Articles

Calorimetric Demonstration of the Potential of Molecular Crowding To Emulate the Effect of an Allosteric Activator on Pyruvate Kinase Kinetics†

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ABSTRACT: A method based on isothermal calorimetry is described for the direct kinetic assay of pyruvate kinase. In agreement with earlier findings based on the standard coupled assay system for this enzyme in the presence of a fixed ADP concentration, the essentially rectangular hyperbolic dependence of initial velocity upon phosphoenolpyruvate concentration is rendered sigmoidal by the allosteric inhibitor phenylalanine. This effect of phenylalanine can be countered by including a high concentration of a space-filling osmolyte such as proline in the reaction mixtures. This investigation thus affords a dramatic example that illustrates the need to consider potential consequences of thermodynamic nonideality on the kinetics of enzyme reactions in crowded molecular environments such as the cell cytoplasm.

There have been several theoretical investigations that have emphasized the need for caution about considering kinetic parameters obtained in dilute solution to describe the enzyme reaction in crowded molecular environments (*1*−*4*). However, a lack of sufficiently striking experimental illustrations to reinforce the theoretical inferences has led to widespread disregard of those warnings. In an endeavor to highlight the experimental relevance of the theoretical predictions, we present results from a kinetic study of rabbit muscle pyruvate kinase which show that the allosteric inhibitory effect of phenylalanine (*5*) can be suppressed by the effects of thermodynamic nonideality arising from the presence of a high concentration of a small inert cosolute: proline has been used as the osmolyte in this instance. The kinetic study has been based on a direct calorimetric assay for pyruvate kinase to avoid the need for distinguishing between effects of thermodynamic nonideality on the pyruvate kinase and lactate dehydrogenase reactions in the standard coupled assay (*6*).

EXPERIMENTAL PROCEDURES

Materials. Rabbit muscle pyruvate kinase, ADP (disodium salt), phosphoenolpyruvate, L-proline, L-phenylalanine, and Trizma base were all Sigma-Aldrich products. The enzyme was routinely dialyzed against Tris−chloride buffer (0.05 M Tris/HCl−0.10 M KCl−0.01 M $MgCl_2$), pH 7.5, to remove ammonium sulfate and to equilibrate the pyruvate kinase with the buffer to be used in subsequent experiments. Concentrations of these stock enzyme solutions (approximately 1 mg/mL) were determined spectrophotometrically on the basis of an absorption coefficient ($A^{1\%}_{1\,cm}$) of 5.4 at 280 nm (*7*).

Enzyme Kinetic Experiments. The activity of rabbit muscle pyruvate kinase was assessed on the basis of the thermal power required to maintain constant temperature (20 °C) during the initial stages of enzyme reaction in the cell of a Microcal isothermal calorimeter (MCS-ITC unit). In the initial series of experiments, the assay mixture comprised 4 mM ADP, 0.001−1.14 mM phosphoenolpyruvate, and enzyme (0.1 μg/mL) either in the above Tris−chloride buffer or in the same buffer supplemented with 6 mM phenylalanine. In a second series of experiments, the buffer was supplemented additionally with 0.1 M proline (occasionally 0.1 M glycine, sorbitol, or sucrose).

Although use of calorimetry for the characterization of enzyme kinetics by means of progress curves and the integrated rate equation has been recommended (*8*, *9*), the collection of steady-state (initial velocity) information (*10*, *11*) is the preferred procedure for the present investigation. Because the thermal power measurement monitors not only the heat of reaction but also any heat of dilution, there is clearly a requirement either to ensure that the heat of dilution is essentially zero, or to conduct the experiment in a manner that allows unequivocal identification of the reaction contribution. Although addition of the last reactant (enzyme) in a medium different from that present in the calorimeter cell inevitably gives rise to a heat of dilution, the thermal power contribution to combat the heat of dilution is transitory. Consequently, the contribution from the steady-state enzyme reaction is recognized as a time-independent rate of heat flow, dQ/dt (Figure 1). The difference between this steady-state

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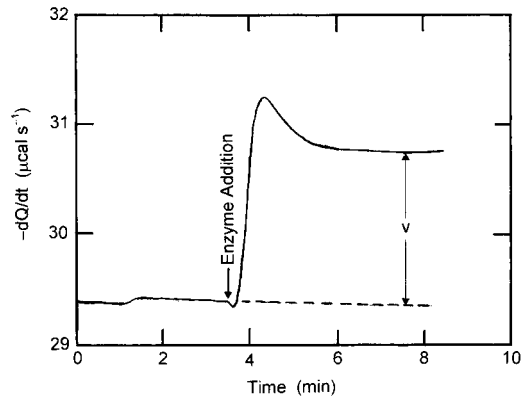

FIGURE 1: Calorimetric trace (heat flow dQ/dt as a function of time) obtained at 25 °C after addition of rabbit muscle pyruvate kinase (10 μL, 20 μg/mL) in Tris−chloride buffer, pH 7.5, to the mixture in the isothermal calorimeter cell comprising phosphoenolpyruvate (0.72 mM), ADP (4 mM), and proline (0.1 M) in the same Tris−chloride buffer. The initial change in dQ/dt reflects compensation of the heat of dilution, after which dQ/dt attains a steady-state value corresponding to the initial velocity of enzyme reaction.

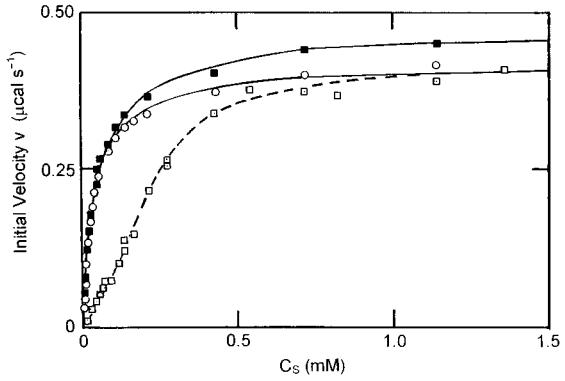

FIGURE 2: Calorimetric measurements of the dependence of initial velocity upon phosphoenolpyruvate concentration in mixtures containing fixed concentrations of ADP (4 mM) and rabbit muscle pyruvate kinase (0.1 μg/mL) in Tris−chloride buffer, pH 7.5 (○), in the same buffer with phenylalanine (6 mM) as an additional reactant (□), and in the latter reaction mixtures supplemented with 0.1 M proline (■). Solid lines denote best-fit rectangular hyperbolic relationships, whereas the broken line merely emphasizes the sigmoidal nature of the saturation curve.

value of dQ/dt and the baseline value prior to enzyme addition thus corresponds to the initial velocity pertaining to the concentration of variable substrate (phosphoenolpyruvate) present in the reaction mixture.

Difference Sedimentation Velocity. In accordance with the established protocol (*12*), solutions of pyruvate kinase (1.0−1.5 mg/mL) in the Tris−chloride buffer (pH 7.5) and in the same medium supplemented with 6 mM phenylalanine were centrifuged simultaneously at 20 °C and 35 000 rpm in a Beckman XL-I ultracentrifuge. In another series of experiments, the Tris−chloride buffer also contained 0.1 M proline. Concentration distributions, monitored spectrophotometrically at 280 nm, were recorded at 10-min intervals for each cell, there being a delay (Δt) of 5 min between the recording of distributions for enzyme alone (cell 1) and enzyme−phenylalanine mixture (cell 2).

Results obtained at angular velocity ω were analyzed in terms of the expression (*13*):

$$\ln(r_1)_{t_1} - \ln(r_2)_{t_2} = (s_1 - s_2)\omega^2 t_1 + [\ln(r_m)_1 - \ln(r_m)_2 - s_2\omega^2\Delta t] \quad (1)$$

where r_1 and r_2 denote the respective boundary positions (midpoints) in cells 1 and 2, whereas $(r_m)_1$ and $(r_m)_2$ are the corresponding positions of the air−liquid menisci: t_1 refers to the duration of centrifugation pertinent to recording of the distribution for cell 1. The slope of the dependence of $[\ln(r_1)_{t_1} - \ln(r_2)_{t_2}]$ upon $\omega^2 t_1$ thus defines $(s_1 - s_2)$, the difference (Δs) between the sedimentation coefficients for pyruvate kinase in the absence and presence of phenylalanine.

RESULTS AND DISCUSSION

Although rabbit muscle pyruvate kinase is not usually considered to be an allosteric enzyme, the system meets the requirements for such a classification in the sense that there is a preexisting equilibrium between isomeric states of the enzyme (*14*, *15*). The essentially Michaelis−Menten kinetic behavior exhibited by rabbit muscle pyruvate kinase (*5*, *16*, *17*) reflects an 11-fold preponderance of the enzymically active isomer (*14*, *15*), whereupon the extent of displacement of the equilibrium position by preferential binding of phosphoenolpyruvate to the active form is insufficient to effect detectable deviation from Michaelis−Menten behavior. On the other hand, supplementation of the assay mixtures with phenylalanine, which binds preferentially to the other isomeric state (*14*, *15*), gives rise to a sigmoidal dependence of initial velocity upon phosphoenolpyruvate concentration (*5*). Because the enzymically more active state of rabbit muscle pyruvate kinase is slightly smaller than its less active counterpart (*5*, *13*−*15*), the outcome of molecular crowding by an inert cosolute should be an increased proportion of active isomer and hence a reversion toward Michaelis−Menten kinetic behavior. Displacement of the preexisting equilibrium in the direction of the smaller isomeric state of pyruvate kinase has been demonstrated previously with sucrose as the molecular crowding agent (*15*), but the kinetic consequences of the displacement remain to be illustrated.

Results of the present kinetic study of rabbit muscle pyruvate kinase with a fixed concentration of ADP (4 mM) and phosphoenolpyruvate as variable substrate are summarized in Figure 2. In keeping with the earlier studies (*5*, *17*), the kinetics in the absence of phenylalanine (○) are well-described by the Michaelis−Menten equation with $K_m = 42$ (± 2) μM, whereas the inclusion of 6 mM phenylalanine gives rise to distinctly sigmoidal kinetic behavior (□). This allosteric inhibition of rabbit muscle pyruvate kinase is essentially suppressed by supplementing the reaction mixtures with 0.1 M proline (■). Effects of other cosolutes have not been investigated in such detail, but it is relevant that similar extents of activation were observed with the same concentration of glycine or sorbitol. On the other hand, enzyme activity was inhibited by 0.1 M sucrose.

Classical interpretation of the results in Figure 2 would require the designation of proline as an allosteric activator of pyruvate kinase, but the activation is merely reflecting physical displacement of the preexisting isomerization equilibrium in favor of the smaller, more active isomeric state of the enzyme. This effect of thermodynamic nonideality, which has already been demonstrated with sucrose as the space-filling solute (*15*), is confirmed by difference sedimentation velocity studies (Figure 3). In agreement with

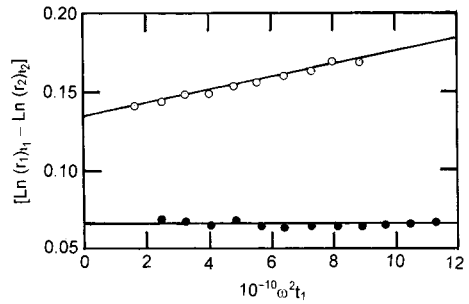

FIGURE 3: Difference sedimentation velocity plots obtained in studies at 35 000 rpm and 20 °C of the effect of phenylalanine on the sedimentation coefficient of rabbit muscle pyruvate kinase in the absence (○) and presence (●) of 0.1 M proline. Results are plotted in accordance with eq 1.

earlier studies (*5*, *13*, *15*), the sedimentation coefficient of rabbit muscle pyruvate kinase is decreased demonstrably in the presence of phenylalanine—a factor indicated by the positive slope of the plot of results according to eq 1 (Figure 3). However, the inclusion of proline (0.1 M) in the two samples subjected to sedimentation velocity effectively eliminates the difference in sedimentation coefficient (Figure 3). The effect of molecular crowding has thus opposed the phenylalanine-mediated displacement of the isomeric equilibrium to such an extent that the pyruvate kinase is present solely as the smaller, more active isomer in both centrifuge cells.

There has been a tendency for biochemists to regard enzyme kinetics as a field that is immune from consequences of thermodynamic nonideality because of the low enzyme concentrations that effect catalysis. However, the thermodynamic activity coefficient of a given species depends not only upon its own concentration but also upon those of all other solutes (reacting or nonreacting) in the solution. Under conditions of constant temperature and chemical potential of solvent, the thermodynamic activity coefficient of species i, γ_i, is given by the expression:

$$\gamma_i \approx \exp[2B_{ii}C_i + \sum_{j\neq i} B_{ij}C_j + ...] \qquad (2)$$

where C_i and C_j denote molar concentrations, and where B_{ii} and B_{ij} are osmotic second virial coefficients reflecting physical (excluded volume) interactions between like and dissimilar species, respectively (*18*, *19*). Strictly speaking, because enzyme kinetic experiments are conducted under the constraint of constant pressure (rather than constant chemical potential of solvent), they require description in terms of molal concentrations and activity coefficients (*18*, *19*). However, relatively minor discrepancies should be introduced by resort to descriptions of thermodynamic nonideality based on eq 2, which have the advantage of being expressed in terms of a more familiar concentration scale.

In a conventional kinetic assay, the concentrations of species are indeed sufficiently small for the activity coefficients of all reacting species to be effectively unity, in which case the characterization refers to the system under thermodynamically ideal conditions. However, this situation ceases to apply to characterization of the system in the presence of an unrelated solute at high concentration. For the present isomerizing system, E ⇄ E*, the term in concentration of the inert solute, C_M, is the dominant contributor to the activity coefficients of the two enzyme species, whereupon

$$\gamma_E \approx \exp(B_{EM}C_M) \qquad (3a)$$

$$\gamma_{E^*} \approx \exp(B_{E^*M}C_M) \qquad (3b)$$

$$X_M \approx X \exp[(B_{EM} - B_{E^*M})C_M] \qquad (3c)$$

X_M is the apparent isomerization constant expressed as the appropriate ratio of species concentrations, whereas X is the true thermodynamic constant. On the basis of spherical geometry for enzyme and an uncharged cosolute species, B_{EM} and B_{E^*M} are the covolumes of enzyme isomer and cosolute [$B_{iM} = 4\pi N(R_i + R_M)^3/3$ where N is Avogadro's number and R denotes radius]. The effect of a concentration of inert cosolute (proline) on the present system with $R_E < R_{E^*}$ and hence $B_{EM} < B_{E^*M}$ is therefore to increase the proportion of more active enzyme isomer, E, at the expense of its less active counterpart, E* ($X_M \rightarrow 0$ as $C_M \rightarrow \infty$). To substantiate this conclusion that molecular crowding by proline has the potential to provide a plausible explanantion of the results presented in Figures 2 and 3, we now examine the consequences of thermodynamic nonideality in greater quantitative detail.

In the enzyme kinetic study (Figure 2), the sigmoidal dependence of initial velocity upon phosphoenolpyruvate concentration, C_S, refers to reaction mixtures containing fixed concentrations of ADP (C_T) and phenylalanine (C_I). Under those circumstances, the distribution of enzyme between its two conformational states is describable in terms of a constitutive isomerization constant, X, defined as the ratio of the sum of the concentrations of all species with enzyme in the E state to the corresponding sum of concentrations of species with enzyme in the E* state (*20*). Specifically:

$$\bar{X} = X\frac{(1 + K_{E^*S}C_S)^4(1 + K_{E^*T}C_T)^4(1 + K_{E^*I}C_I)^4}{(1 + K_{ES}C_S)^4\,(1 + K_{ET}C_T)^4(1 + K_{EI}C_I)^4} \qquad (4a)$$

where K_{E^*i} and K_{Ei} are intrinsic binding constants for the interactions of species i (i = S, T, or I) with four equivalent and independent binding sites on the respective isomeric states of the tetrameric enzyme. Provided that consideration is restricted to a single substrate concentration, $\bar{X}$ becomes a constant which can be treated in the same fashion as X. By analogy with eq 3c, its magnitude in the presence of a concentration C_M of inert cosolute is considered to be described by the relationship:

$$\bar{X}_M = \bar{X} \exp[(B_{EM} - B_{E^*M})C_M] \qquad (4b)$$

which involves the reasonable approximation that the binding of substrate, coenzyme, and/or effector to a particular enzyme state has minimal effect on its excluded volume interaction with cosolute.

In the absence of cosolute, the measured initial velocity, $\bar{v}$, at the selected substrate concentration C_S may be expressed in terms of $\bar{X}$ as

$$\bar{v} = (v_E + \bar{X}v_{E^*})/(1 + \bar{X}) = v_E(1 + \alpha\bar{X})/(1 + \bar{X}) \qquad (5a)$$

where v_E is the initial velocity for this substrate concentration if all enzyme were active isomer, whereas $v_{E^*} = \alpha v_E$ is the corresponding quantity for the other isomer. For a

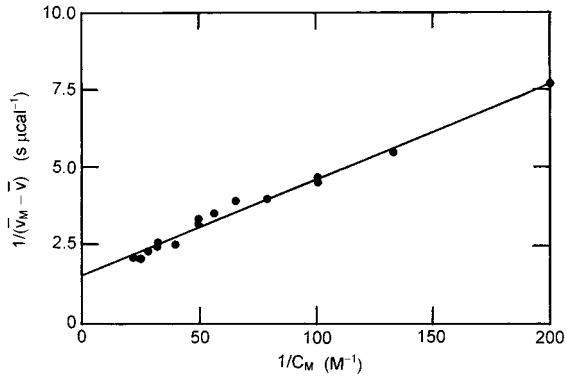

FIGURE 4: Effect of proline concentration on initial velocity of the pyruvate kinase reaction in mixtures containing 6 mM phenylalanine, 0.14 mM phosphoenolpyruvate, and 4 mM ADP (pH 7.5, 20 °C), the results being plotted in the manner suggested by eq 6.

reaction mixture supplemented with a concentration C_M of inert solute, the corresponding initial velocity, v_M, is given by

$$\bar{v}_M = v_E(1 + \alpha\bar{X}_M)/(1 + \bar{X}_M) \qquad (5b)$$

After expression of the exponential terms in series format, algebraic manipulation of eqs 3c−5b yields the relationship:

$$\frac{1}{(\bar{v}_M - \bar{v})} = -\frac{(1 + \bar{X})}{v_E(1 - \alpha)} + \frac{(1 + \bar{X})^2}{v_E(1 - \alpha)\bar{X}(B_{E^*M} - B_{EM})C_M} \qquad (6)$$

as the predicted consequence of thermodynamic nonideality arising from the presence of proline in the current experiments. Initial velocities measured for a phosphoenolpyruvate concentration C_S of 0.14 mM (Figure 4) are clearly consistent with such interpretation in the sense that they exhibit the predicted linear dependence of $1/(\bar{v}_M - \bar{v})$ upon $1/C_M$.

Adoption of the alternative viewpoint that specific chemical interaction is responsible for the proline-mediated displacement of the isomerization equilibrium toward the more active enzyme isomer can be accommodated by expressing the constitutive isomerization constant as

$$\bar{X}_M = \bar{X}[(1 + K_{E^*M}C_M)/(1 + K_{EM}C_M)]^4 \qquad (7)$$

whereupon the counterpart of eq 6 becomes

$$\frac{1}{(\bar{v}_M - \bar{v})} = \frac{(1 + \bar{X})[(1 + K_{EM}C_M)^4 + \bar{X}(1 + K_{E^*M}C_M)^4]}{v_E(1 - \alpha)\bar{X}[(1 + K_{EM}C_M)^4 - (1 + K_{E^*M}C_M)^4]} \qquad (8)$$

Subject to validity of the approximation that $(1 + K_{EM}C_M)^4 \approx 1 + 4K_{EM}C_M$, this expression can certainly be simplified to

$$\frac{1}{(\bar{v}_M - \bar{v})} = \frac{(1 + \bar{X})^2}{4v_E(1 - \alpha)\bar{X}(K_{EM} - K_{E^*M})C_M} + \frac{(1 + \bar{X})(K_{EM} + \bar{X}K_{E^*M})}{v_E(1 - \alpha)\bar{X}(K_{EM} - K_{E^*M})} \qquad (9)$$

A linear dependence of $1/(\bar{v}_M - \bar{v})$ upon $1/C_M$ is thus also potentially feasible for this preferential binding model. However, this description is conditional upon the assumption that $K_{EM}C_M \ll 1$, which would only pertain if K_{EM} were extremely small (a dissociation constant in excess of 1 M). Although the concept of a chemical interaction governed by such a small binding constant is extremely difficult to disprove, an explanation of the present results in terms of a specific proline interaction with the smaller isomer is rendered unlikely by the finding that similar kinetic effects are observed with glycine and sorbitol, and by the earlier observation that similar displacement of the isomerization equilibrium can be effected by comparable concentrations of sucrose (*15*). On the other hand, the inhibitory rather than activatory effect of sucrose on pyruvate kinase kinetics must reflect a superimposed specific interaction of this cosolute with the coenzyme.

Inasmuch as the kinetic and sedimentation velocity results presented above seemingly find rational explanation in terms of thermodynamic nonideality arising from molecular crowding by high concentrations of an inert solute, no chemical interaction of proline with the more active pyruvate kinase isomer is required to account for its suppression of the sigmoidal kinetics observed for pyruvate kinase in the presence of phenylalanine.

CONCLUDING REMARKS

As well as introducing a direct rather than a coupled enzyme assay for pyruvate kinase, this investigation has served to reinforce the need to consider potential effects of thermodynamic nonideality on the kinetics of enzyme reactions in crowded molecular environments. For reactions involving enzyme isomerization, be it preexisting (*21*) or substrate-induced (*22*), a crowded molecular environment favors the smaller isomeric state. Whether this displacement of the isomeric equilibrium position gives rise to activation (*23*−*25*) or inhibition (*26*, *27*) depends on the role of the smaller isomer in the enzyme mechanism. For rabbit muscle pyruvate kinase in the presence of phenylalanine, this effect of thermodynamic nonideality is extremely dramatic in that it changes the nature of the enzyme kinetic behavior at the level of qualitative interpretation (Figure 2).

It is hoped that this study may lead to greater awareness of the need to consider effects of thermodynamic nonideality in the prediction of physiological behavior such as metabolic flux in the crowded molecular environment of the cell cytoplasm. The usual practice of basing such predictions on kinetic parameters obtained under conditions approaching thermodynamic ideality is clearly open to question.

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