# Energetics of Allosteric Regulation in Muscle Pyruvate Kinase†

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ABSTRACT: The regulatory mechanism of rabbit muscle pyruvate kinase has been studied as a function of temperature in conjunction with phenylalanine, the allosteric inhibitor. The inhibitory effect of phenylalanine is modulated by temperature. At low temperatures, the presence of phenylalanine is almost inconsequential, but as the temperature increases so does the phenylalanine-dependent inhibition of the kinetic activity. In addition, the presence of phenylalanine induces cooperativity in the relation between velocity and substrate concentration. This effect is especially pronounced at elevated temperature. The kinetic data were analyzed using an equation that describes the steady-state kinetic velocity data as a function of five equilibrium constants and two rate constants. Van't Hoff analysis of the temperature dependence of the equilibrium constants determined by nonlinear curve fitting revealed that the interaction of pyruvate kinase with its substrate, phosphoenolpyruate, is an enthalpy-driven process. This is consistent with an interaction that involves electrostatic forces, and indeed, phosphoenolpyruvate is a negatively charged substrate. In contrast, the interaction of pyruvate kinase with phenylalanine is strongly entropy driven. These results imply that the binding of phenylalanine involves hydrophobic interaction and are consistent with the basic concepts of strengthening of the hydrophobic effect with an increase in temperature. The effect of phenylalanine at high temperatures is the net consequence of weakening of substrate–enzyme interaction and significant strengthening of inhibitor binding to the inactive state of pyruvate kinase. The effects of salts were also studied. The results show that salts also exert a differential effect on the binding of substrate and inhibitor to the enzyme. When the results of temperature, pH, and salt perturbations on the regulatory mechanism of muscle pyruvate kinase are combined, it is apparent that the regulatory behavior of pyruvate kinase observed in different solution environments is the net result of differential perturbation of substrate and inhibitor binding to the two conformational states of the enzyme.

Mammalian pyruvate kinase (PK)[1] is a key regulatory glycolytic enzyme which exhibits allosteric kinetic behavior (Boyer, 1962; Kayne, 1973). The basic mechanism of regulation at the molecular level is still not clear. There are reports that PK undergoes conformational changes (Kwan & Davis, 1980; Kayne & Price, 1972; Kayne & Suelter, 1965) and that the changes involve domain movements (Consler et al., 1988a). Although these structural changes have been observed, there is only limited knowledge on the quantitative linkage between structural changes and the regulation of the catalytic function of PK. In an attempt to provide information on this specific issue, Oberfelder et al. (1984a,b) studied the thermodynamic linkages in muscle PK by a combination of kinetic, equilibrium, and structural studies. Based on these results, a concerted, allosteric model was developed. The model depicts PK to be distributed between two structural states, active and inactive, which exhibit differential affinities for substrate or inhibitor. Hence, the model is characterized by a total of five equilibrium constants—two defining substrate affinity, two for inhibitor affinity to these two states, and one defining the distribution of these two states. The validity of the model was verified independently by monitoring the global structural changes of PK as a function of Phe concentration and a combination of PEP/Phe concentrations (Heyduk et al., 1992). Establishment of the model enables one to quantitate the various equilibria governing the activity of PK and to test the role played by each specific equilibrium in the control of enzyme activity.

With the establishment of a model and the development of procedures to acquire data and to evaluate the multiple equilibria involved in this model, Consler et al. (1990) studied the synergistic effects of protons and phenylalanine on the enzyme activity. It is interesting to note that this synergism can be explained as the net result of the interplay among the strong proton-linked binding of phenylalanine to the inactive state and the proton insensitivity of the other equilibrium parameters. Hence, by application of these approaches, it seems possible to dissect the system in order to identify the particular functional step(s) that is(are) the target of specific solution perturbation.

On the basis of the encouraging results of the pH effect, it is then useful to employ different solution conditions to probe the specific step(s) that is(are) most sensitive to environmental perturbation and determine which may be involved in the regulation of this enzyme. Previous study has shown that a change in cooperativity as monitored by steady-state kinetics, similar to that in the pH study, is also observed with change in temperature. In order to determine if the same specific steps are sensitive to pH and temperature, a steady-state kinetic and equilibrium binding study was initiated

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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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to probe the effects of temperature and inorganic salts in accordance with the Hofmeister series (1888).

## MATERIALS AND METHODS

Rabbit muscle PK, Tris base, phoshoenolpyruvate, adenosine 5-diphosphate, and nicotinamide adenine dinucleotide (reduced form) were obtained from Boerhinger-Mannheim. Lactate dehydrogenase, Trizma hydrochloride, potassium chloride, and magnesium sulfate were purchased from Sigma Chemical Co. Phenylalanine was from Schwartz-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 and Tris-HCl containing 72 mM KCl and 7.2 mM $MgSO_4$ to pH 7.5 at the particular experimental temperature. This corrected for any pH change with temperature due to the relatively high temperature coefficient of Tris buffers.

*Steady-State Kinetic Measurements.* The kinetic activity of rabbit msucle PK was monitored by the lactate dehydrogenase coupled enzyme assay as originally described by Buchler and Pfleiderer (1955). The specific details are identical to the published procedures (Consler et al., 1990). 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 absorptivities of 0.54 and 1.23 mL/(mg·cm) for pyruvate kinase and lactate dehydrogenase, respectively (Boyer, 1962).

The reaction was monitored by observing the time course of the absorbance change at 340 nm 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. If the values for these control experiments are known, the determination of the extent of binding of ligand to protein can be accomplished according to the following relationships:

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

where $\beta'$ and $\beta$ are the partitioning coefficient of ligand in the absence and presence of protein, respectively and $\alpha$ is the partitioning coefficient of protein in the absence of ligand. $[\ell]$ or $[\ell]'$ and $[\ell]_0$ or $[\ell]'_0$ 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 following relationship:

$$Y = (\ell/P)(\beta - \beta')/(\alpha - \beta') \quad (2)$$

where the partitioning coefficients are as described above, and $\ell$ and P are the total amounts of ligand and protein (in millimoles) present in the assay. The specific details of the procedure are identical to a previously published one (Consler et al., 1990).

*Data Analysis.* 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 (Hill, 1910)

$$V/V_{max} = [S]^n/(K_m + [S]^n) \quad (3)$$

where $n = 1$ in the Michaelis–Menten equation, $V$ and $V_{max}$ are the observed and maximum initial velocities, respectively, $K_m$ is the Michaelis constant, and [S] is substrate concentration. All data sets were normalized by the 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 and temperature. The nonlinear least-squares analysis fitted the data to eq 4, which describes a concerted allosteric 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 temperature) was represented by duplicates of 250–300 experimental determinations.

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

The global analysis yielded values for the five equilibrium and two kinetic parameters that describe the allosteric behavior of PK in the presence of Phe. These parameters are as follows: $L$, the equilibrium constant governing the state transition and defined as [T-state]/[R-state]; $K_S^R$ and $K_S^T$, the dissociation constants for PEP binding to the R- and T-states, respectively; $K_I^T$ and $K_I^R$, the dissociation constants for Phe binding to the T- and R-states, respectively; $k_2^R$ and $k_2^T$, the fractional rate constant of the catalysis for the R-state and T-state, respectively. A constraint was applied so that $k_2^R + k_2^T = 1$.

Equilibrium binding data were initially analyzed as described above, yielding saturation curves of moles of PEP bound per mole of PK ($\bar{Y}$) vs PEP concentration. These data were subsequently subjected to a nonlinear least-squares analysis similar to that for the steady-state kinetic data. Equation 5 describes the binding of a substrate to an enzyme existing in two states 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.

$$Y = [4[S]/K_S^R(1 + [S]/K_S^R)^3(1 + [I]/K_I^R)^4 + 4L[S]/K_S^T(1 + [S]/K_S^T)^3(1 + [I]/K_I^T)^4]/[(1 + [S]/K_S^R)^4 \times (1 + [I]/K_I^R)^4 + L(1 + [S]/K_S^T)^4(1 + [I]/K_I^T)^4] \quad (5)$$

## RESULTS

The steady-state kinetic behavior of rabbit muscle PK was examined as a function of temperature in order to determine the thermodynamic influences underlying the chemical equilibria responsible for the catalytic activity of the enzyme. These equilibria include interactions of PK with its substrate, PEP, as well as the allosteric inhibitor, Phe. In addition, there is a conformational equilibrium between alternate states of the enzyme. These equilibria serve to define the fundamental interactions of PK in the steady-state kinetic assay.

Temperature was varied from 10 to 37 °C. At each temperature, the initial velocity was measured at 16–20 different PEP concentrations ranging from 0 to 4 mM. The effect of

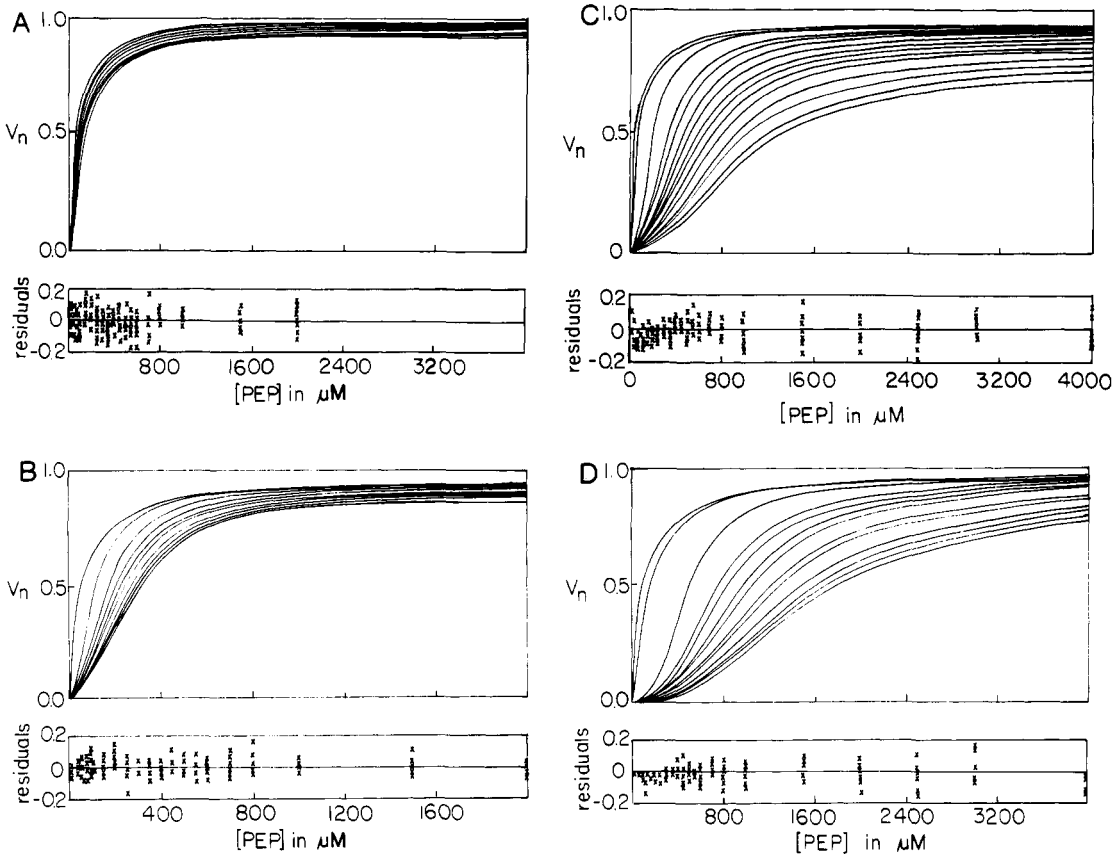


FIGURE 1: Steady-state kinetic data for PK as a function of PEP and Phe concentrations. The normalized data were fitted by nonlinear least-squares analysis using eq 4. Residuals are displayed below the data at (A) 14, (B) 23, (C) 30, and (D) 37 °C. Data points were omitted for clarity. The concentrations of Phe that are common in all sets of data are 0, 6, 8, 10, 12, 14, 16, 18, and 20 mM. Additional Phe concentrations (mM) at each specific temperature: (A) 2 and 4; (B) 3; (C) 1, 3, 5, 7, and 9; (D) 1, 3, 5, and 7.

Phe on the kinetic behavior of PK was also tested. Hence, at each temperature, kinetic data were collected for 11–15 different Phe concentrations ranging from 0 to 20 mM. Thus, for each temperature a total of 200–300 data points were available for simultaneous data analysis.

The most striking observation from these steady-state kinetic data is that the effect of Phe is significantly attenuated by lowering the temperature. At 10 and 14 °C, the data are hyperbolic with respect to PEP concentration, even at 20 mM Phe. There is a slight decrease in activity, but there is no hint of cooperativity (i.e., sigmoidicity) in the relation between velocity and PEP concentration. The data obtained at 17 °C start to exhibit sigmoidicity at intermediate Phe concentrations (5–7 mM). At 23 °C and above, the cooperative nature of enzyme activity is evident even at 1 mM Phe, the lowest inhibitor concentration examined, as shown in Figure 1, which consists of families of curves describing the kinetic behavior of PK in the presence of Phe at each temperature.

These results were then subjected to quantitative analysis to extract the effect of temperature and Phe on the specific reaction steps in the regulation of PK activity. The parameters most often used in the description of catalytic activity of enzymes are the maximal velocity, the Michaelis constant, and the Hill coefficient. These three parameters were obtained by nonlinear fitting of the kinetic data to the fundamental mathematical relationships that describe hyperbolic (the Michaelis–Menten equation) and both hyperbolic and sigmoidal (the Hill equation) substrate dependencies, as shown in eq 3. These analyses are being used initially to mathematically describe the steady-state kinetic behavior being examined and to reflect the degree of cooperativity in PK kinetic behavior as a function of solution variables. The maximal velocity for the PK-catalyzed reaction increases linearly with respect to increasing temperature. Since the maximal velocity is not affected by Phe inhibition and its temperature dependence is simple, the data obtained at each temperature have been normalized.

The substrate dependencies become increasingly sigmoidal with increasing Phe concentration, so the Hill equation can be used to describe all of the individual steady-state kinetic experiments. The value of $K_{M,app}$ obtained from Hill analysis varies with temperature. The limiting cases are the values in the absence and presence of 20 mM Phe. The dependency of $K_{M,app}$ resulting from intermediate Phe concentrations falls in between these two cases. It is interesting to note that the temperature dependence of the two limiting cases intersects at a point representing 0 °C, indicating that the Phe effect disappears at this temperature. Potentially, the behavior of PK at 0 °C can then be described by a simple one-state mechanism; i.e., it conforms to the Michaelis–Menten kinetic scheme.

When the data are analyzed by the Hill equation, it is found that increasing temperature leads to an increase in the Hill coefficient in the absence of Phe, as shown in Figure 2A. This implies that the enzyme exists in more than one state and that equilibria among these states are temperature dependent. In the presence of Phe (at any concentration studied), the Hill coefficient again increases with increasing temperature. At low temperatures, Phe has little effect, and the Hill coefficient remains equal to ~1, but as the temperature is increased, the effect of Phe is strengthened, and the Hill coefficient correspondingly increases to a maximal value of ~2. The

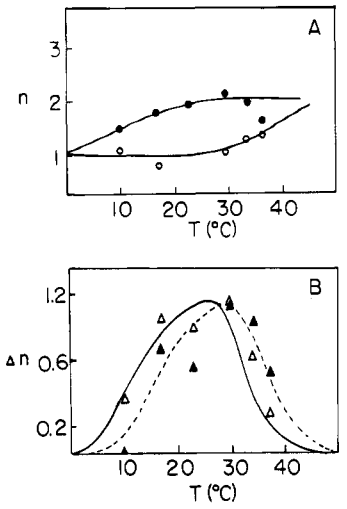


FIGURE 2: (A) Relation between Hill coefficient and temperature. The open and closed circles represent data in the absence and presence of 20 mM Phe, respectively. The lines indicate the trend of the data. (B) Difference in Hill coefficient as a function of temperature. The change in Hill coefficient refers to that observed in the presence and absence of 20 (solid line) and 12 mM Phe (dashed line).

Table I: Fitted Parameters as a Function of Temperature at pH 7.5[a]

| $T$ (°C) | $K_S^R$ | $K_S^T$ | $K_I^R$ | $K_I^T$ | $L$ |
|---|---|---|---|---|---|
| 37 | 109.7 | 2293.0 | 8897 | 330 | 0.086 |
| 34 | 94.4 | 1898.0 | 53184 | 1130 | 0.279 |
| 30 | 57.7 | 847.0 | 15283 | 547 | 0.099 |
| 23 | 51.9 | 580.0 | 7514 | 646 | 0.060 |
| | (0.03–0.07) | (0.05–0.07) | (0.08–0.16) | (0.20–0.34) | (0.50–1.0) |
| 17 | 44.0 | 309.0 | 27764 | 1822 | 0.098 |
| 14 | 56.4 | 166.0 | 84585 | 6371 | 0.026 |
| 10 | 26.2 | 97.2 | 16035 | 1565 | 0.074 |

[a] Binding constants are expressed in micromolar. Numbers in parentheses are simulated errors associated with each of these parameters expressed as a fraction of the parameter at 23 °C and are taken from Consler et al. (1989).

Hill coefficient attains its greatest value at a much lower temperature in the presence of Phe than in the absence of this allosteric ligand. This coupled effect of Phe and temperature can be examined by determining the change in the Hill coefficient as a function of temperature. The change in the Hill coefficient is tabulated by subtracting the value observed in the absence of Phe from that at a constant Phe concentration. The results of such an analysis are shown in Figure 2B. The relationship appears to be almost a symmetric distribution, wherein the difference in the Hill coefficient is zero at low temperature, increases with increasing temperature, and finally decreases to zero at higher temperatures. The temperature at which the maximum difference in the Hill coefficient is observed changes with the Phe concentrations, e.g., in the presence of 20 and 12 mM Phe, the temperature is 25 and 30 °C, respectively.

In order to identify quantitatively the effects of temperature and Phe, the kinetic results were analyzed by nonlinear least-squares fitting in accordance with eq 4, which describes a two-state model of allosteric regulation involving an allosteric inhibitor. Typical results of this analysis are shown in Figure 1. It is evident that the residuals for the complete set of curves are randomly distributed, although in a small number of individual curves the residuals are not random. The resulting equilibrium constants are summarized in Table I. The most reliable parameter of the five equilibrium constants is $K_S^R$. This parameter is the one most directly measured by steady-state kinetics, because it refers to the binding of PEP to the "active", R, state of PK. Therefore, it is the most sensitive parameter in describing the kinetic velocity vs substrate concentration curves. The binding constant of substrate to the "inactive", T, state of PK and the two binding constants for Phe binding to the two states of PK are less directly accessed by this experimental measurement and, therefore, are less reliable and have a higher degree of error. The equilibrium constant governing the state change is the least directly measured parameter and accordingly is the least precise of the five equilibrium constants that are fitted. A more detailed analysis of the reliability of the determined parameters is presented in the Appendix. An error analysis of the approach has been reported [Consler et al. (1989)], and the results are summarized in Table I.

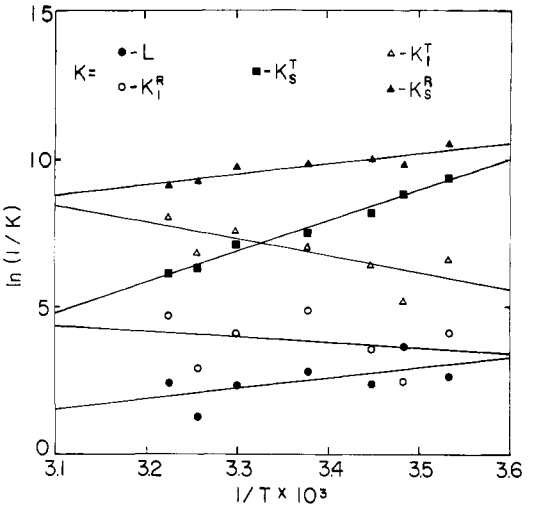


FIGURE 3: Van't Hoff plots of various equilibrium constants resolved from steady-state kinetic data.

It is readily seen in Table I that the affinity of PK (both the R-state and the T-state) for PEP increases with decreasing temperature. Less obvious, due to the error in the estimates, is the fact that the affinity of both states of PK for Phe decreases with decreasing temperature and that $L$ tends to favor the R-state at lower temperatures. These trends are more easily envisioned when the data are presented in the form of van't Hoff plots, as shown in Figure 3. The pertinent thermodynamic parameters involved in each particular interaction were extracted according to

$$\Delta G = -RT(\ln K) = \Delta H - T\Delta S$$

$$\ln K = -(\Delta H/R)(1/T) + \Delta S/R \qquad (6)$$

where $R$ is the gas constant, $T$ is the temperature in kelvin and $\Delta G$, $\Delta H$, and $\Delta S$ are the changes in free energy, enthalpy, and entropy of the system. The slope of the van't Hoff plot is $-(\Delta H/R)$ and the $y$-intercept is $\Delta S/R$.

To the best approximation, all of the van't Hoff relationships are linear within the temperature range studied, as shown in Figure 3. This indicates that there is no significant change in heat capacity involved in any of these interactions. The values for the thermodynamic parameters derived from the van't Hoff analysis are summarized in Table II. This analysis indicates that the state change is an enthalpically driven process, due to the negative value for the enthalpy change. The free energy change involved in this structural transition is small, ~1.5 kcal/mol, and this is consistent with previous estimates for the difference in free energy between the two conformers of PK as determined by the energy of denaturation

Table II: Summary of Thermodynamic Parameters for PK

| reaction | $\Delta H$ (kcal/mol) | $\Delta S$ (eu) | $\Delta\bar{\nu}$ | ln $K^a$ |
|---|---|---|---|---|
| PK isomerization ($L$) | −7.0 ± 4.0 | −18.6 ± 1.5 | 0.1 ± 0.5 | 4.1 |
| substrate binding to R-state ($K_S^R$) | −7.1 ± 0.2 | −4.7 ± 0.4 | 0 ± 0.3 | 8.4 |
| substrate binding to T-state ($K_S^T$) | −19.8 ± 0.5 | −52.0 ± 0.5 | 0.5 ± 0.3 | 8.1 |
| inhibitor binding to R-state ($K_I^R$) | 4.0 ± 0.5 | 21 ± 2.0 | −1.0 ± 0.7 | 0.5 |
| inhibitor binding to T-state ($K_I^T$) | 12.1 ± 1.0 | 54 ± 2.0 | −0.2 ± 0.1 | 6.1 |

[a] Extrapolated values when ln $a_x$ is 0.

of the two states (Consler et al., 1989). These thermodynamic parameters also reveal that the binding of PEP to PK is an enthalpy-driven process, whereas the binding of Phe to PK is an entropy-driven one. These conclusions are based upon the large negative values for $\Delta H$ obtained in the analysis of PEP binding constants and the large positive values for $\Delta S$ obtained in the analysis of Phe binding constants. This simple description of the driving force of binding holds true for the binding of a particular ligand to either state of PK. That is, it is the identity of the ligand that correlates with the thermodynamic characterization of the binding, not the conformational state of the enzyme.

The binding of the allosteric inhibitor to the T-state is characterized by large positive entropic and enthalpic changes, resulting in a relatively small negative free energy change. A similar entropy–enthalpy compensation occurs for the binding of Phe to the R-state, resulting in an even smaller negative free energy change. Thus, the binding of Phe is relatively weak with a preference for the T-state.

In an effort to measure more directly the equilibrium constant governing the binding of PEP to PK, the method of gel partitioning was used to monitor this parameter. These experiments were performed as a function of temperature and as a function of Phe concentration in an attempt to define the linkage between these two ligands of interest. The binding isotherms of PEP at pH 7.5, 23 °C, in the presence of 5 mM Phe are shown in Figure 4A, whereas those at pH 7.5, 37 °C, in the presence of 0 and 10 mM Phe, are shown in Figure 4B. The binding results are compared to the expected binding isotherms based on the parameters determined by steady-state kinetic measurement and nonlinear parameter estimation. Considering the low precision of the data, the binding results qualitatively are in agreement with the kinetic data. That is, the presence of Phe caused a decrease in the affinity of PK for PEP, and this effect was more significant at elevated temperature. The binding data were fitted by a nonlinear least-squares procedure to eq 5, which describes the binding of PEP to the two states of PK in the presence of Phe. The resulting parameter estimates for $K_S^R$ determined from the binding data were consistently lower than those obtained from the fitting of steady-state kinetic data, e.g., at 23 °C. $K_S^R$ assumes values of 32 and 50 $\mu$M as determined from direct binding and steady-state kinetics, respectively. The difference in $K_S^R$ determined by these types of data was only noticeable in the data sets measured in the presence of high Phe concentration. The difference is small, however, and especially when the error involved in the binding measurements is considered, it can be concluded that these binding isotherms yield results that are consistent with those determined by steady-state kinetics.

To further probe the regulatory mechanism of PK, a series of studies was conducted as a function of salt concentrations. In the presence of increasing concentrations of KCl, the

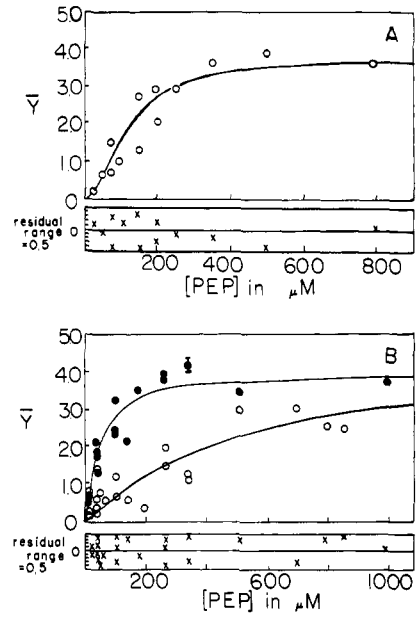


FIGURE 4: PEP binding isotherms as a function of temperature and Phe concentration: (A) at 23 °C in the presence of 5 mM Phe; (B) at 37 °C in the presence of 0 (●) and 10 mM Phe (○). The lines are simulated curves using the appropriate parameters summarized in Table I.

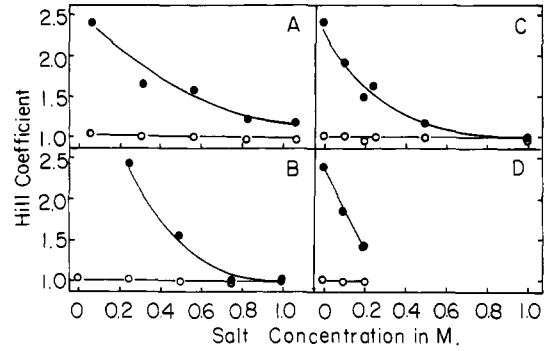


FIGURE 5: Relation between Hill coefficient and salt concentration. The open and closed circles represent data in the absence and presence of 20 mM Phe, respectively. Salt: (A) KCl; (B) NaCl; (C) $Na_2SO_4$; (D) NaSCN.

inhibitory behavior induced by Phe is obliterated. This is evidenced by the superimposition of activity vs substrate concentration curves at high KCl concentration at all Phe concentrations examined (up to 20 mM). The Hill coefficient describing the substrate dependencies at 20 mM Phe decreases as the concentration of KCl is increased, until it assumes a value equal to that for data in the absence of Phe, as shown in Figure 5A. In addition, the values of $K_m$ determined in the presence or absence of 20 mM Phe approach each other as the KCl concentration is raised and assume equal magnitudes as the concentration of KCl is increased to above 0.5 M, as shown in Figure 6A. These kinetic data were further analyzed to obtain the various equilibrium constants as in eq 4. $K_S^R$ increases while $K_S^T$ decreases with increasing KCl concentration, resulting in a ratio of 1 for $K_S^T/K_S^R$ at 1.072 M KCl, as summarized in Table III. While the values for $K_S^R$ and $K_S^T$ converge with increasing KCl concentration, the values for $K_I^R$ and $K_I^T$ diverge. It is interesting to note that at 1.072 M KCl the value of $L$ is significantly higher, i.e., in favor of the T-state.

The effect of the neutral salt NaCl was also tested in order to clarify the results obtained with KCl. The relation between activity and substrate concentration did not reveal any sig-

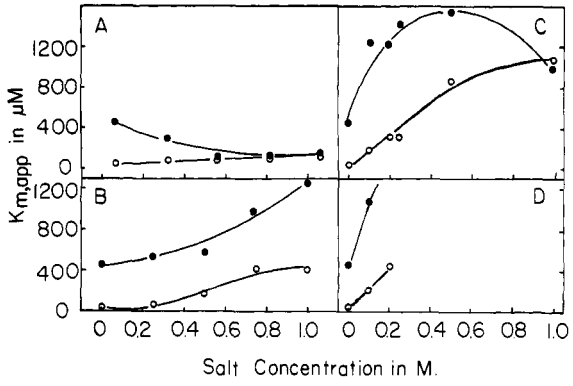


FIGURE 6: Relation between $K_{m,app}$ and salt concentration. The open and closed circles represent data in the absence and presence of 20 mM Phe, respectively. Salt: (A) KCl; (B) NaCl; (C) $Na_2SO_4$; (D) NaSCN.

moidicity at high salt concentrations even in the presence of Phe, and the Hill coefficient was seen to decrease to a value of unity as the NaCl concentration was increased, as shown in Figure 5B. The values of $K_M$ determined in the presence or absence of 20 mM Phe vary as a function of NaCl, but they parallel each other and do not attain the same value, even at 1.0 M NaCl, as shown in Figure 6B. The results of fitting these data to eq 4 show that $K_S^R$ increases with increasing salt concentration while $K_S^T$ is not significantly perturbed. The net result is that the values for $K_S^R$ and $K_S^T$ start to approach each other in magnitude as NaCl concentration is increased, but the values for these binding constants reach a plateau and remain significantly different in magnitude, as summarized in Table III. The ratio of $K_I^T$ and $K_I^R$ does not decrease monotonically like that in KCl, but it appears to approach a minimum value at 0.25 M. In addition, NaCl does not seem to perturb the value of $L$.

The effect of $Na_2SO_4$ was also tested. The anion is characterized as a stabilizer of proteins, and it has properties which tend to increase the water structure in the bulk solvent. It is classified at one extreme of the Hofmeister series and has been referred to as a "kosmotroph" or water-structure maker (Collins & Washabaugh, 1985). $Na_2SO_4$ was found to eliminate the inhibitory effect of Phe at high concentrations, just as observed with other salts. The Hill coefficient decreased to a value of 1.0 as the concentration of this salt was increased, as shown in Figure 5C, and was very similar to the effect observed in KCl. This salt did not have the same effect on the $K_M$, as seen in Figure 6C. In fact, the apparent affinity for PEP was decreased as the concentration of $Na_2SO_4$ was increased in both the presence and absence of 20 mM Phe. The $K_M$ values become equal at high salt concentration, again indicating a relaxation to a simple one-state model. This salt exerted its effect by decreasing the affinity of both R- and T-states of PK for the substrate PEP and inhibitor Phe, as summarized in Table III. The state-specific binding constants were all affected in parallel; i.e., both substrate and inhibitor affinities are decreased with increasing salt concentration. It appears that, in this case, the Phe inhibition was eliminated by a decreased affinity of the enzyme for the effector.

Another sodium salt, NaSCN, the anion of which is classified at the opposite extreme of the Hofmeister series (compared to sulfate), has chaotropic properties and has been called a water-structure breaker (Collins & Washabaugh, 1985). Thiocyanate tends to destabilize protein structure as a mild denaturant. The specific activity of PK is most sensitive to this salt. Because of this, only a small range of NaSCN concentrations was examined. This salt also decreases the sigmoidicity in the substrate dependencies in the presence of Phe and the Hill coefficient, as shown in Figure 5D. The effect of thiocyanate on the $K_M$ is seen in Figure 6D and is similar to that seen with $Na_2SO_4$; i.e., in either the presence or absence of 20 mM Phe, the value of $K_M$ increases with higher salt concentration. This again indicates that the affinity for the substrate may be decreased. The various binding parameters for substrate and inhibitor appear to be affected in a similar manner and lowered affinity for PEP and Phe for both states with increasing salt concentrations, as summarized in Table III.

The data on salt dependence were further analyzed by the Wyman linked function theory (Wyman, 1964):

$$-(\partial \ln K/\partial \ln a_X)_{T,P} = \Delta\bar{\nu}_X \quad (7)$$

where $K$ is the apparent dissociation constant affected by ligand X at an activity of $a$ and $\Delta\bar{\nu}_X$ is the difference in binding of ligand X to the two end states of the reactions under investigation, i.e., liganded and unliganded R- and T-states of PK. Values of activity coefficients for salts are obtained from the literature (Vanysek, 1990). The effects of salts on the various equilibrium constants were analyzed, as shown in Figure 7. Based on the results of an analysis on the reliability of parameters determined as presented in the Appendex and the precision of parameters by Consler et al. (1989), the data are deemed not precise enough to warrant an analysis on the effect of individual salts on these equilibrium constants; hence, the salt-dependence data were analyzed in total by linear least-squares analysis assuming equal weight on each data point. The values of $\Delta\bar{\nu}$ for each equilibrium reaction are summarized in Table II. It is evident that $L$ and $K_S^R$ are not sensitive to salt concentrations since the values of $\Delta\bar{\nu}$ are $0.1 \pm 0.5$ and $0 \pm 0.3$, respectively. It is interesting to note that the other equilibria are more susceptable to perturbation by salt which exerts its differential effect to these equilibria both qualitatively and quantitatively. $K_S^T$ is favored by higher salt concentrations whereas $K_I^T$ and $K_I^R$ are not. $K_I^R$ is more significantly inhibited; i.e., the affinity of substrate is favored by increasing salt concentration, whereas salt has an opposite effect on the binding of inhibitor.

## DISCUSSION

After the energetics of the allosteric regulation of muscle pyruvate kinase are elucidated, it is interesting to note that a pattern has emerged. The effect of low temperature is to diminish the differences in binding affinities of both PEP and Phe for the two states of the enzyme; e.g., the ratio of $K_S^T/K_S^R$ decreases from 21 to 4 when the temperature decreases from 37 to 10 °C, while the ratio of $K_I^T/K_I^R$ changes from 0.04 to 0.1. PEP binding is strengthened by low temperature. It will bind to the R- or T-state of PK with almost equal affinity. Another observation is that the binding of PEP is enthalpically driven, whereas Phe binding is entropically driven, regardless of the conformational state of the enzyme. The validity of these observations depends on the level of confidence on the estimated parameters. A systematic study was conducted to determine the tolerance of variation, and the results are shown in the Appendix. The values for $K_S^R$ and $K_I^T$ are determined with high degree of confidence, whereas those for $K_I^R$ and $K_S^T$ should be accepted as good estimates of the minimal values for these parameters. The values for $L$ are the least reliable. The conclusion of this series of tests for precision is consistent with an earlier one (Consler et al., 1989). Hence results from this study can be accepted to reflect on the intrinsic behavior of PK.

Table III: Fitted Parameters as a Function of Salt Concentration[a]

| [salt] | $L$ | $K_S^R$ | $K_S^T$ | $K_S^T/K_S^R$ | $K_I^R$ | $K_I^T$ | $K_I^T/K_I^R$ ($\times 10^3$) |
|---|---|---|---|---|---|---|---|
| KCl | | | | | | | |
| 0.072 | 0.085 | 38 | 539 | 14 | 16 000 | 655 | 41 |
| 0.322 | 0.001 | 94 | 508 | 5 | 180 000 | 1172 | 7 |
| 0.572 | 0.003 | 94 | 237 | 3 | 140 000 | 2296 | 2 |
| 0.822 | 0.003 | 110 | 251 | 2 | 540 000 | 3256 | 6 |
| 1.072 | 0.117 | 155 | 166 | 1 | 1 000 000 | 327 | 0.3 |
| NaCl | | | | | | | |
| 0.000 | 0.085 | 38 | 539 | 14 | 16 000 | 655 | 41 |
| 0.250 | 0.020 | 99 | 869 | 9 | 670 000 | 1869 | 3 |
| 0.500 | 0.004 | 218 | 936 | 4 | 62 000 | 2846 | 5 |
| 0.750 | 0.024 | 409 | 735 | 2 | 99 000 | 6192 | 63 |
| 1.00 | 0.023 | 424 | 778 | 2 | 23 000 | 4064 | 177 |
| $Na_2SO_4$ | | | | | | | |
| 0.000 | 0.085 | 38 | 539 | 14 | 16 000 | 655 | 41 |
| 0.100 | 0.004 | 229 | 1561 | 7 | 38 000 | 1044 | 28 |
| 0.200 | 0.037 | 328 | 1033 | 3 | 34 000 | 3392 | 100 |
| 0.250 | 0.005 | 338 | 1310 | 4 | 36 000 | 1656 | 46 |
| 0.500 | 0.001 | 819 | 1511 | 2 | 39 000 | 2444 | 63 |
| 1.000 | 0.745 | 1042 | 982 | 1 | 15 000 | 218 | 15 |
| NaSCN | | | | | | | |
| 0.000 | 0.085 | 38 | 539 | 14 | 16 000 | 655 | 41 |
| 0.100 | 0.012 | 215 | 1153 | 5 | 25 000 | 1397 | 56 |
| 0.200 | 0.088 | 395 | 1313 | 3 | 150 000 | 6028 | 40 |

[a] Concentrations of salts are in molar, and binding constants are expressed as micromolar.

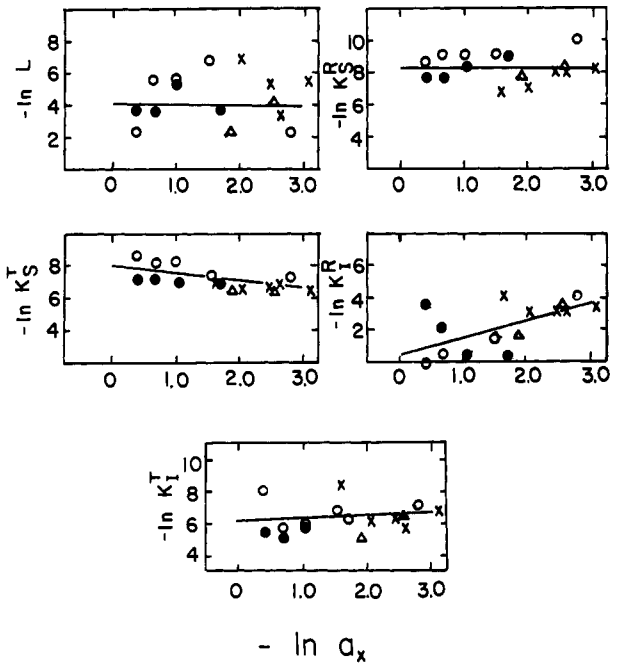


FIGURE 7: Linked function relations between various equilibrium constants and activity of salts. Salt: (○) KCl; (●) NaCl; (×) $Na_2SO_4$; (△) NaSCN.

It is useful to examine the quantitative data shown in Tables I and II 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 behavior at higher temperatures. Under these experimental conditions one needs only to consider the interplays among $K_S^R$, $K_S^T$, and $L$. Between the temperature range of 14–37 °C, the temperature dependence of $L$ indicates a favoring of the T-state at increasing temperatures. Binding of PEP to the R-state is consistently stronger than that to the T-state. Actually, the differences between the values of $K_S^R$ and $K_S^T$ increased with increasing temperature. At high temperature binding of PEP to the R-state becomes even more favorable; for example, the ratios of $K_S^T/K_S^R$ are 3.7 and 20.9 at 10 and 37 °C, respectively. The net result with the addition of substrate is that PEP will bind to the R-state, which is active, and shift the equilibrium toward the R-state. As a consequence of a shift in the distribution of states in PK from the inactive T-state to the active R-state, sigmoidal kinetics are observed. Hence, the increased sigmodicity observed at increasing temperature is a consequence of the greater distribution of PK in the T-state and greater difference in the affinities of PEP for the two states with a preference for the R-state, resulting in a net shift of PK from T- to R-state upon interacting with PEP. At low temperatures the basic kinetics conform to that of Michaelis–Menten. The observation is a reflection on the decrease in the ratio of $K_S^T/K_S^R$ and subsequently a decrease in efficiency in shifting the equilibrium on the state distribution of PK.

In the presence of Phe, one needs to consider the contributions from $K_I^R$ and $K_I^T$ also. With increasing temperature, the difference between $K_I^R$ and $K_I^T$ is enhanced, with binding of inhibitor to the T-state always being more favorable. Thus, the presence of Phe leads to a preferential binding of the inhibitor to the T-state. This preferential interaction is enhanced by increasing temperature because the ratio of $K_I^T/K_I^R$ increases in favor of $K_I^T$, and $L$ is in favor of a distribution toward the T-state. Upon addition of substrate, PEP, which binds more favorably to the R-state, an ultimate shift of the state distribution would result with increasing amount of PEP added. In accordance with the formulation of Monod et al. (1965), such a shift in the distribution of state would lead to sigmoidal relationships in the plots of velocity vs substrate concentration. Such expectation is matched by the experimental observations (Figures 1 and 2).

The same basic rationale is applied to the kinetic observations in the presence of salts. The general effect of salts is to decrease sigmoidicity with increasing salt concentration. Using Figure 7, one may extrapolate the data to ln $a_X = 0$, i.e., to a concentration of about 1 M, and the values for the various equilibrium constants can be obtained, as summarized in Table II. It is evident that the effect of high salt is to reduce the difference in $K_S^T$ and $K_S^R$ so that they are essentially identical since ln $K_S^T$ and ln $K_S^R$ assume values of 8.1 and 8.4, respectively. In contrast, the effect on $K_I^T$ and $K_I^R$ is to accentuate the difference so that the binding of the T-state is favored with a concomitant reduction of affinity to the R-

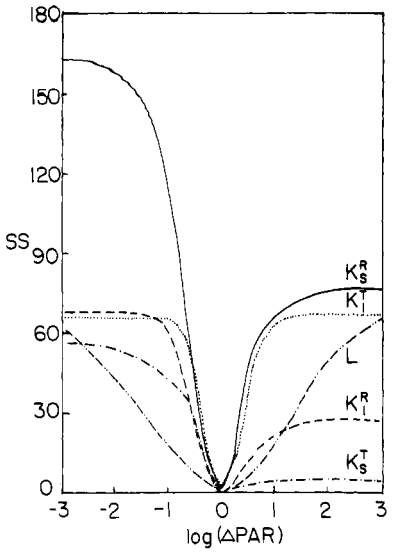


FIGURE 8: Tolerance of variation for the equilibrium constants. The data set is that for pH 7.5 and 37 °C. The curves represent the sensitivity of each equilibrium constant to variations in absolute value.

state since ln $K_I^T$ and ln $K_I^R$ assume values of 6.1 and 0.5, respectively. In the absence of Phe and at high salt, the isomerization reaction of PK is in favor of the R-state. PEP binds equally to the R- or T-state; hence, with the addition of PEP no shift of state will be observed. As a consequence, hyperbolic kinetics will be observed as shown by Figure 5. In the presence of Phe, binding to the T-state is highly favored and would lead to a more favorable distribution of PK in the T-state. Upon addition of PEP, the substrate has equal affinity for both R- and T-states; hence, again no significant shift in state distribution is expected. The activity observed should reflect only that of the R-state. Consequently, the kinetics should conform to a hyperbolic behavior, an expectation supported by the actual experimental results.

In spite of the apparent success in presenting a consistent model of the regulatory mechanism of muscle PK, a word of caution is necessary. An assumption was involved in the analysis of the kinetic data; i.e., there is no cooperative effect between bindings of PEP in the presence of ADP. The small difference between the values of $K_d$ (PEP) determined by steady-state kinetics and direct ligand binding may be a reflection of the invalidity of this assumption. The uncertainty of the present study does not enable one to make a definitive conclusion. Future experiments with higher precision and accuracy may provide the necessary data.

Having examined the effect of temperature and salt on the regulatory mechanism of muscle PK, it is useful to review the effects of various perturbations on the different linked equilibria that govern the activity of the enzyme. Consler et al. (1990) have shown that pH exhibits differential effects on these linked equilibria. Binding of substrate is weakly or not at all linked to proton concentration, whereas the binding of inhibitor to the T-state is significantly favored by protonation but is only weakly influenced in the binding to the R-state. In this study, both temperature and salt have been demonstrated to differentially influence the binding of substrate and inhibitor. Low temperature enhances the binding of substrate to either conformational state of PK, whereas the binding of inhibitor is weakened. In addition, high salt concentration again exerts a differential effect on the bindings of substrate and inhibitor; namely, substrate binding is enhanced while inhibitor binding is weakened. Hence, by application of these different solution conditions to perturb the inhibitory reaction of Phe on PK activity, it becomes evident that the control of PK activity is a fine balance of multiple linked equilibria which are differentially perturbed by each solution variable studied.

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## APPENDIX

*Determination of the Tolerance of Variation.* It is gratifying to obtain consistent results derived from different approaches. There is no reason to suspect that the curve-fitting procedure using eq 4 would result in parameter estimates that are unrealistic, or that the model is too soft, especially since the model is quantitatively verified by independent measurements (Heyduk et al., 1992). However, when the curve-fitting procedure allows a large number of parameters to float, one cannot be certain that the final estimate for each parameter truly represents the best value for that parameter. In an effort to examine the reliability of the fitted parameters, the effect of varying each parameter individually on the sum of the squares of the residuals of the data set was determined.

The shape of the function describing the derivative factor vs the resulting sum of the squares of the residuals indicates the degree of tolerance to variation of that particular parameter. A sharp, steep function, for instance, means that the model tolerates very little deviation in that parameter, and the final estimate obtained is a reliable value for that parameter. On the other hand, a broad, shallow function shows that the model can tolerate a wider range of estimates, indicating that particular parameter may be less accurately determined. This function will be referred to as the tolerance of variation for a particular parameter. It must be mentioned that, by definition, the final results of any particular curve fitting have attained the minimal sum of squares of the residuals for each parameter; i.e., it has found the "best fit." So, this analysis is not intended to test the fitting procedure; it does minimize the error for each data set. The reason for this analysis is to determine if this "best fit" contains estimates for parameters that could be nearly equally well fit by significant variations from those estimates.

One example of the results for this analysis is seen in Figure 8. The results are plotted as the logarithm of the derivative factor vs the resulting sum of the squares of the residuals. These results refer to the data obtained at 37 °C, but the tolerance of variation functions for each parameter was very similar for data sets obtained at other temperatures.

$K_S^R$ and $K_I^T$ seem to tolerate very little variation in their magnitudes. Either increase or decrease in the absolute values for these two parameters results in the propagation of large errors; i.e., the sum of the squares of the residuals increases sharply on both sides of the best estimate. This is an expected result, since these are two of the more directly measured parameters by the experimental protocol.

$K_S^T$ and $K_I^R$ exhibit different degrees of tolerance depending on the direction of changes in their values. They can tolerate variations with an increase in values, but the goodness of fit cannot withstand a decrease in magnitude. This means that there is a threshold value for that parameter above which deviations cause no significant change in the sum of the squares of the residuals. This is particularly true for $K_S^T$, the binding constant for substrate binding to the T-state. The reason for the phenomenon is that this parameter is a dissociation constant, and as such, it represents the inverse of the ligand's

affinity for that state of the enzyme. This parameter is found only in the denominator of terms in eq 4; thus, as its value increases (representing a decrease in affinity), the value of these terms approaches zero. Hence, values above a certain magnitude result in no significant change in the value of the total equation. A similar but not as extreme a relationship is found for $K_1^R$. These two parameters are fitted fairly reliably; at least their values represent a minimal estimate, because below that threshold, the sum of the squares of the residuals increases rather steeply.

The equilibrium constant for the protein isomerization, $L$, exhibits a symmetrical tolerance of variation, but this particular function is not nearly as steeply defined as those described above. The consequence of this relationship is that $L$ is more tolerable of significant variations in magnitude. Hence, this parameter is probably not very accurately estimated. This is again consistent with the method of measurement; steady-state kinetics alone do not contain very reliable structural information.

The final two parameters representing the catalytic rate constants for the two states of PK are quite reliably estimated by the fitting procedure. $k_2^R$ can tolerate very little variation, especially if it is an increase in magnitude. This is an expected outcome, considering the importance of this parameter in the determination of the value for eq 4, and it is clearly the most directly assayed parameter of the seven. $k_2^T$ is very sensitive to increases in value, but it can tolerate any value below its best estimate. Since this parameter nearly always attains a value close to zero, it can be easily seen why its tolerance of variation has this property. Indeed, the fact that this parameter can tolerate decreases in magnitude tends to support the notion that the T-state is "inactive"; i.e., the catalytic rate constant governing the T-state is nearly equal to zero.

## REFERENCES


Boyer, P. D. (1962) in *The Enzymes* (Boyer, P. D., Lardy, H., & Myrback, K., Eds.) Vol. 6, p 95, Academic Press, New York.

Boyer, P. D., Lardy, H. A., & Phillips, P. H. (1942) *J. Biol. Chem. 146*, 673.

Bucher, T., & Pfleiderer, G. (1955) *Methods Enzymol. 1*, 435–440.

Collins, K. D., & Washabaugh, M. W. (1985) *Q. Rev. Biophys. 18*, 323–422.

Consler, T. G., Uberbacher, E., Bunick, G., Liebman, M. N., & Lee, J. C. (1988a) *J. Biol. Chem. 263*, 2787–2793.

Consler, T. G., Uberbacher, E., Bunick, G., Liebman, M. N., & Lee, J. C. (1988b) *J. Biol. Chem. 263*, 2794–2801.

Consler, T. G., Woodard, S. H., & Lee, J. C. (1989) *Biochemistry 28*, 8756–8764.

Consler, T. G., Jennewein, M. J., Cai, G.-Z., & Lee, J. C. (1990) *Biochemistry 29*, 10765–10771.

Heyduk, E., Heyduk, T., & Lee, J. C. (1992) *J. Biol. Chem. 267*, 3200–3204.

Hill, A. V. (1910) *J. Physiol. (London) 40*, 190–224.

Hirose, M., & Kano, Y. (1971) *Biochim. Biophys. Acta 251*, 376–379.

Hofmeister, F. (1888) *Arch. Exp. Pathol. Pharmakol. 24*, 247.

Kayne, F. J. (1973) in *The Enzymes* (Boyer, P. D., Ed.) Vol. 8, p 353, Academic Press, New York.

Kayne, F. J., & Suelter, C. (1965) *J. Am. Chem. Soc. 87*, 897–900.

Kayne, F. J., & Price, N. C. (1972) *Biochemistry 11*, 4415–4420.

Kwan, C.-Y., & Davis, R. C. (1980) *Can. J. Biochem. 58*, 188–193.

Monod, J., Wyman, J., & Changeux, J. P. (1965) *J. Mol. Biol. 12*, 88–118.

Oberfelder, R. W. (1982) Ph.D. Thesis, Department of Biochemistry, St. Louis University.

Oberfelder, R. W., Lee, L. L.-Y., & Lee, J. C. (1984a) *Biochemistry 23*, 3813–3821.

Oberfelder, R. W., Barisas, B. G., & Lee, J. C. (1984b) *Biochemistry 23*, 3822–3826.

Rossmann, M. G., & Argos, P. (1975) *J. Biol. Chem. 250*, 7525–7532.

Vanysek, P. (1990) in *Handbook of Chemistry and Physics* (Lide, D. R., Ed.) pp 5-99–5-100, CRC Press, Boca Raton, FL.

Wyman, J. (1964) *Adv. Protein Chem. 19*, 224–285.

Yphantis, D. A. (1964) *Biochemistry 3*, 297–317.