 1.0 mM largely restored the difference spectrum (Figure 3, curve D), again in accord with the fluorescence titration behavior. A final addition of 2.0 mM Ala again abolished the appearance of this tryptophan difference spectrum (Figure 3, curve E). Curve F shows the expected negative difference spectrum obtained by the subsequent addition of Phe to the reference protein solution. In agreement with the fluorescence measurements, Ala in the absence of divalent cation gives rise to a difference spectrum similar to that caused by Phe, but there is no evidence for this in the presence of Mg^{2+}.

Kinetics. The inhibition of pyruvate kinase activity under conditions similar to those used in the optical studies is shown in Figure 4. These results are qualitatively similar to those of Carminatti *et al.* (1971), but two differences are evident. Firstly, 50% inhibiton is observed at a higher concentration of Phe under our conditions. Secondly, the inhibition is also more cooperative as evidenced by the greater *n* value, *ca.* 2.3, compared to 1.6 as reported by Carminatti *et al.* (1971). Our experiments were performed at a lower temperature (24.5° compared to 30°) and it has been demonstrated that the conformational equilibria of the enzyme are quite temperature-dependent (Kayne and Suelter, 1968). Carminatti *et al.* (1971)

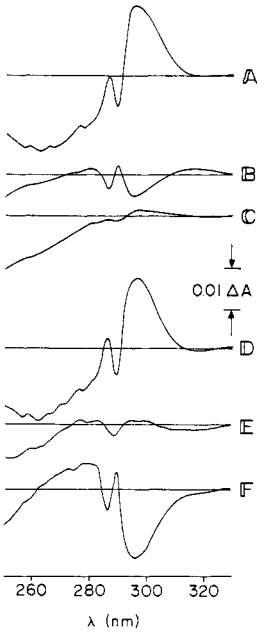

FIGURE 3: Difference spectra of pyruvate kinase in the presence of various ligands. Enzyme was at a concentration of 2.2 mg/ml in 50 mM potassium ion–*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (75 mM KCl added) at pH 7.5 and a temperature of 25°. (A) Pyruvate kinase + 170 μM Phe *vs.* pyruvate kinase, (B) pyruvate kinase + 170 μM Phe + 5 mM $MgCl_2$ *vs.* pyruvate kinase, (C) pyruvate kinase + 170 μM Phe + 5 mM $MgCl_2$ *vs.* pyruvate kinase + 5 mM $MgCl_2$, (D) pyruvate kinase + 1.0 mM Phe + 5 mM $MgCl_2$ *vs.* pyruvate kinase + 5 mM $MgCl_2$, (E) pyruvate kinase + 1.0 mM Phe + 5 mM $MgCl_2$ + 2 mM Ala *vs.* pyruvate kinase + 5 mM $MgCl_2$, and (F) pyruvate kinase + 1.0 mM Phe + 5 mM $MgCl_2$ + 2 mM Ala *vs.* pyruvate kinase + 1.0 mM Phe + 5 mM $MgCl_2$. The overall dilutions were less than 5% and the spectra are uncorrected for this factor.

used a commercial preparation of pyruvate kinase and this could also be responsible for some of the differences.

In view of the dramatic difference in the behavior of the complexes, pyruvate kinase–Mg^{2+}–P-enolpyruvate and pyruvate kinase–Mn^{2+}–P-enolpyruvate in the fluorescence titration, it was expected that some differences might also appear in the kinetic behavior of these complexes. This expectation was borne out by the observation that Phe at concentrations up to 75 mM caused less than 5% inhibition under the same conditions used for the assay in Figure 4 with Mn^{2+} in place of Mg^{2+} as the divalent cation. Due to the enzyme's higher affinity for Mn^{2+} over Mg^{2+}, the addition of Mn^{2+} to a 92%

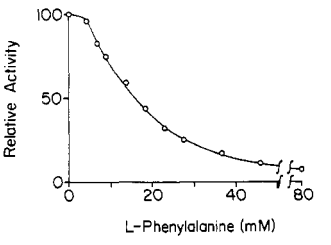

FIGURE 4: Inhibition of pyruvate kinase by Phe. Assay conditions were ADP = 1.5 mM, P-enolpyruvate = 100 μM, $MgSO_4$ = 5 mM, NADH = 200 μM, lactate dehydrogenase = 25 μg/ml. The reactions were initiated by the addition of about 0.15 μg of pyruvate kinase. Assays were performed in 50 mM potassium ion–*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (75 mM KCl added) at pH 7.5. The temperature was 24.5°.

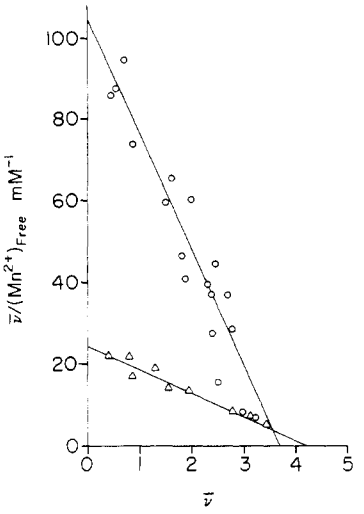

FIGURE 5: Scatchard plots for the binding of Mn^{2+} to pyruvate kinase in the absence (○) and presence (△) of 10 mM Phe. Data were obtained as described in the text. $\bar{\nu}$ is the moles of Mn^{2+} bound per mole of enzyme. Conditions as in Table II.

Phe inhibited (Mg^{2+}) assay system allowed the enzymatic reaction to accelerate to a velocity typical of the Mn^{2+}-activated reaction.

Manganese(II) Binding Studies. The effects of Phe and Ala on the binding of Mn^{2+} to pyruvate kinase were studied by epr titrations. The concentration of free Mn^{2+} is proportional to the amplitude of the epr lines since the bound Mn^{2+} does not contribute significantly to the observed signal (Mildvan and Cohn, 1965). Two types of titrations were performed: one in which the total Mn^{2+} concentration was held constant at 100 μM and the concentration of pyruvate kinase varied between 4 and 250 μM, and the other in which Mn^{2+} (up to 800 μM) was added to a fixed concentration of pyruvate kinase (40 μM). The titrations were performed in the absence of effectors and in the presence of 10 mM Phe, 10 mM Ala, and 10 mM Phe plus 10 mM Ala. In the presence of 100 μM Mn^{2+}, 10 mM Phe effectively saturates the enzyme and 10 mM Ala is nearly saturating (Table I). The data were analyzed according to the method of Scatchard and there was no apparent change in the number of Mn^{2+} sites (1/subunit) with any of these effectors. Figure 5 shows the Scatchard plots in the absence and presence of 10 mM Phe.

The dissociation constants for the Mn^{2+}–pyruvate kinase complex are listed in Table II. Phenylalanine weakens the affinity of the enzyme for Mn^{2+} and this effect is substantially reversed upon addition of Ala. L-Alanine alone has only a small effect on Mn^{2+} binding. Correction for binding of Mn^{2+} to Phe or Ala is unnecessary, since under our conditions dissociation constants of *ca.* 180 mM for these complexes are suggested by epr measurements.

TABLE II: Dissociation Constants and Enhancement Parameters (ϵ_b) for Mn^{2+}–Pyruvate Kinase under Various Conditions.[a]

Sample	K_d (μM)	ϵ_b
Pyruvate kinase	40	18.9
Pyruvate kinase + 10 mM Phe	145	18.7
Pyruvate kinase + 10 mM Phe + 10 mM Ala	95	20.8
Pyruvate kinase + 10 mM Ala	65	19.4

[a] The buffer was 50 mM potassium ion-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (75 mM KCl added) at pH 7.5. For the epr and PRR measurements, the temperatures were 24 and 22°, respectively. The ϵ_b was calculated from the observed PRR enhancements and the per cent Mn^{2+} bound as described in the text.

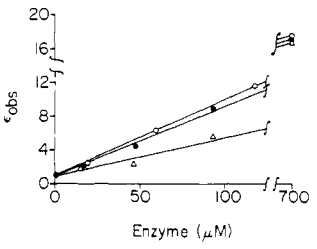

FIGURE 6: The PRR enhancement parameter (ϵ_{obsd}) of Mn^{2+} bound to pyruvate kinase as a function of enzyme concentration. Mn^{2+} = 100 μM throughout. (○) Pyruvate kinase, (△) pyruvate kinase + 10 mM Phe, (●) pyruvate kinase + 10 mM Phe + 10 mM Ala. The buffer was 50 mM potassium ion-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (75 mM KCl added) at pH 7.5, and the temperature at 22°. Measurements were made at 24.3 MHz.

Proton Relaxation Rate (PRR) Measurements. Measurements of the PRR of water were performed in an attempt to establish whether a change in the environment of the Mn^{2+} bound to pyruvate kinase occurred upon addition of Phe. Figure 6 presents the data in the form of the observed enhancement parameter (ϵ_{obsd} as defined by Mildvan and Cohn, 1965), as a function of enzyme concentration in the presence of the various effectors. Clearly, at low concentrations of enzyme, Phe causes an apparent deenhancement of the PRR and this effect is reversed by the addition of Ala. These effects disappear at higher enzyme concentrations (Figure 6). Since the amount of bound Mn^{2+} could be calculated from the epr data, it was possible to calculate the enhancement parameter characteristic of the bound Mn^{2+} in the various complexes. These results are included in Table II and show little if any change in this parameter upon addition of the various effectors.

High-Resolution Nuclear Magnetic Resonance Measurements. Preliminary experiments were performed to see if any paramagnetic effects of Mn^{2+} bound to the enzyme were observed on either the aromatic protons of Phe or the methyl and α-carbon protons of Ala in the ternary complexes. The addition of Mn^{2+} (20–80 μM) to solutions of pyruvate kinase (1 mM subunits) with 30 mM Phe or Ala resulted in only small decreases in the T_1 values of these protons, whereas in the absence of enzyme substantially larger effects were observed. Unequivocal interpretation of these data is not possible without a detailed study of the temperature and frequency dependencies of these effects. However, assuming a correlation time, τ_c, for Mn^{2+} bound to pyruvate kinase of *ca.* 5×10^{-9} sec (Reuben and Cohn, 1970) and rapid exchange of bound and free Phe, a lower limit for the distance between the Mn^{2+} and the various protons could be set at approximately 12 Å using the equation of Solomon and Bloembergen (1956).

Discussion

Allosteric interactions are presumed to be mediated *via* ligand-induced protein structural changes. Recently, Car-

minatti *et al.* (1971) have observed these types of interactions in the inhibition of muscle pyruvate kinase by Phe. This was the first report of allosteric behavior in this multisubunit enzyme. These authors have listed the criteria by which the Phe site can be considered as distinct from the active site. Our nmr measurements, although only preliminary, suggest that the Phe site is some distance away from the Mn^{2+} (and therefore, probably the active) site.

Optical properties of pyruvate kinase (chiefly ultraviolet absorption and fluorescence) have been used to indicate that the conformation of the enzyme is influenced by various factors, including activating cations and substrates (Suelter, 1967; Kayne and Suelter, 1968; Price, 1972). In this paper, we have shown that these optical properties can be used to detect and monitor the changes in protein structure brought about by binding Phe. It is clear that the changes in fluorescence and absorption are in the opposite direction to those observed on addition of activating cations and substrates (Suelter, 1967; Kayne and Suelter, 1968; Price, 1972). By analogy with the previous observations, our results suggest that certain tryptophan residues of the enzyme are transferred to a less polar environment on binding Phe.

These optical studies provide a powerful tool to study conformational changes and subunit interactions over a wider range of conditions than is possible in kinetic studies. Our results, summarized in Figures 1 and 2 and Table I, demonstrate that the strength of subunit interactions in pyruvate kinase as well as the affinity of the enzyme for Phe are markedly affected by the presence of the divalent metal ions and substrate. The subunit interactions are strengthened (as reflected in the higher value of n in the Hill equation) in the presence of divalent activators (Mg^{2+} or Mn^{2+}). At low concentrations, the greater effect of Mn^{2+} is due to its stronger affinity for the divalent metal ion site (Suelter *et al.*, 1966). At saturating levels, the effects of the two cations are very similar. P-enolpyruvate in the absence of divalent metal ions does not appear to change the subunit interactions greatly, but shifts the titration curve to higher Phe concentrations. In the presence of both substrate and divalent metal activator, subunit interactions as well as affinities for Phe are markedly changed (Table I). These values are in reasonable agreement with the inhibition studies shown in Figure 4. Since the kinetic studies are carried out in the presence of ADP and NADH as well as the coupling enzyme, the slight differences observed are not surprising. Both the fluorescence and kinetic studies point to significant differences between the complexes pyruvate kinase–Mn^{2+}–P-enolpyruvate and pyruvate kinase–Mg^{2+}–P-enolpyruvate. With the former complex, the effects of Phe are very small and suggest either that the conformational equilibrium in this case is driven further away from the form which binds Phe preferentially or that Phe binds, but the conformational change giving rise to the fluorescence increase and kinetic inhibition does not occur. In view of this, one might exercise caution in extrapolating some observations in systems where the normal activator, Mg^{2+}, is replaced by Mn^{2+}.

Carminatti *et al.* (1971) showed that the Phe inhibition could be relieved by Ala. The observation that the changes in the optical properties of pyruvate kinase induced by Phe can be fully reversed by subsequent addition of Ala (in the presence of divalent metal ion and/or substrate) implies that the proposed structural changes are related to the kinetic observations. At present, the significance of the effect of Ala on the enzyme's optical properties in the absence of divalent metal ion and substrate is unclear. Since this effect is not observed in the presence of divalent cation or substrate, it probably has little kinetic significance and may be related to differences in the stabilization of the various conformations of the enzyme by Phe and Ala.

The above studies have demonstrated the effects of the divalent metal ion on the interaction of the enzyme with Phe. The reciprocal heterotropic effects have been studied by magnetic resonance methods (Table II). Phenylalanine weakens the binding of Mn^{2+} to the enzyme by a factor of 3.5, an effect at least partially reversed by Ala. Complete reversal would not be expected at this concentration of Ala, since the fluorescence titration data (Table I) show that Ala has only a relatively low affnity for the enzyme in the presence of 100 μM Mn^{2+}.

The enhancement of the PRR of water by the bound Mn^{2+} is not significantly changed by addition of Phe and/or Ala to the enzyme. This suggests that no major changes occur in the environment of the bound Mn^{2+}, which would cause either a displacement of water molecules from the hydration sphere of the metal ion or a change in the dominant correlation time of the Mn^{2+}. The change in the dissociation constant in the presence of Phe corresponds to a lowering of the free energy of interaction of Mn^{2+} and pyruvate kinase by only *ca.* 10% of the total (6 kcal/mole). It is possible that more subtle changes in the environment of the bound Mn^{2+} than can be observed by the PRR method could be detected by epr studies such as those of Reed and Cohn (1972a,b).

It should be pointed out that the studies reported in this paper were carried out in the presence of *ca.* 100 mM K^+. Preliminary studies carried out in the presence of $(CH_3)_4N^+$, a nonactivating cation, have shown Phe and Ala to have qualitatively similar effects on the fluorescence of the enzyme.

While the physiological significance of the interactions between these amino acid effectors and muscle pyruvate kinase is not clear, these studies do show that the interactions critically depend on the concentrations of divalent metal ion and substrate. A number of reports have implicated these amino acids as well as other effectors (*e.g.*, fructose 1,6-diphosphate) in the regulation of the pyruvate kinase reaction in other tissues (Carminatti *et al.*, 1971). It is hoped that the present study will provide a reasonable basis for the further investigation of the action of effectors in the regulation of the enzyme from the various sources.

Acknowledgment

The authors thank Karin Norlen for help with the nmr measurements.

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