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Synergistic Effects of Proton and Phenylalanine on the Regulation of Muscle Pyruvate Kinase†

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ABSTRACT: Steady-state kinetic studies of muscle pyruvate kinase were conducted as a function of pH and phenylalanine concentrations. Results show that at a pH below 7.0, there is no observable effect of phenylalanine on the kinetic properties of muscle pyruvate kinase. When the results at a pH below 6.5 are used as the state for comparison, the kinetic results show that phenylalanine and proton exert a synergistic effect on the allosteric properties of the enzyme. A significantly greater change in Hill coefficients at high pH can be detected in the presence of phenylalanine than in its absence. To pinpoint the specific mechanism that leads to the synergistic effect, the kinetic data were resolved into the five equilibrium and two rate constants that characterize the basic two-state model. It can be shown that K_I^T, the binding constant of phenylalanine to the inactive T state, is strongly proton-linked. The affinity of phenylalanine for the T state increases with increasing pH. When the pH dependence of K_I^T was analyzed by the linked-function theory [Wyman, J. (1964) *Adv. Protein Chem. 19*, 224–285], it was shown that deprotonation favors phenylalanine binding to the T state. K_I^R (the binding constant of phenylalanine to the active R state), K_S^T (the binding constant of substrate to the T state), and L (the isomerization constant of the two states) not only are all weakly proton-linked but also it was shown that protonation favors the ligand–pyruvate kinase complex. K_S^R, the binding constant of substrate for the R state, shows no observable linkage to proton concentration. Thus, pH exhibits differential effects on these equilibrium constants both qualitatively and quantitatively. Knowing the proton linkage relationships, it is possible to conclude that the synergistic effect of phenylalanine and proton can be explained by the interplay among the strong proton-linked effect on the affinity of phenylalanine to the T state and the apparently weak or insignificant proton linkage in the other equilibrium parameters.

The basic molecular mechanism of regulation for muscle pyruvate kinase (PK)[1] has yet to be elucidated, although results from numerous studies have helped to establish a correlation between enzyme conformation and its function. It has been reported that PK undergoes a conformational change upon substrate binding (Kayne & Suelter, 1965; Mildvan & Cohn, 1965, 1966). These conformational transitions in PK can be affected by temperature and pH changes. These changes can be monitored by spectroscopic and hydrodynamic measurements (Kayne & Suelter, 1965, 1968). The structural transition(s) can be reversed by Phe, an allosteric inhibitor (Consler

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[1] Abbreviations: PK, pyruvate kinase; TKM buffer, 50 mM Tris buffer that contains 72 mM KCl and 7.2 mM $MgSO_4$; PEP, phosphoenolpyruvate.

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et al., 1988; Kwan & Davis, 1980; Kayne & Price, 1972; Conslor & Lee, 1988). Recently, Consler et al. (1988) and Consler and Lee (1988) reported that this reversible conformational transition involves domain movement. More specifically, the domain movement results in the closure of a cleft in which the active site is located. This domain–domain interaction is mediated by a "flexible" hinge region about which a domain rotates in order to effect this cleft closure. Since substrate binding is involved with the closure of the active-site cleft and inhibitor binding causes the opening of this region, it is likely that it is the structural condition of PK which dictates the potential of the enzyme for catalysis; i.e., if the substrates are present and bound, PK is in the cleft-closed state and is able to carry out enzymatic catalysis; if Phe is bound, however, the active-site cleft is opened up, and catalysis cannot proceed. In order to provide further insights into the regulatory mechanism, it is necessary to study these processes as a function of solution properties, e.g., temperature, pH, and ionic strength.

In the present study, the combined effect of proton and Phe was investigated. The effect of pH on the kinetic properties of PK has been characterized thoroughly (Phillips & Ainsworth, 1977; Gregory & Ainsworth, 1981; Dougherty & Cleland, 1985a,b). Results of these studies identify the pK_a's of functional groups in the active sites and enhance the understanding in the chemical nature of the enzymatic reaction catalyzed by PK. These studies, however, do not provide insights into the effects of pH on the various thermodynamically linked reactions that lead to the ultimately measured parameters in steady-state kinetics. For example, muscle PK exhibits a higher degree of cooperativity and is more susceptible to Phe inhibition at high pH. Since these kinetic observations are the composite consequence of differential bindings of substrate and inhibitor to the conformational states of PK and the distribution of these states, it would be useful to determine the specific effects of pH on these various binding equilibria. In order to gain further insights into the molecular mechanism of regulation, this study was initiated to characterize the effects of pH and Phe on muscle PK activity. These results were initially analyzed by the Wyman linked-function theory (Wyman, 1964) and subsequently resolved into five equilibrium constants characterizing the affinities of PEP and Phe for the two macrostates of PK and the isomerization constant of these states. Only one of these five equilibrium constants is significantly sensitive to pH. These conclusions are consistent with the results from PEP binding studies.

Materials and Methods

Rabbit muscle PK, Tris base, phosphoenolpyruvate, adenosine 5′-diphosphate, and nicotinamide adenine dinucleotide (reduced form) were obtained from Boehringer-Mannheim. Lactate dehydrogenase, Trizma hydrochloride, potassium chloride, and magnesium sulfate were purchased from Sigma Chemical Co. Phenylalanine was from Schwarz/Mann; [^{14}C]PEP was purchased from Amersham and Opti-Fluor scintillation cocktail from Packard Instruments.

Buffers were made up by titrating 50 mM Tris base against 50 mM Tris-HCl, each of which contains 72 mM KCl and 7.2 mM $MgSO_4$, to the desired pH at the particular experimental temperature. This corrected for any pH change with temperature due to the relatively high temperature coefficient of Tris buffers. The ammonium sulfate precipitate of PK was subjected to centrifugation and resuspended before being exhaustively dialyzed into the desired buffer.

Steady-State Kinetic Measurements. The kinetic activity of rabbit muscle PK was monitored by the lactate dehydrogenase coupled enzyme assay as originally described by Buchler and Pfleiderer (1955). Enzymes were desalted by passage over Sephadex G-25 columns that had been equilibrated with the experimental buffer. Protein concentrations were determined by measuring the absorbance at 280 nm and using the absorptivity values of 0.54 and 1.23 mL/(mg·cm) for PK and lactate dehydrogenase, respectively (Boyer, 1962).

The assay mix contained 2 mM ADP, 0.3 mM NADH, and 10 μg/mL lactate dehydrogenase, and PEP was the substrate, the concentration of which was varied. Under specified conditions, the concentration of ADP was kept constant but lower than 2 mM. When necessary, the pH of the assay mix was corrected after addition of ADP by back-titrating with KOH before addition of NADH or LDH. This assay mix accounted for 0.8 mL of the 1.0-mL assay volume. PEP was added in 0.1 mL of TKM buffer, and phenylalanine, when present, was added in 0.1 mL of TKM buffer. The reaction was initiated by the addition of PK, in a minimal volume of TKM buffer (usually 2–3 μL), to obtain a final concentration of 0.3 μg/mL.

The reaction was monitored by observing the absorbance change at 340 nm/min in a Gilford 250 spectrophotometer equipped with a multiple sample changer and a Gilford 6051 chart recorder. The temperature of the assay was controlled by a Thermoset electronic thermocontroller from Gilford.

Equilibrium Binding. The binding of PEP to PK was measured by the method of Hirose and Kano (1971). This technique relies on the partitioning of ligand and protein in a Sephadex G-50 gel slurry. For each experimental determination, the partitioning of ligand or protein in the absence of each other was measured. These controls allowed the determination of the extent of binding of ligand to protein, according to the relationships:

$$\beta' = [L]/[L]_0$$
$$\alpha = [P]/[P]_0 \quad (1)$$
$$\beta = [L]/[L]_0$$

where β' and β are the partitioning coefficients of ligand in the absence and presence of protein, respectively, and α is the partitioning coefficient of protein in the absence of ligand. [L] and $[L]_0$ are the concentrations of ligand in the presence and absence of gel, respectively. Likewise, [P] and $[P]_0$ are the concentrations of protein in the presence and absence of gel, respectively. The extent of binding of ligand to protein is determined by the relationship:

$$\bar{Y} = (L/P)(\beta - \beta')/(\alpha - \beta') \quad (2)$$

where the partitioning coefficients are as described above and L and P are the total amounts of ligand and protein (in millimoles) present in the assay.

Sephadex G-50 (coarse) was washed extensively with distilled deionized water, and the fines were decanted. This was repeated until no fines remained and the gel had swollen fully. The gel was then washed with 95% ethanol several times, and the excess ethanol was removed by decanting. Finally, the gel was dried in a 40 °C oven overnight. Fifty milligrams of prepared Sephadex G-50 was weighed out into disposable glass test tubes. For each ligand concentration, one tube was used for the determination of β', and the other for β,: 0.350 mL of TKM buffer was pipetted into each test tube with gel; in addition, the same volume of TKM buffer was pipetted into a blank test tube (no gel) for the determination of $[L]_0$. Each test tube was sealed tightly with parafilm, and the gel was allowed to swell for 6 h at room temperature.

The ligand solutions were made up by dilution of a concentrated stock solution of PEP that had been spiked with an

aliquot of [^{14}C]PEP, generally 3–5 μCi/mL total radioactivity in the stock solution. By making the solutions by serial dilution, it was assured that the final stock solutions had the same ratio of labeled to unlabeled ligand.

After the gel was swollen, the protein (PK) and radioactive ligand ([^{14}C]PEP) were added to the appropriate tubes. The tubes were again sealed tightly with parafilm and allowed to incubate in a temperature-controlled water bath at the desired temperature for 15 min. Samples were removed from the tubes with a 100-μL Hamilton syringe; this allowed the solution outside of the gel matrix to be sampled without contamination of gel beads. Two such samples could be obtained from each assay tube; these samples were injected into separate scintillation vials and served as duplicate measurements. In addition, duplicate tubes were prepared at each ligand concentration. Nine milliliters of Opti-Fluor scintillation cocktail was added to each sample, and the vial was vigorously shaken. The amount of ligand in each sample was determined by scintillation counting of [^{14}C]PEP in a Beckman scintillation counter.

Data Analysis. For the kinetic studies in which the concentration of ADP varies from 0.05 to 2.0 mM, steady-state kinetic rates were determined at various concentrations of PEP at each ADP concentration. The results can be analyzed by eq 3 (Cornish-Bowden, 1979) where V_{max} is the maximum

$$v = \frac{V_{max}[S_A][S_B]}{K_i^A K_m^B + K_m^B[S_A] + K_m^A[S_B] + [S_A][S_B]} \quad (3)$$

velocity, $[S_A]$ and $[S_B]$ are the concentrations of substrate A (PEP) and substrate B (ADP), respectively, K_m^A and K_m^B are the Michaelis constants for substrates A and B, respectively, and K_i^A and K_i^B are the dissociation constants of EA and EB complexes, respectively.

In the studies in which ADP was present in excess (i.e., 2 mM) and PEP was the variable substrate, steady-state kinetic data were initially analyzed graphically as the change in absorbance per minute as a function of PEP concentration. Each data set was subjected to a nonlinear least-squares fitting routine to determine the maximal velocity of that particular data set. In this preliminary analysis, data sets were fitted to the Michaelis–Menten equation or the Hill equation as shown in eq 4 (Hill, 1910) where $n = 1$ in the Michaelis–

$$v = \frac{V_{max}K^n[S]^n}{1 + K^n[S]^n} \quad (4)$$

Menten equation. All data sets were normalized by their maximal velocity so determined. This procedure allowed a global nonlinear least-squares analysis to be performed on multiple data sets obtained under similar experimental conditions, e.g., at one particular pH. The nonlinear least-squares analysis fit the data to eq 5 that describes a concerted allosteric

$$V = \{k_2^R[S]/K_S^R(1 + [S]/K_S^R)^3(1 + [I]/K_I^R)^4 + k_2^T L[S]/K_S^T(1 + [S]/K_S^T)^3(1 + [I]/K_I^T)^4\}/\{(1 + [S]/K_S^R)^4(1 + [I]/K_I^R)^4 + L(1 + [S]/K_S^T)^4(1 + [I]/K_I^T)^4\} \quad (5)$$

mechanism for PK interacting with an allosteric inhibitor (Oberfelder, 1982; Consler et al., 1989). Thus, each global fit included a family of data sets obtained at a series of inhibitor concentrations under one particular experimental condition. In general, each experimental condition (i.e., at one pH value) was represented by duplicates of 250–300 experimental determinations.

The global analysis yielded values for the seven kinetic parameters that describe the allosteric behavior of PK in the presence of Phe. These parameters are the following: L, the equilibrium constant governing the state transition, and is defined as [T state]/[R state]; K_S^R and K_S^T the dissociation constants for PEP binding to the R and the T state, respectively; K_I^T and K_I^R, the dissociation constants for Phe binding to the T and the R state, respectively; k_2^R and k_2^T, the rate constants of catalysis for the R state and the T state, respectively.

Equilibrium binding data were initially analyzed as described above, yielding saturation curves of moles of PEP bound per mole of PK ($\bar{Y}$) versus PEP concentration. These data were subsequently subjected to a nonlinear least-squares analysis similar to that for the steady-state kinetic data. Equation 6 describes the binding of a substrate to an enzyme

$$\bar{Y} = 4\{[[S]/K_S^R(1 + [S]/K_S^R)^3(1 + [I]/K_I^R)^4 + 4L[S]/K_S^T(1 + [S]/K_S^T)^3(1 + [I]/K_I^T)^4]/[(1 + [S]/K_S^R)^4(1 + [I]/K_I^R)^4 + L(1 + [S]/K_S^T)^4(1 + [I]/K_I^R)^4]\} \quad (6)$$

in the presence of an allosteric inhibitor and was employed for the curve fitting of the binding data (Oberfelder, 1982). The same five equilibrium constants described above for the steady-state kinetic analysis are the parameters being estimated.

Results

A majority of the data presented herein were obtained by steady-state kinetic measurements using the coupled enzyme assay described (Materials and Methods). It is essential to ascertain that the observed kinetic behavior is a true reflection of PK and not influenced by the coupling enzyme lactate dehydrogenase. Control experiments were conducted in all of the experimental conditions by monitoring the activity–substrate concentration relation as a function of lactate dehydrogenase concentration ranging from 5 to 20 μg/mL. In all of the experimental conditions, namely, pH ranging from 6.0 to 9.0 in the absence and presence of Phe, there are no quantitative differences in the activity–substrate concentration relations regardless of the concentration of lactate dehydrogenase. These results show that the coupling enzyme is not a limiting factor, and the kinetic results do reflect the intrinsic behavior of PK.

In earlier studies from this laboratory, the dissociation constants of PEP determined independently are, within the experimental uncertainties, identical with the K_m values derived from the kinetic data (Oberfelder et al., 1984; Consler et al., 1989). These results provide credence to the proposal that it is valid to analyze the kinetic data by the proposed two-state model. However, the equilibrium binding data were obtained in the absence of the second substrate ADP, whereas the kinetic data were obviously acquired in its presence. Hence, an implicit assumption was made; i.e., there is no cooperative interaction between the binding of both substrates to PK. In an effort to test the validity of this assumption, kinetic measurements were made by varying concentrations of both substrates at pH 7.5, 8.0, and 9.0. Typically, at each pH, 120–150 data points were acquired. These include 22 different PEP concentrations ranging from 0 to 2 mM and 6 ADP concentrations of 0.2–2.5 mM. These results were analyzed in accordance with eq 3 by global fitting, i.e., by taking all data points at one pH condition and searching for the best fit for the total data set. The various fitted constants are summarized in Table I. Within experimental uncertainties of ±30% (maximum deviations), at each pH value, the corresponding values of K_m and K_i for PEP and ADP are identical; e.g., at

Table I: Summary of Steady-State Kinetic Data as a Function of pH

	PEP		ADP	
pH	K_m (μM)	K_i (μM)	K_m (μM)	K_i (μM)
7.5	50 ± 13	40 ± 16	410 ± 10	290 ± 10
8.0	46 ± 6	41 ± 4	234 ± 5	240 ± 5
9.0	70 ± 5	60 ± 8	117 ± 2	93 ● 5

pH 8.0, the values of K_m and K_i for PEP are 46 ± 6 and 41 ± 4 μM, respectively. These results provide evidence that there are no cooperative interactions between these two substrates. Since the values of K_m and K_i for PEP are identical in all pH values, it implies that for a substrate involved in a random mechanism, the K_m determined is a good approximation of K_D. Furthermore, these observations provide strong evidence that the detailed data analysis conducted in previous and present studies can yield valid equilibrium parameters characterizing the proposed two-state model.

The steady-state kinetics of muscle PK were studied as a function of pH ranging from 6.0 to 9.0 in the presence of Phe concentrations ranging from 0 to 20 mM. At pH values at or below 6.5, Phe does not seem to affect the kinetic behavior, as it does at pH values greater than 7.0. Figure 1 shows some of the typical kinetic data. At pH 7.0, as at all higher pH values studied, the relation between enzymic activity and substrate concentration becomes more sigmoidal with increasing concentration of Phe. As the pH is increased from 7.0 to 9.0, the sigmoidicity of this relation becomes more pronounced. To quantitate these observations, the kinetic data were fitted to the Hill equation to determine the values of K_m^{app}, the apparent Michaelis constant, and n, the Hill coefficient. In the absence of Phe, the value of K_m^{app} shows only a slight dependence on pH. However, the presence of Phe significantly affects the value of K_m^{app}, which increases with increasing pH. The magnitude of change becomes more pronounced at higher concentrations of Phe. The data, as shown in Figure 1, show that the effect of Phe is attenuated by high proton concentration and that kinetic data obtained at low pH can be adequately described by a simple one-state model; i.e., PK exists exclusively in the active conformation under acidic conditions. In order to be consistent, even the data obtained at pH values below 7 were "forced" to fit a two-state model equation. In Table II, it can be seen that, in effect, the best fit of these data (at pH 6.5 and 6.0) equally supports a one-state model. At pH 6.5, the parameters for the binding of substrate, K_S^R and K_S^T, are best fit by essentially the same value (i.e., $K_S^R = K_S^T$), and those for the binding of inhibitor, K_I^R and K_I^T, assume unrealistically high values (concentrations much higher than the solubility of Phe). These estimates illustrate that the "two-states" of PK at pH 6.5 bind substrate equally well and bind inhibitor poorly or not at all. In the fitting of the data obtained at pH 6.0, the low value fit for K_S^R combined with the nearly equal binding parameters for Phe does not mean

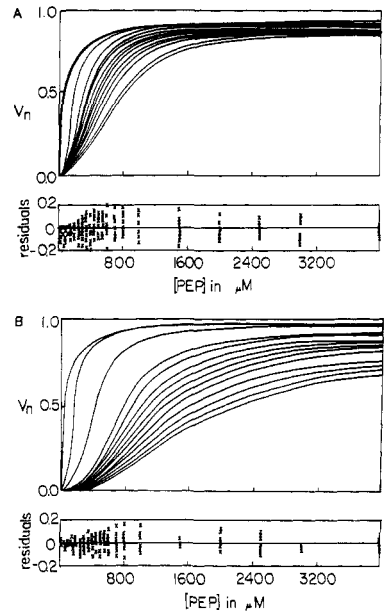

FIGURE 1: Steady-state kinetic data for PK as a function of PEP and Phe concentrations. The normalized data were fitted by nonlinear least-squares analysis using eq 5. Residuals are displayed below the data. The data were obtained at (A) pH 7.8 and (B) pH 9.0. Data points were omitted for clarity.

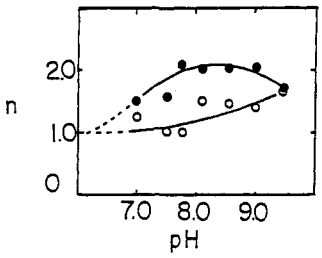

FIGURE 2: Relation between Hill coefficient and pH. The open and closed cycles represent data in the absence and presence of 20 mM Phe, respectively. The lines indicate only the trend of the data.

that the actual regulatory mechanism changes with pH but rather that at the extremes of experimental conditions the regulatory properties are limited and the mathematical model describing the kinetic behavior can be more simply expressed as a one-state model.

In addition to changes in the value of K_m^{app}, the degree of cooperativity exhibited in the kinetic data also changes with pH, as shown in Figure 2. In the absence of Phe, the value of the Hill coefficient, n, increases from 1.0 ± 0.06 to 1.7 ± 0.15 with increasing pH. The presence of Phe significantly enhances the effect; e.g., at pH 7.8, n is 2.1 ± 0.14 and 1.0 ± 0.1 in the presence and absence of 20 mM Phe, respectively. The effect of Phe as a function of pH can be expressed as Δn, the difference in the Hill coefficient in the presence and absence of 20 mM Phe. In this context, a smaller Δn implies

Table II: Fitted Steady-State Kinetic Parameters as a Function of pH at 23 °C[a]

pH	K_S^R	K_S^T	K_I^R	K_I^T	L
6.0	34	404	648	314	0.553
6.5	27	23	2.8×10^{10}	2.1×10^5	0.145
7.0	82	1303	4869	740	0.051
7.5	52	580	7511	646	0.060
7.8	65	1253	15655	591	0.090
8.1	43	715	11362	340	0.096
8.5	52	2029	8257	167	0.094
9.0	55	1744	11807	220	0.093
simulated error	0.03–0.07	0.05–0.07	0.08–0.16	0.20–0.34	0.5–1.0

[a] Binding constants are expressed in micromolar. L is unitless.

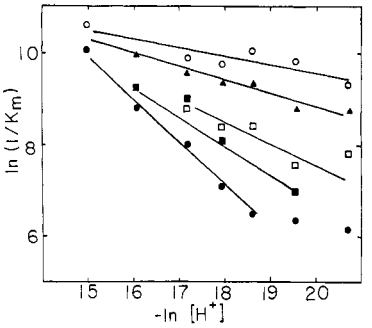

FIGURE 3: Linked function relations between apparent Michaelis constants and proton concentrations. The symbols and Phe concentrations in millimolar are as follows: (○) 0; (▲) 1; (□) 3; (■) 5; (●) 20.

a decrease in the effectiveness of Phe in inducing a change in the cooperativity observed by kinetics. It is evident that the effect of Phe decreases at a pH higher or lower than pH 7.8. These observations serve to illustrate the linkages involved among the state change (as monitored by the Hill coefficient), pH, and Phe. It is seen that both protons and Phe influence the cooperativity of the system. For example, at high pH, the state change has at least partially occurred; thus, the Δn (with 20 mM Phe) is decreased relative to that at a lower pH. These linkages will now be examined quantitatively.

In earlier studies, it has been shown that $1/K_m^{app}$ can be approximated as the apparent association constants of PEP, K_{app} (Oberfelder, 1982; Oberfelder et al., 1984a,b). Thus, the dependence of K_m^{app} on pH was analyzed by the Wyman linked function (1964) which states

$$\left(\frac{\partial \ln K_{app}}{\partial \ln a_x}\right)_{T,p,a_j} = \Delta\bar{\nu}_x = \bar{\nu}_{x,active} - \bar{\nu}_{x,inactive} \qquad (7)$$

where a_x is the activity of component x, which in this case is a proton, K_{app} is the association constant that is perturbed as a function of a_x, and $\Delta\bar{\nu}_x$ is the difference in proton binding to the active and inactive states of PK in the assay mixture. Figure 3 shows the relation between ln $(1/K_m^{app})$ and ln $[H^+]$ at different concentrations of Phe. In all cases, the slope yields positive values for $\Delta\bar{\nu}_{H^+}$ and increases with increasing Phe concentration. The values of $\Delta\bar{\nu}_{H^+}$ range from 0.16 ± 0.03 to 0.83 ± 0.08 within the Phe concentration range of 0–20 mM, as shown in Figure 4A. These results imply that in converting PK to the active form, a net release of proton is observed, although the magnitude of change depends on the amount of Phe present. An analogous analysis was applied to the data with Phe as the variable ligand. Figure 4B shows that, under all pH values tested, $\Delta\bar{\nu}_{Phe}$ assumes a negative value which becomes more negative with increasing pH until it assumes an apparently maximum value of 1.0. These results imply that conversion of PK to the active form in the assay mixture requires a release of Phe, the amount of which is pH dependent.

At best, K_m^{app} is an apparent equilibrium constant which is a composite of all the equilibrium constants characterizing the various linked reactions that ultimately lead to the measured enzymic activity. In order to probe further into the coupled effects of Phe and pH, the kinetic data were analyzed by nonlinear least-squares analysis in accordance with eq 5. The dependence of the five equilibrium constants on pH was again analyzed by the Wyman linked function relationship, and the results are shown in Figure 5. It is interesting to observe that K_S^R is not pH dependent while K_S^T, K_I^R, and $1/L$ are all slightly pH dependent, yielding a value of 0.19 ± 0.02 for $\Delta\bar{\nu}_{H^+}$ in all cases. The single equilibrium constant that shows a unique

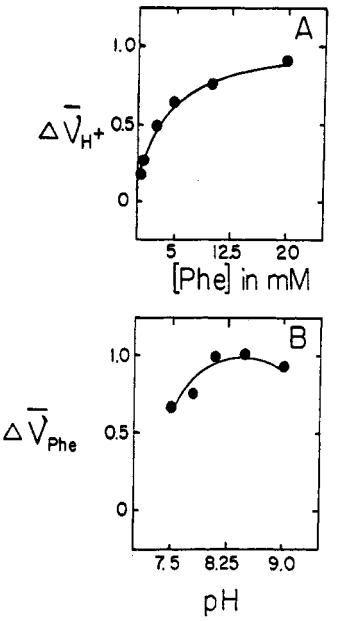

FIGURE 4: Relations between preferential binding parameter and ligand concentration. (A) Binding of proton as a function of Phe concentration. (B) Binding of Phe as a function of proton concentration.

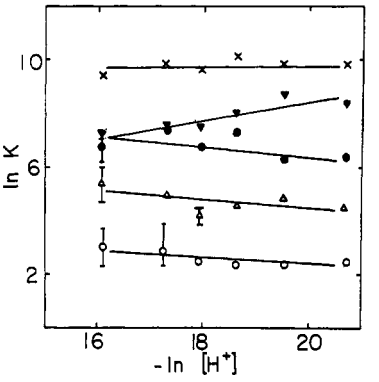

FIGURE 5: Linked function relations between association constants and proton concentrations. The symbols and dissociation constants are as follows: (×) K_S^R; (▼) K_I^T; (●) K_S^T; (△) K_I^R; (○) L.

pH dependence is K_I^T which yields a value of −0.35 ± 0.02 for $\Delta\bar{\nu}_{H^+}$. These results imply that different equilibrium reactions are susceptible to pH changes in different manners and the observed kinetic behaviors shown in Figure 1 are composites of these reactions, as they are influenced by both Phe and proton concentrations.

Since the results shown in Figure 5 indicate that K_S^R is pH independent, it would be useful to have a direct assessment of this parameter. Hence, the binding of PEP to PK was monitored as a function of pH. Binding isotherms were determined for five pH values ranging from 7.0 to 9.0, and typical results are shown in Figure 6. It is evident that, within experimental uncertainties, these binding isotherms are essentially identical, yielding a K_D^{app} of $(4 \pm 2) \times 10^{-5}$ M. K_D^{app} is an apparent parameter which is a composite of K_S^R, K_S^T, and T as shown in eq 8. K_D^{app} can be calculated by using the values

$$K_D^{app} = \frac{1 + L}{1/K_S^R + L/K_S^T} \qquad (8)$$

for K_S^R, K_S^T, and L resolved from the kinetic data and eq 8. It can be shown that the calculated values range from 4.7×10^{-5} to 7.1×10^{-5} M with an average deviation of 15%. Thus, it may be concluded that the PEP binding data are consistent with the values for K_S^R, K_S^T, and L derived from steady-state kinetic data.

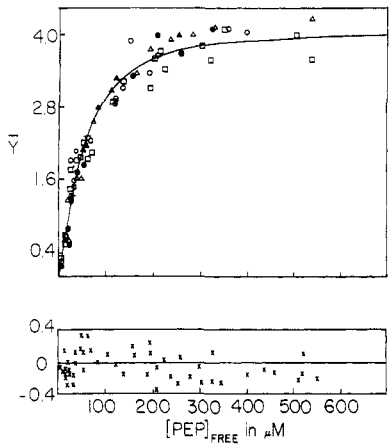

FIGURE 6: PEP binding isotherms as a function of pH. The symbols and pH are the following: (○) 7.0; (□) 7.5; (●) 8.1; (△) 8.5; (▲) 9.0. The line is a fitted curve for $K_D^{app} = 4 \times 10^{-5}$ M.

DISCUSSION

Results from this study show that at pH below 7.0, there is no observable effect of Phe on the kinetic properties of muscle PK. Using the data obtained under these conditions (at one of the limiting cases of the model, i.e., all R state) as the state for comparison, it is most interesting to note that Phe and protons exhibit a synergistic effect on the allosteric properties of muscle PK. Most studies reported in the literature have focused on the effect of proton alone; thus, a systematic evaluation has not been reported on the possible cooperative effect by these two ligands. The significant changes in Hill coefficients at high pH in the presence of Phe are most intriguing. Quantitative analysis of the kinetic data makes it possible to pinpoint the specific mechanism that leads to the observation.

By analyzing the kinetic data to resolve the five equilibrium constants, some insights have been provided into the regulatory mechanism of muscle PK. The validity of the proposed mechanism depends on the accuracy of the various constants determined. In two earlier studies, it was established that the same equilibrium constants can be accurately determined by steady-state kinetics alone as are obtained with a combination of kinetic, binding, and structural data (Oberfelder et al., 1984a,b; Consler et al., 1989). Furthermore, in this study, it was shown that the K_D^{app} for PEP binding is in reasonable agreement with the predicted value using the various equilibrium constants determined by the kinetic data alone. Thus, it can be concluded that the two-state model employed in the analysis of data is still valid, and internally consistent results are being obtained.

A rigorous test was conducted in an earlier study to establish the confidence limits for the various parameters derived by global fitting (Consler et al., 1989). The standard deviation of the independent simulation for k_2^R, k_2^T, K_S^R, and K_S^T is less than 7%, as summarized in Table II. The state-specific binding constants for Phe, K_I^R and K_I^T, were less precise with a standard deviation of 10–20%. The least defined parameter is L, which varies by 50–100%.

Having satisfied that the various equilibrium constants do accurately represent the intrinsic properties of muscle PK and knowing their confidence limits, it is useful to examine them in order to provide a rationale for the observed kinetic behavior. In the absence of Phe, the kinetic data show deviation from simple Michaelis–Menten only at pH ≥8.1. Under these experimental conditions, any term that involves Phe can be omitted; thus, one needs only to consider the values of L, K_S^R, K_S^T, k_2^R, and k_2^T. Since k_2^T is best fitted as nearly zero under all conditions studied, one only needs to examine the interplay among L, K_S^R, and K_S^T. Only L and K_S^T are proton-linked, whereas K_S^R shows no observable linkage to proton concentration. On the basis of values of L and K_S^T, as the pH is raised, the T state becomes increasingly favored, whereas the affinity of substrate for the T state decreases. Since the affinity of substrate for the R state is orders of magnitude stronger, especially at high pH, the net result is that with the addition of substrate, PEP will preferentially bind to the R state, and enzyme activity will be observed. With subsequent addition of PEP, the distribution of PK will shift toward the R state. According to the formulation of Monod et al. (1965), such a shift in the distribution of state would lead to a sigmoidal relationship in the plots of velocity vs substrate concentration as indicated by an increase in the Hill coefficient to greater than 1.0. Since L is only weakly proton-linked, one does not expect to observe significant changes of the Hill coefficient with pH under these experimental condition. Such expectation is exactly matched by experimental observation in the absence of Phe (Figures 1 and 2).

In the presence of Phe, the contributions of K_I^R and K_I^T will also need to be considered. The degree of contribution by these parameters will be a function not only of their magnitudes but also of the specific concentration of Phe present. Let us consider the case at 20 mM Phe and at high pH. K_I^T is the parameter that is the most strongly proton-linked, and it indicates an increase in the affinity of Phe for the T state with increasing pH. Thus, at pH 9.0, the presence of 20 mM Phe would lead to essential saturation of the inhibitor binding site in PK by Phe. Since K_I^T is orders of magnitude stronger than K_I^R under these experimental conditions, the distribution of states will be highly in favor of the T state. Addition of substrate, as above, results in its preferential binding to the R state as a consequence of the much greater affinity of PEP for the R state as compared to the T state. Again, a shift of state occurs which is observed as a sigmoidal relationship between enzyme activity and substrate concentration. Due to its strong linkage to proton, the affinity of Phe for the T state changes with pH much more significantly than any of the other parameters. Since K_I^T indicates a decrease in affinity with decreasing pH, the contribution of this parameter to the state change becomes less dominant at lowered pH; thus, even in the presence of Phe, the degree of sigmoidicity in the relationship between velocity and substrate concentration decreases as indicated by the decrease in Hill coefficient.

At present, it can be concluded that the synergistic effect of Phe and proton can be explained by the strong and negative proton-linked effect on the affinity of Phe to the T state and the apparently weak or insignificant positive proton linkage in the other equilibrium parameters.

The validity of this linkage scheme between Phe and proton can be further quantitatively evaluated by considering the Wyman linked-function theory. In Figure 4A, it was shown that a net proton release is associated with the activation of muscle PK. The amount of proton released is apparently dependent on Phe concentration. It increases from 0.2 to 0.8 with Phe concentration ranging from 0 to 20 mM. Let us examine the sources of this net release of proton. In the absence of Phe, one needs to consider only the proton linkages of L, K_S^R, and K_S^T. A conversion of the T to the R state leads to a release of 0.2 mol of proton, and a similar magnitude is associated with the binding of PEP to the T state. However, the interaction between PEP and the R state apparently is not proton-linked; thus, no net proton release or absorption is connected to this reaction. The observed net proton release

of approximately 0.2 mol must be contributed from the weakly proton-linked reactions of L and K_S^T. In the presence of 20 mM Phe, the initial binding of Phe to the T state would lead to an absorption of 0.35 mol of proton. Subsequent titration of the system with substrate would lead to a state change and in the release of Phe (Figure 4B). These two reactions can account for 0.6 mol of the observed 0.8 mol of proton released. Thus, by considering the quantitative proton linkages among these equilibrium constants, it is possible to provide a rationale for the kinetic behavior of PK in solution.

In summary, the synergistic effect of Phe and proton on the kinetic behavior of muscle PK is the composite effects of proton on the multiple equilibria governing the regulatory mechanism of the enzyme. The strong and negative proton linkage observed in the binding affinity of Phe for the inactive T state seems to play a major role.

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The critical review and stimulating discussion with Dr. Tomasz Heyduk are greatly appreciated.

Registry No. PK, 9001-59-6; PEP, 138-08-9; ADP, 58-64-0; L-Phe, 63-91-2; H^+, 12408-02-5.

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Structure and Polymorphism of Saturated Monoacid 1,2-Diacyl-*sn*-glycerols†

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ABSTRACT: The 1,2-diacyl-*sn*-glycerols (1,2-DGs) are the predominant naturally occurring isomer found in cell membranes, lipid droplets, and lipoproteins. They are involved in the metabolism of monoacylglycerols, triacylglycerols, and phospholipids. The 1,2-DGs participate in the activation of protein kinase C, in phosphorylation of target proteins, and in transduction of extracellular signals into the cell. We have undertaken a study of the physical properties of a homologous series of synthetic optically active diacylglycerols. Stereospecific 1,2-diacyl-*sn*-glycerols were synthesized with saturated fatty acyl chains of 12, 16, 18, 22, and 24 carbons in length. Their polymorphic behavior was examined by differential scanning calorimetry and X-ray powder diffraction. The solvent-crystallized form for all the 1,2-DGs packs in the orthorhombic perpendicular subcell (β') and melts with a single sharp endotherm to an isotropic liquid. On quenching, the C_{12}, C_{16} and C_{18} compounds pack in a hexagonal subcell (α), whereas the C_{22} and C_{24} pack in a pseudohexagonal subcell (sub-α). The sub-α phase reversibly converts to the α phase. The long spacings of these compounds in both the α and β' phases increase with chain length. In the α and β' phases, the acyl chain tilts were found to be 90° and 62° from the basal methyl plane. The polymorphic behavior of 1,2-diacyl-*sn*-glycerol is quite different from that of the corresponding monoacid saturated 1,3-diacylglycerols which form two β phases with triclinic parallel subcells.

Diacyl-*sn*-glycerols (DGs) are comprised of two fatty acyl chains esterified to two out of three glycerol carbons. Due to the prochiral nature of the glycerol molecule, esterification of two acyl chains of the same kind results in three isomeric 1,2-, 2,3-, and 1,3-diacyl-*sn*-glycerols (IUPAC–IUB, 1977). The racemic 1,2-diacyl-glycerols are a mixture of 1,2- and 2,3-diacyl-*sn*-glycerols. In this study, the racemic forms are

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