Thermodynamic Linkages in Rabbit Muscle Pyruvate Kinase: Kinetic, Equilibrium, and Structural Studies†

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ABSTRACT: The mechanism of allosteric regulation of rabbit muscle pyruvate kinase (PK) was examined in the presence of the allosteric inhibitor phenylalanine (Phe). Steady-state kinetic, equilibrium binding, and structural studies were conducted to provide a broad data base to establish a reasonable model for the interactions. Phe was shown to induce apparent cooperativity in the steady-state kinetic measurements at pH 7.5 and 23 °C. The apparent K_m for phosphoenolpyruvate was shown to increase with increasing Phe concentrations. These results imply that Phe reduces the affinity of PK for phosphoenolpyruvate. This conclusion was substantiated by equilibrium binding studies which yielded association constants of phosphoenolpyruvate as a function of Phe concentration. The binding constant of Phe was also determined at pH 7.0 and 23 °C. The effect of ligands on the hydrodynamic properties of PK was monitored by difference sedimentation velocity, sedimentation velocity, and equilibrium experiments. The results showed that PK remains tetrameric both in the presence and in the absence of Phe. However, Phe induces a small decrease in the sedimentation coefficient of the enzyme; hence, it suggests a loosening of the protein structure. The accessibility of the sulfhydryl residues of the enzyme also increases in the presence of Phe. Furthermore, the Phe-induced conformational change was approximately 90% complete when only 25% of the binding sites were saturated. This result suggested that the regulatory behavior of PK might satisfactorily be described by the two-state model of Monod–Wyman–Changeux [Monod, J., Wyman, J., & Changeux, J.-P. (1965) *J. Mol. Biol. 12*, 88–118].

Mammalian pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) (PK)[1] exists in multiple isozymic forms of M_1, M_2, L, and R (Hall & Cottam, 1978; Ibsen, 1977). They differ in their regulatory properties and have different chemical and immunological properties (Hall & Cottam, 1978). All isozymes, with the exception of M_1, exhibit allosteric properties. The rabbit muscle PK system, which is classified as an M_1 isozyme, appears to be the least regulated species of the PK isozymes; e.g., it is weakly activated by fructose 1,6-diphosphate only under certain experimental conditions (Phillips & Ainsworth, 1977). The system, however, can be perturbed by Phe, an allosteric inhibitor, as reported by Carminatti and co-workers (Carminatti et al., 1971). The binding of Phe apparently perturbs the protein structure as indicated by an enhancement of the fluorescent intensity of a protein fluorophore (Kayne & Price, 1972). Results from ultraviolet difference spectra measurements are consistent with fluorescence studies which indicate a conformational change in the enzyme upon interacting with Phe (Kayne & Price, 1972; Kwan & Davis, 1980). There is consensus in the literature, therefore, that Phe acts as an allosteric inhibitor of muscle PK and the enzyme can exist in multiple conformations. These results, however, do not yield information on the actual linkage between conformational changes and enzyme activity. At present, the role of subunit interactions in the regulation of the enzyme has not been quantitatively defined.

The PK system becomes an attractive model when one attempts to elucidate the role of quarternary structure in allosteric regulation of enzymes. The effects of regulators on the subunit–subunit interactions can be quantitated by physical, chemical, and kinetic approaches. An evaluation of the results from these studies may reveal the molecular mechanism of allosteric regulation in PK. Furthermore, corresponding studies with other PK isozymes should provide information on the possible role of specific amino acid residues in the regulatory mechanism. Accordingly, a study was initiated to quantitatively define the kinetic and structural properties of rabbit muscle PK as a function of Phe concentration at pH 7.5. The binding affinities of phosphoenolpyruvate and Phe for PK were determined individually and in pairs under experimental conditions which were essentially the same as those employed in the kinetic studies. Results in all these experimental measurements are consistent with a "two-state" model. A preliminary report of this work has been presented earlier (Oberfelder, 1980; Oberfelder et al., 1981).

Experimental Procedures

Materials

Pyruvate kinase from rabbit muscle in 3.2 M $(NH_4)_2SO_4$ suspension was purchased from Boehringer Mannheim. SDS–polyacrylamide gel electrophoresis of 50 μg of protein demonstrated that the protein preparation was greater than 98% pure. [^{14}C]PEP, the tricyclohexylammonium salt, and L-[4-^{3}H]phenylalanine were purchased from Amersham and had specific radioactivities of 12.4 mCi/mmol and 27 Ci/mmol, respectively. The disodium salt of ADP, the tricyclohexylammonium salt of PEP, Tris base, Tris-HCl, reduced nicotinamide adenine dinucleotide (NADH), L-alanine (Ala), phenylalanine (Phe), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), tetramethylammonium chloride (TMA^+Cl^-), lactate dehydrogenase type II, and glass beads of 1-mm diameter were all obtained from Sigma Biochemical. KCl, $MgSO_4$, and Scintiverse were obtained from Fisher, and acrylamide was

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[1] Abbreviations: PK, pyruvate kinase; Tris, tris(hydroxymethyl)-aminomethane; TKM buffer, 50 mM Tris, 72 mM KCl, and 7.2 mM $MgSO_4$ at pH 7.5; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TMA^+Cl^-, tetramethylammonium chloride; PEP, phosphoenolpyruvate; SDS, sodium dodecyl sulfate.

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purchased from Bethesda Research Laboratory. Sephadex G-25 fine and Sephadex G-50 coarse were purchased from Pharmacia, and the plastic eight-well microdialysis apparatus was purchased from Hoeffer Scientific.

Methods

Enzyme Kinetics. The activity of rabbit muscle PK was assayed by the coupled enzyme method of Buchler & Pfleiderer (1955). Results from control experiments assured that the coupling enzyme, lactate dehydrogenase, was not affected by Phe. The assay mix consisted of 72 mM KCl, 7.2 mM $MgSO_4$, 2.0 mM ADP, 0.3 mM NADH, 10 μg/mL lactate dehydrogenase, 0.05 M Tris base, and variable amounts of PEP and Phe as indicated. The entire assay mix was adjusted to pH 7.5 before the addition of PK to initiate the reaction. Both PK and lactate dehydrogenase were desalted over separate columns of Sephadex G-25 fine, equilibrated with 72 mM KCl, 7.2 mM $MgSO_4$, and 0.05 M Tris base at pH 7.5 (TKM buffer), before being added to the assay mixture. The change in absorbance at 340 nm as a function of time was monitored on a Gilford 250 spectrophotometer equipped with a multiple sample changer and a Gilford 6051 recorder. The temperature of the assay mixture was controlled by using either a waterbath and water-jacketed cuvettes or the Thermoset electronic thermocontroller from Gilford.

Ligand Binding. PEP binding was monitored by the method of Hirose & Kano (1971) at pH 7.5. Sephadex G-50 coarse was washed, first with deionized, distilled water and then with 100% ethanol, followed by drying of the gel at 80 °C for 12–16 h prior to the performance of the experiment in TKM buffer. A 0.35-mL aliquot of the buffer was added to 50 mg of the dried gel and allowed to equilibrate for 4 h, although control experiments have established that equilibrium is attained after 1 h. PEP and PK were then added, in a total volume of 0.2 mL, to the slurry and gently mixed by vortexing for 10 min. Two 100-μL aliquots of the solution, free of gel particles, were withdrawn with Hamilton syringes. One aliquot was used for the determination of PEP concentration by quantitation of radioactivity while the other was used for the spectrophotometric determination of protein concentration by measuring the absorbance at 280 nm. An absorptivity value of 0.54 mL/(mg·cm) (Boyer, 1962) was employed. The absorbance of PK at 280 nm was not affected by the presence of PEP. The final protein concentration was approximately 6 mg/mL. Samples for PEP concentration determination were mixed with 0.4 mL of deionized, distilled water and 4.0 mL of Scintiverse (Fisher), and each sample was counted for 5 min in a Packard 3220 liquid scintillation counter. A quench curve was generated by using a set of ^{14}C quenched standards, and the channel ratio method was used to make the necessary efficiency corrections.

The method of Hirose & Kano (1971) is based upon the principle that the protein is completely excluded from the interior of the resin, while the ligand will be distributed throughout the solution. In the presence of a protein which binds the ligand, the establishment of chemical equilibrium will result in an apparent preferential exclusion of the ligand from the interior of the resin. Having determined the concentrations of PEP and PK before and after equilibrium is reached, we can combine these parameters to obtain the amount of bound and free PEP since

$$\alpha = (P/V_o)/(P/V) \tag{1}$$

where α is the ratio of protein concentrations, P is the total amount of protein added to the solution, V is the total volume of solution added to the dry gel, and V_o is the volume of solution outside the resin. P/V is the concentration of the protein added, assuming that it is equally distributed throughout the solution, while P/V_o is the observed concentration of the protein. Similarly

$$\beta = (L_o/V_o)/(L/V) \tag{2}$$

where β is the ratio of ligand concentrations and L and L_o are the total amount of ligand added and the amount of ligand outside the gel, respectively. L/V is the concentration of ligand, assuming it is evenly distributed in the total volume added, while L_o/V_o is the concentration of ligand observed in the gel-free solution. While β is the value of the ratio in the presence of protein, β' is the value in the absence of protein. The amount of bound ligand is

$$L_o(\text{bound}) = L\frac{\beta - \beta'}{\alpha - \beta'} \tag{3}$$

The concentration of free ligand outside the gel is

$$L_o(\text{free}) = \frac{L}{V}\frac{\beta'(\alpha - \beta)}{\alpha - \beta'} \tag{4}$$

The amount of ligand bound per mole of PK tetramer is

$$\bar{Y} = L_o(\text{bound})/P \tag{5}$$

Equilibrium dialysis experiments were carried out to measure the binding of [^{3}H]Phe. The protein concentration used was 12 mg/mL. Solutions containing the ligand and protein were initially placed on opposite sides of the dialysis membrane. Glass beads were placed in each chamber to aid equilibration, and the cells were rotated for 20 h. The time required to reach equilibrium was established by monitoring the concentration of radioactive ligand in the chamber with the protein solution as a function of time. It was observed that equilibrium was established by the 12th h, so the incubation period of 20 h employed was sufficient to assure that the system was at equilibrium. Within an experimental uncertainty of ±5% in the enzyme activity measurement, PK was stable at room temperature over a 36-h period under the present experimental conditions. Hence, protein denaturation should not influence the results obtained.

The concentrations of free, C_F, and bound, C_B, ligands can be calculated since

$$C_F = \frac{C_1 D_F}{D_P + D_F} \tag{6a}$$

$$C_B = C_1\frac{D_P - D_F}{D_P + D_F} \tag{6b}$$

where C_1 is the initial concentration of the ligand and D_P and D_F are the disintegrations per minute (dpm) in the presence and absence of protein, respectively.

Sedimentation Equilibrium. Molecular weights were determined by the high-speed sedimentation equilibrium methods of Yphantis (1964). The experiments were performed in a Spinco Model E analytical ultracentrifuge equipped with electronic speed control, RTIC temperature control, and both an interference optical system and a UV scanner. The experiments were carried out at 14 000 rpm for 24 h at 23 °C. For all experiments, 120 μL of solution was employed using Kel-F coated aluminum double-sector centerpieces with sapphire windows. Interference patterns were recorded on Kodak spectroscopic II G plates and analyzed on a Nikon 6C microcomparator.

Interference patterns or UV scanner tracings obtained at 4-h intervals indicated that equilibrium was achieved after 20 h at speed since the patterns of later time intervals were

identical. Both the number and the weight-average molecular weights at each radial position were obtained. The partial specific volume, $\bar{\nu}$, of PK was calculated by the method of Cohn & Edsall (1943). A value of 0.739 mL/g was obtained on the basis of the amino acid composition reported by Anderson & Randall (1975).

Difference Sedimentation Velocity. The change in hydrodynamic properties of PK induced by ligands was monitored by difference sedimentation velocity according to the procedure of Gerhart & Schachman (1968). All of the experiments were conducted at 60000 rpm, pH 7.5 and 4 °C. Schlieren patterns were recorded as a function of time on Kodak metallographic plates and analyzed on a Nikon 6C microcomparator. Kel-F-coated aluminum centerpieces with sapphire windows were employed for all experiments. For each experiment, two double-sector cells were placed in an AN-D rotor. One cell contained PK in the presence of the ligand being tested, while the other cell contained PK in the presence of tetramethylammonium chloride, the concentration of which was adjusted to an ionic strength equivalent to that of the test ligand. Simultaneous viewing of both cells was achieved by utilizing a 2°-positive wedge window.

The difference in sedimentation coefficients, ΔS, can be computed with the following equation (Howlett & Schachman, 1977):

$$\frac{1}{\omega^2}\frac{d(\Delta r/\bar{r})}{dt} = \Delta S \tag{7}$$

where ω is the angular velocity, $\Delta r = r_2 - r_1$, $\bar{r} = (r_1 + r_2)/2$, and t is time. r_2 and r_1 are the radial positions of the schlieren peaks of the sample and reference solution, respectively. The reported sedimentation coefficients were corrected to the standard conditions of 20 °C in water. The densities of the buffer- and ligand-containing solutions were measured with a Paar-Mettler precision density meter (DMA-02D). The viscosities of these solutions were measured with a Cannon capillary viscometer with a flow time of 137 s for water at 20 °C. Temperature was controlled by a Cannon constant-temperature bath.

Sulfhydryl Titration. The effect of ligands upon the accessibility of sulfhydryl residues in PK was determined by monitoring the rate of their reaction with 5,5′dithiobis(2-nitrobenzoic acid) (DTNB) at 23 °C. Each titration was performed with two tandem cells containing identical solutions. The reference cell remained unmixed while the sample cell was mixed at time zero by inversion. In each cell, 1.0 mL of 4 mM DTNB in TKM buffer at pH 7.5 was added to one compartment, and the other contained 1.0 mL of 0.35 mg/mL PK. Both solutions contained ligand at the concentration indicated by the individual experiment.

The progress of the reaction was followed spectrophotometrically at 412 nm on a Cary 118 spectrophotometer as a function of time. The extent of the reaction can be calculated by using the extinction coefficient of 13600 M^{-1} cm^{-1} for 2-nitro-5-thiobenzoate (Habeeb, 1972), and the data were analyzed in terms of a pseudo-first-order reaction according to the equation

$$\ln\frac{A_\infty - A_t}{A_\infty - A_0} = -kt \tag{8}$$

where A_0 and A_∞ represent the initial and final absorbances, respectively, and A_t represents the absorbance at time t. The semilogarithmic plots of the data can be analyzed for distinguishable classes of sulfhydryl groups by extrapolating the linear portion of the plot of $t = 0$. The slope and intercept of the line yield rate constants and the initial concentration of the slow reactive class, respectively (Frost & Pearson, 1961).

Fluorescence. The effects of ligands on the exposure of tryptophan were monitored by measuring the quenching of the intrinsic fluorescence of tryptophan by acrylamide (Eftink & Ghiron, 1976). The excitation wavelength was 292 nm, and the emission was monitored at 328 nm on a Perkin-Elmer 512 double-beam spectrofluorometer by using 250-μL samples in a microcuvette. The sample solutions included PK at a final concentration of 200 μg/mL, along with appropriate amounts of acrylamide and ligand, all of which were in TKM buffer at pH 7.5.

A constant for quenching can be obtained by using the Stern–Volmer equation (Stern & Volmer, 1919):

$$F_0/F = 1 + K_Q[Q] \tag{9}$$

where F_0 and F are the fluorescence intensities of the fluorophore in the absence and presence of acrylamide, respectively. [Q] is the concentration of acrylamide, and K_Q is the collisional quenching constant. Since PK contains more than one tryptophan residue, each of which probably fluoresces independently, eq 9 can be written as (Bandyopadhyay & Wu, 1979)

$$F_0/F = \sum\frac{1 + K_{Qi}[Q]}{f^i} \tag{10}$$

where f^i is the fraction of the total fluorescence corresponding to the ith tryptophan residue and K_{Qi} is the respective collisional quenching constant. The initial slope of the plot of F_0/F vs. [Q] is approximately equal to the weighted average of the individual quenching constants.

Results

The activity of PK was examined as a function of PEP concentration at several different Phe concentrations. In the absence of Phe, the curve is hyperbolic, while in the presence of Phe the enzyme activity at low PEP concentrations is low and is followed by a rapid increase in activity over a short range of PEP concentrations. Increasing Phe concentration results in successively more sigmoidal curves. Such a sigmoidal relationship is typical for an interaction displaying positive cooperativity. Since Phe and PEP are structurally dissimilar, these ligands most likely bind to separate sites on PK. The data, therefore, suggest that the effect of Phe is allosteric.

The order to facilitate quantitative analysis, the kinetic data were further analyzed in the form of both Eadie (1942) and Hill plots (1910). The Eadie plot was employed to estimate the maximal velocity, V_{max}, and a plot of a representative set of data is shown in Figure 1. Examination of all of the data indicates that the extrapolated value for V_{max} in the presence of Phe did not deviate significantly from the value obtained in the absence of Phe. The V_{max} obtained in this manner was used to determine k_{cat} since $k_{cat} = V_{max}/E_t$, where E_t is the total concentration of PK. Plotting the data in the form of an Eadie plot also provides a sensitive visual method for detecting cooperativity in the PEP–PK–Phe interactions (Hensley et al., 1981). The Eadie plots display a concave-downward trend in the presence of Phe, and the curvature becomes more pronounced as the Phe concentration is increased. The cooperativity observed in the kinetics was further quantitated by the Hill equation (Hill, 1910):

$$\log\frac{V}{V_{max} - V} = h \log [S] - \log K \tag{11}$$

where h is the Hill coefficient, [S] is the concentration of free substrate, and K is a constant.

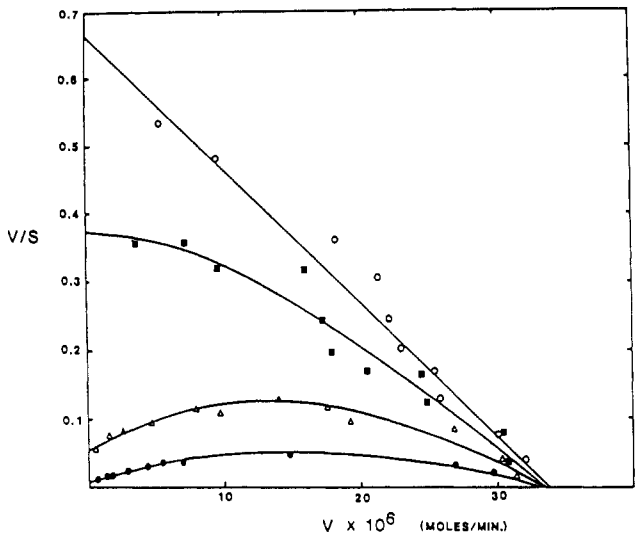


FIGURE 1: Eadie plot as a function of the Phe concentration at pH 7.5 and 23 °C. Phe concentrations in millimolar are (○) 0; (■) 1, (△) 3, and (●) 5. The curves shown have been simulated by utilizing the V_{max} determined from the Eadie plots and the apparent K_m and the Hill coefficient given in Table I.

Table I: Summary of Kinetic Properties of PK at pH 7.5 and 23 °C

[Phe] (mM)	$K_{m,app}$ ($\times 10^5$ M)	h	k_{cat} [mol of product s^{-1} (mol of PK)$^{-1}$]
0	7 ± 3	1.0 ± 0.1	1000 ± 90
1	8 ± 1	1.1 ± 0.1	900 ± 200
3	15 ± 5	1.4 ± 0.2	900 ± 100
5	26 ± 1	1.7 ± 0.3	1000 ± 80
7	33 ± 1	1.9 ± 0.2	900 ± 100
9	43 ± 4	1.9 ± 0.2	900 ± 100
12	57 ± 9	2.0 ± 0.1	1000 ± 70
0[a]	8 ± 1	1.0 ± 0.1	
12[a]	52 ± 6	1.8 ± 0.2	

[a] Measurements were made with 50 μg/mL PK and deoxycytosine diphosphate as a substitute for ADP.

Results of such an analysis are summarized in Table I. The value of $K_{m,app}$ is dependent upon the Phe concentration, and it increases from (8 ± 3) × 10^{-5} to (57 ± 9) × 10^{-5} M as the Phe concentration increased from 0 to 12 mM. Increasing the Phe concentration also results in an increase in the degree of cooperativity as indicated by an increase in the Hill coefficient (h). The Phe-induced effects can be reversed by Ala. Kinetic data indicated that 5 mM Ala can overcome the inhibitory effect of 12 mM Phe.

In order to compare the kinetic and structural studies, it is desirable that these experiments be conducted at comparable protein concentrations. Kinetic assay conditions were devised so that the protein concentrations employed would overlap with those used in the structural studies. It was not practical to increase the protein concentration to 50 μg/mL by using the typical components of the assay mix. The turnover number of PK is too high; thus, the rate of NADH conversion to NAD would be too rapid to be measured. Substitution of the analogue dCDP for ADP produced a 500-fold decrease in the catalytic rate (Plowman & Krall, 1965); therefore, saturating concentrations of dCDP were used in place of ADP. Experiments were carried out as a function of PEP concentration at 50 μg/mL PK in the presence and in the absence of 12 mM Phe, and the results are shown in Figure 2. In the absence of Phe, a hyperbolic curve is observed, and the K_m for PEP is 8 × 10^{-5} M. In the presence of 12 mM Phe, a sigmoidal curve is observed, and the apparent K_m is 5.2 × 10^{-4} M. These values for K_m were equivalent to those shown in Table I for data obtained by utilizing ADP at low protein concentrations.

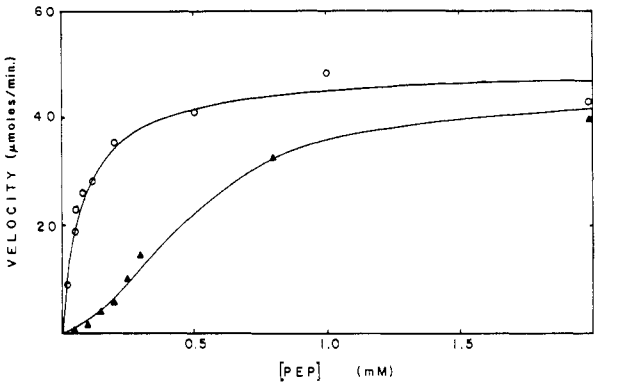


FIGURE 2: Kinetic properties of PK at a high protein concentration. The assay was performed at 23 °C and pH 7.5 by utilizing saturating deoxycytosine diphosphate as a substitute for ADP. The curves shown are simulations determined by using values for V_{max}, K_m, and the Hill coefficient given in Table I. The experimental conditions are (○) 0 and (▲) 12 mM Phe.

Similarity of the kinetic parameters obtained at 0.2 and 50 μg/mL indicates that the effect of Phe on PK must not be dependent upon protein concentration in this concentration range.

In order to provide additional insight into the effects of Phe upon PK, the affinity of PEP for the protein was monitored both in the presence and in the absence of Phe, the inhibitor. Since PEP bound extensively to the dialysis membrane, equilibrium dialysis was rendered unreliable as a technique to measure PEP binding to PK. Consequently, the batch gel partition procedure of Hirose & Kano (1971) was adopted. Results from control experiments show that the time required for complete rehydration of the resin is less than 1 h; hence, an incubation period of 4 h was deemed sufficient to assure hydration of the resin (Oberfelder, 1982). The time required for the binding reaction to reach equilibrium was also tested. Within experimental uncertainties, there were no significant differences in the results obtained from samples with incubation periods ranging from 10 min to 3 h, so equilibrium must have been achieved within 10 min. Results of the binding experiments are shown as a Scatchard plot (Scatchard, 1949) in Figure 3. $\bar{Y}$ is the amount of ligand bound per mole of PK tetramer. It can be concluded that a maximum of 4.4 ± 0.4 mol of PEP was bound to each mol of PK tetramer with an apparent dissociation constant of (7 ± 1) × 10^{-5} M. Further analysis of the data by a Hill plot did not indicate cooperativity. The binding of PEP to PK was also studied at pH 7.5 in the presence of 3.0 mM Phe. A maximum of 4.1 ± 0.4 mol of PEP was bound per mol of PK tetramer, and an apparent dissociation constant of (1.4 ± 0.2) × 10^{-4} M was obtained. This value is in good agreement with that of the $K_{m,app}$ (Table I) determined under identical conditions. The Hill coefficient for PEP binding was 1.1, indicating that the binding was not significantly cooperative under these experimental conditions. Although 3 mM Phe apparently did not significantly induce cooperativity in the binding of PEP, it reduced the apparent affinity of PEP for the enzyme.

Since Phe has an inhibitory effect upon the kinetics of PK and it also reduces the apparent affinity of PEP for PK, it seemed worthwhile to determine the affinity of Phe itself for PK. Kayne & Price (1973) published a binding isotherm at pH 7.5, but these experiments were performed in the presence of 2.5 mM Mg^{2+}. Since the binding of Phe has been reported to be dependent on metal ions and the concentration of Mg^{2+} employed in this study was 7.2 mM, the resuls reported by Kayne & Price (1973) might not be applicable to this study.

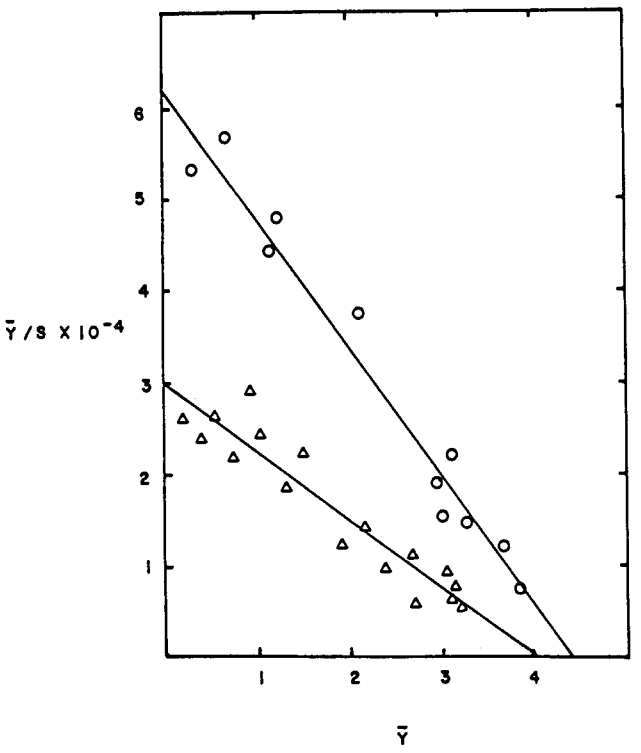


FIGURE 3: Scatchard plots of the PEP binding data in TKM buffer at pH 7.5 and 23 °C in (○) the absence of Phe and (△) the presence of 3.0 mM Phe.

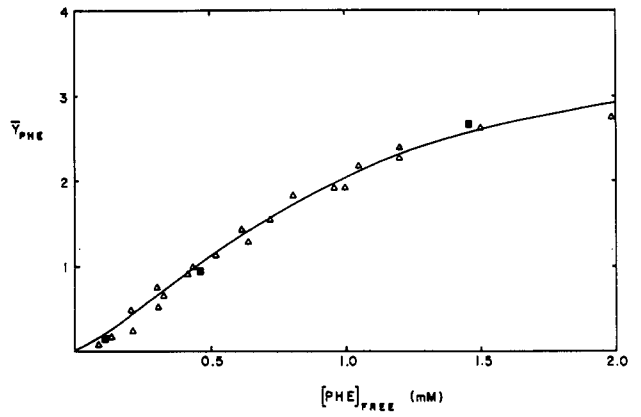


FIGURE 4: Binding of Phe to PK at pH 7.5. Data are from (△) Kayne & Price (1973) and (■) this study.

Several data points were obtained by using equilibrium dialysis in order to assure that the differing Mg^{2+} concentrations did not affect the affinity of Phe. Figure 4 shows the data reported by Kayne and Price along with the additional results obtained by equilibrium dialysis in this study. It appears that both data sets agree within experimental error. The binding isotherm is sigmoidal, suggesting that there are two or more forms of the protein which differ in their affinities for Phe. Since the protein preparation was homogeneous, this implies that Phe induces some sort of state change in the protein. The apparent dissociation constant and Hill coefficient are 1.0×10^{-3} M and 1.3, respectively.

Both the kinetic and ligand binding data indicate that muscle PK exhibits allosteric properties in the presence of Phe; however, the physical identities of the different states of the enzyme implied by these data remained to be determined. These states might be oligomeric forms of PK differing from one another in their degrees of polymerization. This possibility was tested by monitoring the molecular weight of PK in the presence and absence of Phe by sedimentation equilibrium.

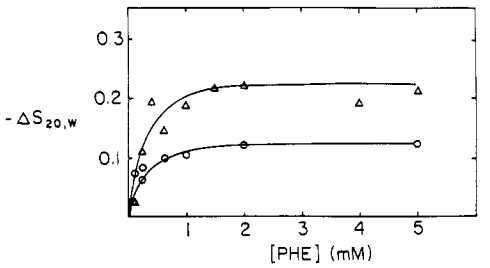


FIGURE 5: Effects of Phe concentration on $\Delta s_{20,w}$. The experiments were performed at 4 °C in 0.05 M Tris at 60000 rpm (△) in the absence of KCl and $MgSO_4$ and (○) in the presence of 72 mM KCl and 7.2 mM $MgSO_4$.

Within the concentration range of 50–750 μg/mL, the value for the weight-average molecular weight is the same as that of the number-average molecular weight, suggesting that the protein in solution was homogeneous. There is little discernible difference between the data sets obtained in the presence or absence of Phe. The average molecular weight of PK is shown to be 220000 ± 5000. These results imply that native PK does not undergo association–dissociation under the conditions tested. This conclusion is further substantiated by sedimentation velocity data at protein concentrations as low as 15 μg/mL. The sedimentation coefficient did not show any evidence of a dependence on protein concentration.

The molecular weight of the PK subunit was measured by SDS–polyacrylamide gel electrophoresis and molecular sieve chromatography in 6 M guanidine hydrochloride. An average value of 57000 ± 3000 was obtained; thus, the native protein of 220000 molecular weight must be a tetramer.

Since the tetrameric PK does not undergo association–dissociation, the different states in the allosteric model are likely to be manifestations of secondary–tertiary structural changes in the enzyme. Evidence for such a change was sought through fluorescence, chemical modification, and difference sedimentation velocity experiments. These methods were chosen to monitor different levels of structural changes which might be ligand dependent; e.g., the sedimentation experiments would yield information on the global structural changes while the other experiments might indicate more localized structural changes.

Difference sedimentation experiments were performed as a function of the Phe concentration. In these experiments, the sample contained PK with Phe in 50 mM Tris at pH 7.5, while the reference contained PK and an appropriate amount of TMA^+Cl^- to match the ionic strength. These experiments were conducted to monitor the effect of Phe on PK in the absence of any activators. Results of these experiments are shown in Figure 5. A decrease in $s_{20,w}$ is observed, and the value of $\Delta s_{20,w}$ approaches a maximum of −0.24 S at infinite concentrations of Phe. The effect of activating cations on the Phe-induced structural change was also monitored. In these experiments, the buffer was TKM, and the results are shown in Figure 5. The sedimentation rates of all of the Phe-containing samples were slower than those of the reference solutions. The change in $s_{20,w}$ is dependent on Phe concentration and approaches a maximum value of −0.13 S at infinite concentrations of Phe.

The effects of a variety of other ligands on the hydrodynamic properties of PK were also tested, and the results are summarized in Table II. PEP apparently produces an increase in the sedimentation rate while the other substrate, ADP, did not appear to induce any significant changes. $MgSO_4$ and KCl individually produce small increases in the sedimentation rate, but together they induce a more substantial increase. Although

Table II: Effects of Ligands upon $\Delta s_{20,w}$[a]

ligand	$\Delta s_{20,w}$ (S)
1 mM PEP	+0.11 ± 0.04
2 mM ADP	−0.02 ± 0.02
7.2 mM $MgSO_4$	+0.05 ± 0.04
72 mM KCl	+0.02 ± 0.01
7.2 mM $MgSO_4$ + 72 mM KCl	+0.12 ± 0.06
12 mM Ala	−0.06 ± 0.04
12 mM Ala + 12 mM Phe	−0.07 ± 0.04
100 mM TMA^+Cl^-	+0.10 ± 0.01
saturating Phe	−0.24 ± 0.02[c]
saturating Phe + 7.2 mM $MgSO_4$ + 72 mM KCl[b]	−0.13 ± 0.02[c]

[a] The standard reference condition was 50 mM Tris buffer at pH 7.5 with varying amounts of TMA^+Cl^- to adjust the ionic strength of the reference solution to match that of the test solution. [b] The reference solution contained 7.2 mM $MgSO_4$ and 72 mM KCl. [c] These are maximum values for $\Delta s_{20,w}$ extrapolated to infinite Phe concentration.

Ala itself does not induce any significant change in the hydrodynamic properties of PK, it does reverse the structural change in PK induce by Phe, as shown in Table II. When PK alone was used as a reference, 100 mM TMA^+Cl^- produced an increase in the sedimentation rate after correction for changes in η and ρ. The result with TMA^+Cl^- suggests that there may be a nonspecific ionic strength effect at relatively high ionic strengths which results in an increase in the sedimentation rate.

Sulfhydryl titrations of PK were performed in the presence of various ligands in an effort to confirm the effects observed in the sedimentation experiments. Both the rate of titration and the number of available sulfhydryl groups were determined. All of the assay solutions contained KCl and $MgSO_4$. In the absence of ligands, an initial rapid reaction rate was followed by a slower rate which in turn was apparently followed by another faster rate. Such a triphasic reaction profile was reproducible and was observed in essentially all of the experimental conditions tested. One millimolar PEP does not affect the rate of the initial portion of the reaction. It does, however, significantly decrease the rate of the slower reactions, suggesting that PEP is making the sulfhydryl groups less accessible to chemical modification through a mechanism not yet defined. Twelve millimolar Phe exerts a pronounced effect on these sulfhydryl residues. These residues are apparently very accessible to chemical modifications in the presence of Phe. Ala does not enhance the reactivity, but at 12 mM it can partially reverse the effect of 12 mM Phe on the slow-reacting sulfhydryl residues. The initial, very rapid phase represents a titration of 4–6 residues per tetrameric PK while completion of the reaction results in a titration of a total of 36 ± 6 residues. According to Cottam et al. (1969), there are 36 sulfhydryl groups per PK tetramer; thus, all of the free sulfhydryl groups present in the protein are accessible to chemical modification under the experimental conditions. These data were further analyzed according to eq 8 to assess the effect of ligands on the apparent pseudo-first-order rate constants. Results of such an analysis are shown in Figure 6. Due to the complex nature of these curves, it is difficult to obtain meaningful quantities for the initial phase of these reactions. Examination of the later phase of these curves provides an indication of the effects of ligands on the accessibility of sulfhydryl residues in PK. The values for the apparent pseudo-first-order rate constants are summarized in Table III. It is evident that Phe, even at low concentrations, increased the rate of titration and the rate increased progressively as the concentration of the inhibitor was increased. The rate observed in the presence of 12 mM Phe is approximately 10-fold faster than that observed for PK alone. A plot

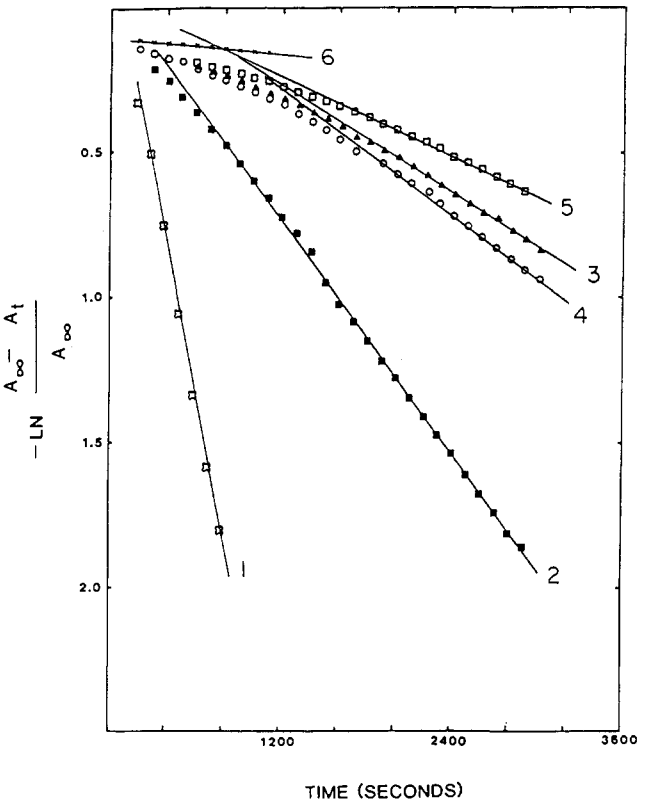


FIGURE 6: Reaction of PK sulfhydryl groups with DTNB in the presence of various effectors. The experiments were performed by utilizing initial concentrations of 4.0 mM DTNB and 0.35 mg/mL PK in pH 7.5 TKM buffer at 23 °C. The titration curves are for (1) 12 mM Phe, (2) 12 mM Phe + 12 mM Ala, (3) 6 mM TMA^+Cl^-, (4) PK + buffer, (5) 12 mM Ala, and (6) 1 mM PEP.

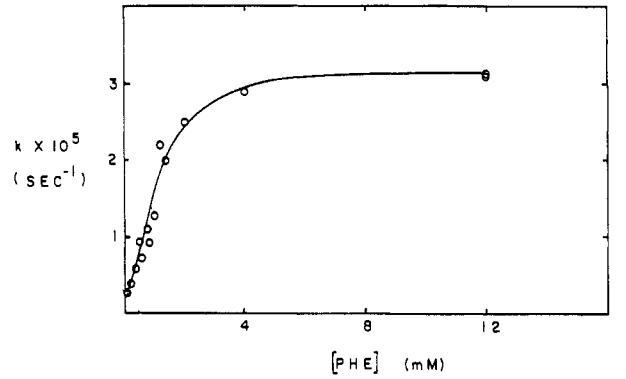


FIGURE 7: Effect of Phe concentration upon the pseudo-first-order rate constant for the titration of the sulfhydryl residues of PK. The line is the best fit by visual inspection.

Table III: Summary of the Pseudo-First-Order Rate Constants[a]

ligand	k ($\times 10^{-4}$ s^{-1})
	3 ± 1
1 mM PEP	0.6 ± 0.1
12 mM Ala	2 ± 1
12 mM Ala + 12 mM Phe	7 ± 1
6 mM TMA^+Cl^-	3 ± 1

[a] All experiments were performed at 23 °C in pH 7.5 TKM buffer.

of the apparent pseudo-first-order rate constant as a function of Phe concentration, as shown in Figure 7, indicates that the change is saturable. The concentrations of Phe required to maximize the change in the rate constant are in the same range as those required to achieve saturation in the binding isotherm. The fact that these concentrations are similar implies that Phe binding probably causes the change in the rate constant. The

results of the sulfhydryl titrations indicate that the accessibility of the sulfhydryl groups is ligand dependent. In the presence of Phe, they are more accessible while incubation of PK with PEP results in a decrease in the accessibility of these groups.

The effects of ligands on the local environments of tryptophan residues in PK were investigated by monitoring the quenching of tryptophan fluorescence by acrylamide (Eftink & Ghiron, 1976), an agent which decreases the fluorescence intensity via physical contact with the excited indole ring. Results of such studies were analyzed by using Stern–Volmer plots. The presence of either 2 mM PEP or 12 mM Phe did not induce an observable *net* change in the environments of tryptophan residues in PK. The values of K_Q, the weight-average collisional quenching constant, are identical and assume a value of 5. The identity of the "exposed" residues might have changed, but the present experimental approach does not resolve changes in the exposure of individual residues unless they result in a net change in the degree of exposure of all of the residues in the protein. Having determined the accessibility of tryptophan residues in the native state of PK, it was of interest to establish an internal reference in order to compare the relative accessibility of these residues. The unfolded state of PK was chosen to be the reference state. The quenching of tryptophan fluorescence was, therefore, measured in the presence of 6 M guanidine hydrochloride, and a steeper slope with a value of 6 as observed in the Stern–Volmer plot. For the PK system, the values of 0 and 6 may be taken as the two extremes, the first representing complete inaccessibility and the second representing total exposure of the tryptophan residues. A value of 5 for K_Q may indicate that the tryptophan residues perturbed are relatively exposed to the solvent even in the absence of effectors.

Discussion

The original definition of "allostery" is that the binding of one effector influences the binding of another ligand molecule. Such a linkage concept was initially formulated mathematically by Wyman (1948, 1964) to describe the observations reported for the hemoglobin system. This linked function concept was further developed in the 1960's as hypotheses to describe the abnormal kinetic behavior of a larger number of regulatory enzymes (Monod et al., 1965; Koshland et al., 1966). In these hypotheses, it was proposed that an enzyme can exist in various states of different affinities for substrates and effectors and the binding of these ligands affects the equilibrium between these states. The physical identities of these states are not defined in these hypotheses, although it has been generally accepted that they are manifested as changes in secondary–tertiary structures of the enzyme. These states may potentially be the products either of a simple redistribution of charges or of association–dissociation resulting from an interaction with ligands. For the rabbit muscle PK system, there is substantial spectroscopic evidence in the literature demonstrating that some chromophores in PK are perturbed by effecors and substrates (Kayne & Price, 1972; Kwan & Davis, 1980, 1981; Kayne & Suelter, 1965; Suelter, 1967). However, there have been no attempts to quantitatively relate structural changes to the kinetic observations. Since we know that kinetic and equilibrium binding data alone *do not* provide enough information to differentiate between the two-state or sequential models (Monod et al., 1965; Koshland et al., 1966), this report constitutes an initial attempt to quantitatively correlate the change in the global PK structure with enzyme kinetic and ligand binding observations and to elucidate the mechanism through which allosteric regulation is elicited.

The results from difference sedimentation velocity experiments provide unequivocal evidence of a transition in the hydrodynamic states of PK. There are several parameters which might account for the observed changes, since (Svedberg & Pederson, 1940)

$$s = \frac{M_w(1 - \bar{v}\rho)}{Nf} \tag{12}$$

where M_w and $\bar{v}$ are the weight-average molecular weight and the partial specific volume of the macromolecule, respectively, ρ is the solvent density, N is Avogadro's number, and f is the frictional coefficient. The observed, ligand-dependent change in the sedimentation coefficient might arise from a change in M_w, $\bar{v}$, or f. Let us examine the source of the observed changes in PK.

Enzyme kinetic measurements at 50 μg/mL PK serve to bridge the gap between the physical studies usually conducted at higher protein concentrations and that of routine kinetic measurements which are carried out at <1 μg/mL PK. Since identical results were obtained regardless of protein concentration, it is reasonable to assume that the presence of substrates or cofactors does not induce PK to undergo association–dissociation. Furthermore, these results indicate that the physical properties of PK monitored at high protein concentrations can be extrapolated to the low protein concentrations employed for enzyme kinetic measurements. Results from sedimentation equilibrium studies indeed show that Phe does not induce PK to undergo association–dissociation. This conclusion is further substantiated by the sedimentation velocity data which indicate no significant change in the sedimentation coefficient at protein concentrations as low as 15 μg/mL. The additional contribution of weight by the ligands upon formation of a PK–ligand complex should yield a higher sedimentation coefficient. However, the maximum change in molecular weight would be 800, assuming four binding sites for Phe or PEP per PK tetramer. This change in molecular weight can only lead to a change of +0.04 S. Hence, the additional weight of ligand in the protein–ligand complex cannot totally account for the observed change in the sedimentation coefficient.

It is possible that a ligand-dependent change in $\bar{v}$ might arise simply through an additive effect of complex formation without involving structural changes in protein. Incorporation of 4 additional mol of Phe in the $\bar{v}$ calculation (Cohn & Edsall, 1943) results in an increase of 0.0007 in the value for $\bar{v}$, which in turn yields a maximum decrease of 0.003 S in the sedimentation coefficient of the PK–ligand complex. Although the direction of the change produced by the $\bar{v}$ effect is consistent with the observed results, the magnitude of the change is not sufficient to account for the observed effect. $\bar{v}$ for PEP or Ala is 0.74, and the contribution of 4 mol of either PEP or Ala has virtually no effect upon the calculated value of $\bar{v}$ for the PK–ligand complex. On the other hand, $\bar{v}$ changes might also arise through a change in the molar volume of PK due to alterations in the packing of the amino acid residues in the protein. If one assumes a change of 0.24 S is totally due to a change in the molar volume, it would lead to a change of 0.007 in the value of $\bar{v}$, which represents a molar volume change of 1500 mL/mol, a magnitude comparable to that usually observed during protein unfolding (Lee & Timasheff, 1974). It does not seem likely that Phe is affecting a change as dramatic as unfolding of the protein. In addition to a volume change, a difference in $s_{20,w}$ may also be a consequence of changes in the frictional coefficient, f. Sedimentation data alone do not provide enough information to resolve the relative contribution of $\bar{v}$ and f changes to the observed $\Delta s_{20,w}$. Nev-

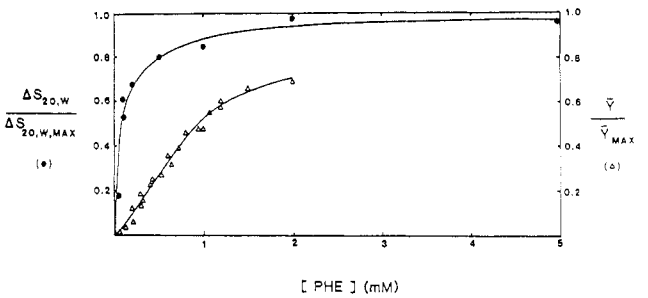


FIGURE 8: Comparison of the Phe binding isotherm with the Phe-induced structural change in the presence of KCl and $MgSO_4$.

ertheless, it can be concluded that the sedimentation data do reflect a change in the hydrodynamic properties of PK.

The decrease in sedimentation coefficient observed in the presence of Phe implies a loosening of the protein structure. This conclusion is consistent with the results of titration of sulfhydryl groups. The cysteine residues, with the possible exception of the four most reactive ones, are more accessible to chemical modification in the presence of Phe, suggesting a less compact structure. In contrast, PEP induces PK to assume a more compact structure, as indicated by an increase in the sedimentation coefficient and a decrease of 5–6-fold in the apparent pseudo-first-order rate of the reaction between sulfhydryl residues and DTNB.

Having established that Phe induces a global structural change in PK, it is important to compare the structural and equilibrium binding data to establish a molecular model which best fits all of the data. In order to compare the state change and the binding data, it is assumed that the fractional change in $\Delta s_{20,w}$ is equal to the fraction of molecules which have undergone a state change. If the Phe-induced state change is completed prior to saturation of the binding sites by the ligand, then the Monod–Wyman–Changeux two-state model (Monod et al., 1965) is the likely explanation for the observed phenomenon. On the other hand, if the state change coincides with the binding isotherm, the sequential model is suggested. In order to facilitate comparison of the data, both the state change and binding data were expressed as fractional changes.

Figure 8 shows the relation between the normalized parameters and Phe concentration in the presence of the cations. It is evident that the completion of state change precedes the saturation of Phe binding. At 0.5 mM Phe, the state change is greater than 80% complete while the protein is only 25% saturated with the ligand. Since there are four binding sites of Phe per PK tetramer, this result indicates that the binding of 1 mol of Phe results in a shift of the state equilibrium to the point where the transition is greater than three-quarters complete. On the basis of this evidence, the two-state model seems to be the most likely one to describe the observed phenomena. It should be pointed out that the difference sedimentation data were performed at 4 °C while the binding data were obtained at 23 °C; however, preliminary binding experiments were conducted at 4 °C, and results of these experiments indicate that the apparent affinity of Phe for PK is not significantly affected by the change in temperature. Hence, the conclusions are valid, despite the difference in the temperatures employed for the two experiments.

Results from sedimentation studies have clearly demonstrated that PK can exist in at least two states, R and T, which differ in their hydrodynamic properties. The state assumed by PK depends upon the ligands present. On the basis of steady-state kinetic evidence, PEP should induce PK to assume the active state (R) whereas Phe should favor the inactive or less active state (T). In addition, the structural studies indicate

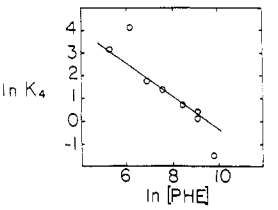
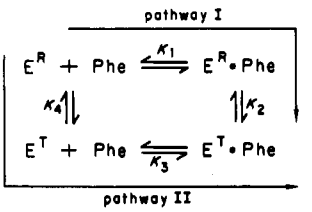


FIGURE 9: Linkage between Phe concentration and the equilibrium constant of state change. The linkage was based upon the difference sedimentation data obtained in the presence of KCl and $MgSO_4$. The line is a linear least-squares fit of the data.

that the transition from R to T is cooperative and occurs in a concerted manner; hence, the interaction between Phe and PK can be described as



(13)

where E^R and E^T represent the free enzyme in the R and T states, respectively. E^R·Phe and E^T·Phe represent the enzyme–Phe complex for each state. K_2 and K_4 are the equilibrium constants characterizing protein isomerization in the presence and absence of bound Phe, respectively. K_1 and K_3 are the equilibrium constants for single-site intrinsic reactions when it is possible for four Phe to bind to E^R and E^T, respectively. Thus, pathway I represents a Phe-mediated state change in PK whereas pathway II is that of a Phe-faciliated one.

Phe binding is clearly linked to the state change in PK, so it is of interest to investigate the relation quantitatively. Let us assume that the fractional change in $\Delta s_{20,w}$ represents the relative distribution between the two states, R and T, so that

$$\frac{\Delta s_{20,w}}{\Delta s_{20,w,max} - \Delta s_{20,w}} = T/R = K_4 \qquad (14)$$

where $\Delta s_{20,w}$ and $\Delta s_{20,w,max}$ are the observed change in the sedimentation coefficient at a particular Phe concentration and the maximum change of the sedimentation coefficient at infinite Phe concentration, respectively. T and R are the concentrations of the two proposed states of PK, and K_4 is the equilibrium constant characterizing the transition between states, as shown in eq 14. The relation between equilibrium constants and Phe concentration can be analyzed by the linked-function theory expressed by Tanford (1969)

$$\frac{\delta \ln K_4}{\delta \ln a_{Phe}} = \Delta\bar{\nu}_{Phe} - \frac{n_{Phe}}{n_{H_2O}}\Delta\bar{\nu}_{H_2O} = \Delta\bar{\nu}_{Phe,pref} \qquad (15)$$

where a_{Phe} is the activity of Phe, $\Delta\bar{\nu}_x$ is the change in the amount of ligand bound per tetrameric PK upon a change in states, and n_{Phe}/n_{H_2O} is the ratio of the number of moles of Phe to H_2O present in the solution. Since n_{Phe}/n_{H_2O} assumes a very small value, this term can be neglected from eq 15. The result of such an analysis is shown in Figure 9. A slope of +0.7 can be calculated, thus suggesting that the occurrence of a state transition from R to T involves a net uptake of about 1 mol of Phe. The exact quantitative interpretation of this parameter depends on the linkage between the equilibrium constants governing the state transition, K_2 and K_4, and the association constants between Phe and these states, K_1 and K_3.

The present study provides evidence that the interactions between PK and ligands are closely linked to the state change. It is essential to monitor the thermodynamic linkage between

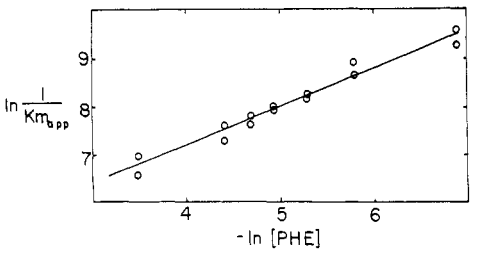


FIGURE 10: Linkage between Phe concentration and PEP binding. The kinetic data employed were obtained at pH 7.5 and 23 °C. The line is a linear least-squares fit of the data.

PEP and Phe. Weber (1975) has expressed the linked-function concept of Wyman in terms of binding energy. The term $\Delta G°_{XY}$, the free energy of interaction between ligands X and Y, is defined as (Weber, 1975)

$$\Delta G°_{XY} = \Delta G°(X/Y) - \Delta G°(X) = \Delta G°(Y/X) - \Delta G°(Y) \quad (16)$$

where $\Delta G°(X)$ and $\Delta G°(Y)$ are the standard free energies of binding to unliganded protein by X and Y, respectively, whereas $\Delta G°(X/Y)$ and $\Delta G°(Y/X)$ are the free energies of binding of X and Y to protein which is fully liganded with the other ligand. The linkage between PEP and Phe binding to PK is investigated in accordance with this scheme. $K_{m,app}$ values for PEP determined by steady-state kinetics were treated as apparent dissociation constants, K_d. Equilibrium binding data for PEP in the presence and absence of 3.0 mM Phe show that K_m and K_d assume identical values, indicating that K_m is a reasonable representation of K_d at least in the presence of low Phe concentrations. Since the K_m for PEP in the presence of 12 mM Phe is 57×10^{-5} M, and $\Delta G° = RT \ln K_d$ where R is the gas constant and T is the temperature in degrees kelvin, a value of −4.4 kcal/mol can be calculated. The corresponding value for PEP in the absence of Phe is −5.6 kcal/mol. Hence, $\Delta G°_{PEP-Phe}$ assumes a value of +1.2 kcal/mol. This result implies that the binding of these ligands in the presence of each other is unfavorable. Energy must be expended in order for one ligand to bind to PK when it is saturated with the other ligand. The source of this unfavorable interaction is not known. It is conceivable that the free-energy change involved in the R to T transition may contribute to it or it may represent the difference in the $\Delta G°$ values for PEP binding to the two states.

The relation between the apparent binding constants of PEP and Phe concentration was further analyzed by the linked function in accordance with eq 15, as shown in Figure 10. The slope was determined by a linear least-sequences fit of the data between 3 and 12 mM Phe, and a value of −0.9 was obtained. This value suggests that the binding of an additional mole of PEP results in a net release of about 1 mol of Phe.

In conclusion, this study has utilized a multifaceted approach in the analysis of the system. Steady-state kinetics, equilibrium ligand binding, and protein structural analysis have been used to demonstrate that the regulatory behavior of PK might satisfactorily be described by the two-state model of Monod–Wyman–Changeux. A detailed development of the model and quantitative analysis of the data are provided in the following paper (Oberfelder et al., 1984).

Registry No. PK, 9001-59-6; PEP, 138-08-9; TMA^+Cl^-, 75-57-0; Phe, 63-91-2; Ala, 56-41-7.

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