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Biophysical Chemistry 104 (2003) 189–198

Biophysical Chemistry

www.elsevier.com/locate/bpc

Further evidence for the reliance of catalysis by rabbit muscle pyruvate kinase upon isomerization of the ternary complex between enzyme and products

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Received 30 September 2002; received in revised form 1 November 2002; accepted 4 November 2002

Abstract

Isothermal calorimetry has been used to examine the effect of thermodynamic non-ideality on the kinetics of catalysis by rabbit muscle pyruvate kinase as the result of molecular crowding by inert cosolutes. The investigation, designed to detect substrate-mediated isomerization of pyruvate kinase, has revealed a 15% enhancement of maximal velocity by supplementation of reaction mixtures with 0.1 M proline, glycine or sorbitol. This effect of thermodynamic non-ideality implicates the existence of a substrate-induced conformational change that is governed by a minor volume decrease and a very small isomerization constant; and hence, substantiates earlier inferences that the rate-determining step in pyruvate kinase kinetics is isomerization of the ternary enzyme product complex rather than the release of products.

Keywords: Enzyme kinetics; Isothermal calorimetry; Molecular crowding; Pyruvate kinase; Substrate-induced isomerization; Thermodynamic non-ideality

1. Introduction

Results of kinetic studies of enzyme catalysis by rabbit muscle pyruvate kinase were considered initially to conform with a rapid equilibrium mechanism involving random order of substrate addition [1,2]. However, subsequent investigations of exchange reactions between enzyme–substrate and enzyme–product complexes [3–5] have invalidated interpretation of pyruvate kinase kinetics in terms of the classical rapid–equilibrium–random scheme. Instead, it was proposed that the rate-determining step may be isomerization of the ternary enzyme–product complex rather than the release of products.

As demonstrated elsewhere [6–15], molecular crowding by high concentrations of an inert cosolute provides a powerful probe of isomeric transitions within a protein because of displacement of the equilibrium position towards the smaller (or more symmetrical) isomer. By demonstrating that the inclusion of a relatively high concentration (0.1 M) of proline, glycine or sorbitol enhances

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PII: S0301-4622(02)00366-6

catalysis by rabbit muscle pyruvate kinase, this investigation provides additional support for a reaction mechanism involving isomerization of the ternary enzyme–product complex [3–5].

2. Experimental

2.1. Materials

Rabbit muscle pyruvate kinase (ammonium sulfate suspension), ADP, ATP, phosphoenolpyruvate, L-proline and Trizma base were all Sigma-Aldrich products. The suspension of pyruvate kinase 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 (approx. 1 mg/ml) were determined spectrophotometrically on the basis of an absorption coefficient ($A^{1\%}_{1\,cm}$) of 5.4 at 280 nm [16].

2.2. Enzyme kinetic studies

Initial velocities for the pyruvate kinase reaction were measured directly by isothermal calorimetry [17–19] in a Microcal MCS-ITC unit thermostatically maintained at 20 °C. The advantage of initial velocity measurements is that the contribution from the steady-state enzyme reaction is recognized as a time-independent rate of heat flow (dQ/dt), irrespective of any transient contribution from heat of dilution that may arise from the addition of enzyme in a medium differing from that in the calorimeter cell [19]. On the basis of 10 measurements of the heat release during the complete conversion of known amounts (0.4–2.0 μmol) of phosphoenolpyruvate to pyruvate in the presence of enzyme and excess MgADP, $\Delta H = 5550\ (\pm 50)$ cal mol^{-1}. For a 1.3-ml reaction mixture, the calibration factor relating calorimetrically determined velocities (μcal s^{-1}) to their molar counterparts (M s^{-1}) is thus 7.15 cal M^{-1}. This calorimetric method has been preferred to the classical spectrophotometric procedure because it avoids complications arising from the effects of molecular crowding on lactate dehydrogenase [6,10], the enzyme added to reduce the pyruvate at the expense of NADH in the standard coupled assay.

Assay mixtures (1.3 ml) in the first series of experiments comprised 1.4 mM phosphoenolpyruvate, 0.1–4.0 mM MgADP and enzyme (0.25 μg ml^{-1}) in either the above Tris–chloride buffer, pH 7.5, or in the same buffer supplemented with 0.1 M proline, glycine or sorbitol as molecular-crowding osmolyte [20–22]. In experiments designed to test the effect of 0.1 M osmolyte on the kinetics of rabbit muscle pyruvate kinase with phosphoenolpyruvate as variable substrate, the reaction mixtures comprised 4.5 mM MgADP, 0.01–1.14 mM phosphoenolpyruvate and enzyme (0.05 μg ml^{-1}).

2.3. Analysis of kinetic results obtained in the presence of osmolyte

Effects of thermodynamic non-ideality in kinetic studies have been analyzed by a procedure analogous to those described previously for single-substrate systems with 1:1 stoichiometry [6,23]. The application of theory written in terms of a single substrate to a two-substrate system such as pyruvate kinase is an approximation that is considered to be justified by the use of a saturating concentration of the fixed substrate for an enzyme exhibiting a random-sequential reaction mechanism [1,2,24,25]. In the effective absence of any free enzyme, enzyme-catalyzed conversion of small substrate to product P has been analyzed in terms of the reaction scheme

$$\mathrm{ET} + \mathrm{S} \overset{K}{\rightleftarrows} \mathrm{ETS} \overset{X}{\rightleftarrows} \mathrm{EPQ} \overset{X^{\ddagger}}{\rightleftarrows} \mathrm{EPQ}^{\ddagger} \overset{k}{\rightarrow} \mathrm{E} + \mathrm{P} + \mathrm{Q} \qquad (1)$$

where ET refers to the binary complex between enzyme and fixed substrate, and S to the substrate whose concentration is varied. An ETS ⇄ EPQ step is included to cover any conformational change prior to that associated with formation of the activated enzyme–product complex encountered in absolute reaction rate theory [26]. The equilibrium constant for the latter transition is thus considered to be extremely small ($X^{\ddagger} \ll 1$). Inherent in the analysis [6,23] is the assumption that

the rate constant for product formation (k) is sufficiently small to render feasible the approximation that reactants and the various enzyme–substrate and enzyme–product complexes are in a state of equilibrium governed by the following association constants

$$K = \psi_K C_{ETS}/(C_{ET}C_S);\ \psi_K = \gamma_{ETS}/(\gamma_{ET}\gamma_S) \quad (2a)$$

$$X = \psi_X C_{EPQ}/C_{ETS};\ \psi_X = \gamma_{EPQ}/\gamma_{ETS} \quad (2b)$$

$$X^{\ddagger} = \psi^{\ddagger} C_{EPQ^{\ddagger}}/C_{EPQ};\ \psi^{\ddagger} = \gamma_{EPQ^{\ddagger}}/\gamma_{EPQ} \quad (2c)$$

Thermodynamic equilibrium constants are thus described as products of the appropriate ratios of molar species concentrations (C_i) and the corresponding ratios of activity coefficients (γ_i). Standard algebraic manipulation of the rate equation based on steady-state kinetics ($dC_{EPQ}{}^{\ddagger}/dt = 0$) and a small value of $X^{\ddagger}$ allows the expression for initial velocity in the presence of molecular crowding cosolute, v_M, to be written as:

$$V_M = \frac{kKXX^{\ddagger}(C_E)_{tot}C_s}{\psi_x\psi^{\ddagger}\{\psi_k + KCs[1 + (X/\psi_X)]\}} \quad (3)$$

where $(C_E)_{tot}$ refers to the total enzyme concentration. This expression describes a rectangular hyperbolic dependence of initial velocity upon substrate concentration that is characterized by the following Michaelis parameters:

$$V_M = kXX^{\ddagger}(C_E)_{tot}/\{\psi_X\psi^{\ddagger}[1 + (X/\psi_X)]\} \quad (4a)$$

$$(K_m)_M = \psi_K/\{K[1 + (X/\psi_X)]\} \quad (4b)$$

$$(V/K_m)_M = kKXX^{\ddagger}(C_E)_{tot}/(\psi_X\psi^{\ddagger}\psi_K). \quad (4c)$$

The maximal velocity (V) and the Michaelis constant (K_m) are the two independent parameters obtained by curve-fitting the untransformed data to the Michaelis–Menten equation. However, an expression for their ratio is also included because of its relevance to the specificity constant (k_{cat}/K_m), the apparent second-order rate constant that refers to the properties and reactions of the free enzyme and free substrate [27].

Advantage is then taken of the fact that the thermodynamic activity coefficient of a macromolecular species, γ_i, is given by:

$$\gamma_i = \exp[2B_{i,i}C_i + \sum_{j \neq i} B_{i,j}C_j + \ldots], \quad (5)$$

where $B_{i,i}$ and $B_{i,j}$ are osmotic second virial coefficients reflecting non-ideality arising from the presence of like and dissimilar species, respectively. These virial coefficients are described rigorously on the statistical-mechanical basis of excluded volume [28–31]. Because the molar concentrations of all enzyme species are negligibly small in comparison with the concentration of inert cosolute, C_M, the activity coefficient ratios may be approximated by:

$$\psi_K \approx (1/\gamma_S)\exp[(B_{ETS,M} - B_{ET,M})C_M], \quad (6a)$$

$$\psi_X \approx \exp[(B_{EPQ,M} - B_{ETS,M})C_M], \quad (6b)$$

$$\psi^{\ddagger} \approx \exp[(B_{EPQ^{\ddagger},M} - B_{EPQ,M})C_M]. \quad (6c)$$

Provided that the cosolute (M) bears no net charge, the second virial coefficient equates with the molar covolume: thus $B_{i,M} = 4\pi N(R_i + R_M)^3/3$ for spherical species with radii R_i and R_M. Explicit expressions for ψ_X, $\psi^{\ddagger}$ and ψ_K in terms of covolumes are thus available, but ψ_K also contains the activity coefficient of substrate (γ_S)—a quantity requiring separate experimental determination. For illustrative purposes γ_S has been taken as unity, whereupon $\psi_K \approx \exp[(B_{ETS,M} - B_{ET,M})C_M]$. Substitution of the expressions for the various ψ_i in Eqs. (6a), (6b) and (6c) then gives:

$$V_M = \frac{kXX^{\ddagger}(C_E)_{tot}}{\exp[(B_{EPQ^{\ddagger}M} - B_{ETS,M})C_M]\ \{1 + X\exp[(B_{ETS,M} - B_{EPQ,M})C_M]\}} \quad (7a)$$

$$(K_m)_M = \frac{\exp[(B_{\mathrm{ETS},M} - B_{\mathrm{ET},M})C_M]}{K\{1 + X\exp[(B_{\mathrm{ETS},M} - B_{\mathrm{EPQ},M})C_M]\}}, \quad (7b)$$

$$(V/K_m)_M = \frac{kXX^{\ddagger}(C_{\mathrm{E}})_{\mathrm{tot}}}{\exp[(B_{\mathrm{EPQ}^{\ddagger}M} - B_{\mathrm{ET},M})C_M]}. \quad (7c)$$

There are several aspects of the application of these expressions that require comment. (i) Although the theory has been developed specifically for systems with 1:1 enzyme–substrate stoichiometry, it also applies to results obtained with a tetrameric enzyme such as rabbit muscle pyruvate kinase because of the equivalence and independence of the active sites on the four subunits. Violation of that requirement would be manifested as a departure from Michaelis kinetic behavior. The only qualification to the application of Eqs. (7a), (7b) and (7c) to pyruvate kinase kinetic data is that K_{m} becomes an intrinsic constant [32] and the covolume differences refer to changes within a single subunit rather than the whole enzyme [23]. (ii) There is an implicit assumption in the derivation of these expressions that the experimental measurements are made under the constraints of constant temperature and chemical potential of solvent [30,31]. Because enzyme kinetic experiments are conducted with constant pressure as the second constraint, their rigorous description should be in terms of molal concentrations and activity coefficients [12]. However, relatively minor quantitative discrepancies should arise from description of thermodynamic non-ideality based on Eqs. (2a), (2b) and (2c), which have the advantage of being expressed in terms of the conventional concentration scale (molar) in kinetic studies. (iii) Finally, as noted above, the analysis of the effect of thermodynamic non-ideality is based on the premise that product formation is the rate limiting step—an assumption made to allow consideration of the previous steps [Eq. (1)] in equilibrium terms. Although the results of early kinetic studies were considered to justify such action [1,2], the validity of assuming equilibrium attainment between the various species has been questioned [3,24,25]. This point is addressed further after consideration of the results in terms of Eqs. (7a), (7b) and (7c).

2.4. Difference sedimentation velocity

As in previous studies with this enzyme [8,33,34], different sedimentation velocity [35] was used to monitor displacements of isomerization equilibria effected by ligand binding to rabbit muscle pyruvate kinase. Solutions of pyruvate kinase (approx. 2 mg/ml) in the Tris–chloride buffer (pH 7.5) and in the same medium supplemented with ligand were centrifuged simultaneously at 20 °C and 35 000 rev./min in a Beckman XL-I ultracentrifuge. The resulting absorbance scans (280 nm) at angular velocity ω were analyzed by means of the expression [34]:

$$\ln(r_1)_{t_1} - \ln(r_2)_{t_2} = (s_1 - s_2)\omega^2 t_1 + [\ln(r_m)_1 - \ln(r_m)_2 - s_2\omega^2(t_2 - t_1)], \quad (8)$$

$(r_1)_{t_1}$ and $(r_2)_{t_2}$ denote the respective boundary positions (midpoints) in cells 1 and 2 at times t_1 and t_2, respectively, whereas $(r_m)_1$ and $(r_m)_2$ are the corresponding positions of the air–liquid meniscus in the two cells: t_1 refers to the duration of centrifugation for the distribution in 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 (cell 1) and presence (cell 2) of ligand.

3. Results and discussion

Kinetic studies of pyruvate catalysis with MgADP as variable substrate are summarized in Fig. 1a, where the measurements of initial velocity in the absence (○) and presence (●) of 0.1 M proline are described adequately by the best-fit rectangular hyperbolic dependencies of initial velocity upon substrate concentration (—). A similar situation applies to the results of corresponding studies with phosphoenolpyruvate as variable substrate (Fig. 1b). Essentially, the same changes in kinetic behavior are observed when 0.1 M concentrations of glycine (■) or sorbitol (▲) are substituted for the proline. The consistent observation

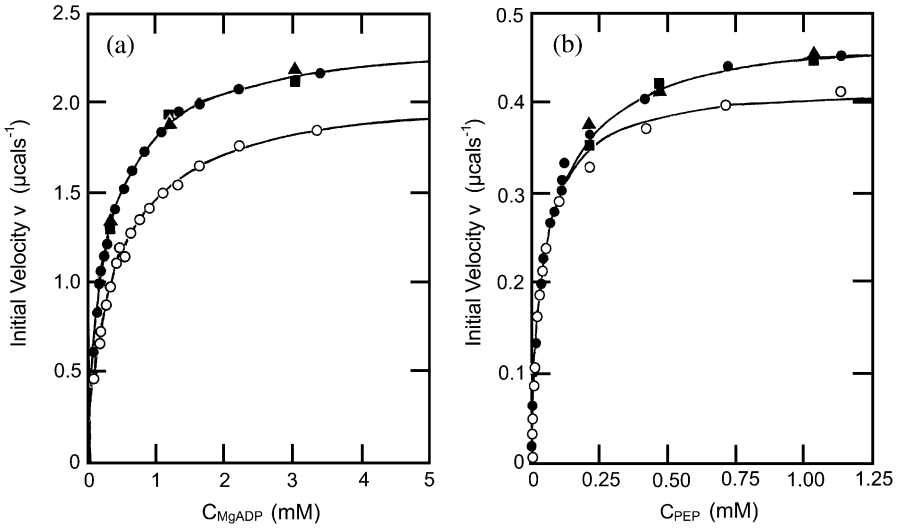

Fig. 1. Effect of osmolytes on the kinetics of the rabbit muscle pyruvate kinase reaction. (a) Initial velocity studies with 1.4 mM phosphoenolpyruvate and MgADP as variable substrate in the absence (○) and presence of 0.1 M concentrations of proline (●), glycine (■) or sorbitol (▲). (b) Corresponding studies with 4.5 mM MgADP and phosphoenolpyruvate as variable substrate.

of a comparable increase in maximal velocity (approx. 15%) with 0.1 M proline, glycine or sorbitol as cosolute is of particular note inasmuch as specific interaction between these chemically different osmolytes and an enzyme species is rendered an unlikely explanation of the velocity enhancement. We, therefore, examine the findings in the light that the enhanced catalysis may be a consequence of thermodynamic non-ideality arising from molecular crowding by the space-filling cosolute.

As noted previously [6,7,10,23], an enhanced maximal velocity under conditions of molecular crowding is symptomatic of an equilibrium step within the mechanistic pathway that involves a decrease in size and/or asymmetry of an intermediate species. Consideration of the phenomenon to be an effect of thermodynamic non-ideality upon the preexisting equilibrium between isomeric states of rabbit muscle pyruvate kinase [8,33,36] is precluded because molecular crowding has no effect on V for such systems [9,23]. The isomerization(s) being detected must, therefore, be substrate-induced.

Values of maximal velocity (V) and Michaelis constant (K_m) obtained from the four sets of [v, C_S] data in Fig. 1 are summarized in Table 1, which also includes the corresponding values of their ratio V/K_m. Although the magnitude of the ratio emanates directly from Lineweaver–Burk and

Table 1
Kinetic parameters for catalysis by rabbit muscle pyruvate kinase in the absence [V, K_m] and presence [V_M, $(K_m)_M$] of 0.1 M proline

Variable substrate	Initial velocity (μcal s^{-1})		Michaelis constant (μM)		Ratio	
	$V^{a,b}$	$V_M{}^{a,b}$	$K_m{}^{a,b}$	$(K_m)_M{}^{a,b}$	$10^3\ (V/K_m)^b$	$10^3\ (V/K_m)_M{}^b$
MgADP	2.05 (±0.05)	2.36 (±0.04)	410 (±40)	310 (±20)	5.0 (±0.6)	7.6 (±0.6)
Phosphoenolpyruvate	0.42 (±0.01)	0.48 (±0.01)	42 (±7)	71 (±6)	10.0 (±0.2)	6.8 (±0.1)

[a] Deduced by non-linear regression analysis of the results (Fig. 1) in terms of a rectangular hyperbolic dependence of initial velocity upon substrate concentration.

[b] Numbers in parentheses denote the uncertainty (±2 S.D.) of the estimate.

Hanes analyses of kinetic data, their use is open to criticism because of the distortion of experimental uncertainty effected by linear transformation of the rectangular hyperbolic dependence of initial velocity upon substrate concentration [37]. Consequently, the experimental uncertainties (±2 S.D.) listed in Table 1 are based on those inherent in the estimates of V and K_m obtained by non-linear regression analysis of the untransformed [v, C_S] data in terms of a rectangular hyperbolic dependence of initial velocity upon substrate concentration. Uncertainties in V/K_m have then been inferred from the sums of relative errors in the two independent curve-fitting parameters, V and K_m.

Because the use of cosolutes as viscogenic agents is a well-established protocol in mechanistic studies of enzyme action [38–42], a decision needs to be made whether the correction factor should be applied to V_M to take into account the greater relative viscosity of the buffer supplemented with 0.1 M proline. Such action would clearly be necessary for a reaction subject to diffusion control. However, the magnitudes of k_{cat}/K_m deduced for rabbit muscle pyruvate kinase in the absence of proline are only 1.7×10^5 $M^{-1}s^{-1}$ and 3.5×10^6 M^{-1} s^{-1} in the experiments with MgADP and phosphoenolpyruvate as varied substrate, respectively. On the grounds that these specificity constants are considerably smaller than the second-order rate constants of 10^7–10^8 M^{-1} s^{-1} that are symptomatic of diffusion control, catalysis by rabbit muscle pyruvate kinase is not considered to be diffusion-limited. Consequently, no allowance has been made for the effect of viscosity.

From the first line of entries in Table 1 it is evident that inclusion of 0.1 M proline has enhanced the maximal velocity V by 15% (±6%) and the biospecificity parameter (V/K_m) by 52% (±30%) in experiments with MgADP as variable substrate. The corresponding comparison of results obtained with phosphoenolpyruvate as variable substrate (bottom line of Table 1) reveals a similar effect of 0.1 M proline on maximal velocity inasmuch as V increases by 14% (±6%). However, V/K_m undergoes a 32% (±15%) decrease in this instance.

To facilitate the reconciliation of these findings with the theoretical consequences of thermodynamic non-ideality we rewrite Eq. (7a) and Eq. (7c) as:

$$\frac{V_M}{V} = \frac{(1+X)}{\exp[(B_{EPQ^{\ddagger},M}-B_{ETS,M})C_M]\{1+X\exp[(B_{ETS,M}-B_{EPQ,M})C_M]\}} \quad (9)$$

$$(V/K_m)_M/(V/K_m) = \exp[(B_{ET,M}-B_{EPQ^{\ddagger},M})C_M]. \quad (10)$$

The general expression for the ratios of maximal velocities is considerably more complicated than its counterpart in terms of V/K_m. However, Eq. (9) can be simplified for the pyruvate kinase system by taking advantage of the observation [5] that phosphoenolpyruvate is a 'sticky substrate'—the term used to signify that any phosphoenolpyruvate involved in ternary enzyme–substrate complex formation is committed to pyruvate production. Such substrate 'stickiness' implies that the conversion of EST to EPQ is characterized by a large isomerization constant. With that proviso ($X \gg 1$), Eq. (9) becomes:

$$V_M/V = \exp[(B_{EPQ,M}-B_{EPQ^{\ddagger},M})C_M]. \quad (11)$$

An effect of cosolute concentration on V/K_m thus monitors the volume change between the initial (ET) and final ($EPQ^{\ddagger}$) enzyme species [Eq. (10)], whereas the corresponding effect on V only reflects the volume change associated with isomerization of the ternary enzyme–product complex to its activated state ($EPQ \rightleftarrows EPQ^{\ddagger}$).

The fact that similar enhancements of maximal velocity are observed with either MgADP (Fig. 1a) or phosphoenolpyruvate (Fig. 1b) as variable substrate is seen to be a mandatory consequence of molecular crowding by virtue of its effect on a volume change associated with the $EPQ \rightleftarrows EPQ^{\ddagger}$ transition. Interpretation of the 15% enhancement of maximal velocity by 0.1 M proline in terms of Eq. (11) signifies a magnitude of 1.4 l/mol for the covolume difference ($B_{EPQ,M}-B_{EPQ,M}{}^{\ddagger}$). On the other hand, the disparity between

the effects of osmolyte on the V/K_m ratios (Table 1) implies the existence of a difference between the radius of ET in the two series of experiments [Eq. (10)]. Because pyruvate kinase catalysis occurs via a random-sequential mechanism [1,2,23,24], the initial enzyme species [ET in Eq. (1)] is the binary enzyme–phosphoenolpyruvate complex in the first series of experiments, and E-MgADP in the other. Interpretation of the V/K_m enhancement with MgADP as variable substrate in terms of Eq. (10) signifies a magnitude of 4 (± 2) l/mol for ($B_{ET,M} - B_{EPQ^{\ddagger},M}$), whereas a magnitude of -3.9 (± 0.5) l/mol for the corresponding covolume difference is inferred from the V/K_m decrease in experiments with phosphoenolpyruvate as variable substrate. The credibility of such interpretation is clearly conditional upon the feasibility of a difference of 8 (± 3) l/mol between the covolumes ($B_{ET,M}$) for proline and the two binary enzyme-substrate complexes.

On the basis of a molecular mass of 240 kDa and a sedimentation coefficient ($s_{20,w}$) of 10.0 S for rabbit muscle pyruvate kinase [43], the isomeric state of the enzyme to which phosphoenolpyruvate binds [33,34] has a Stokes radius of 5.52 nm and hence, a hydrated volume of 424 l/mol. Upon noting that the covolumes in Eq. (10) refer to an enzyme subunit (not the whole enzyme) [23], a value of 106 l/mol can be ascribed to the subunit volume, which corresponds to an effective subunit radius, r_{ET}, of 3.48 nm: the covolume, $B_{ET,M}$, is thus 126 l/mol for a small cosolute with a radius (r_M) of 0.2 nm. The consequent magnitude of 130 l/mol for $B_{EPQ^{\ddagger},M}$ leads to a calculated covolume radius, ($r_{EPQ^{\ddagger}} + r_M$), of 3.72 nm and hence a radius of 3.52 nm for the activated EPQ complex (cf. 3.48 nm for r_{ET}). On the other hand, a putative covolume, $B_{ET,M}$, of 134 l/mol for proline and the other binary complex signifies a subunit radius, r_{ET}, of 3.57 nm for E-MgADP (cf. 3.48 nm for the enzyme–phosphoenolpyruvate binary complex).

A difference of this magnitude is certainly within the realm of feasibility inasmuch as a subunit radius of 3.59 nm applies to the larger isomer ($s_{20,w} = 9.7$ S) involved in the preexisting isomerization undergone by rabbit muscle pyruvate kinase [8,33,34]. Difference sedimentation velocity

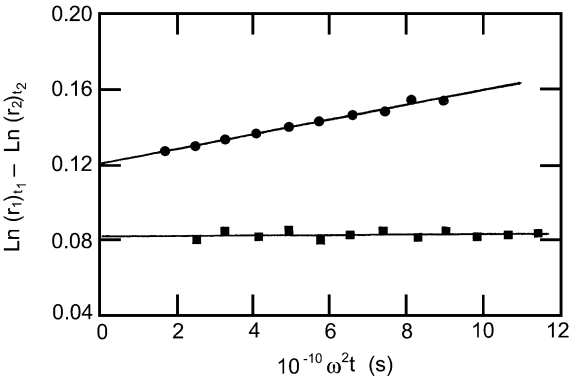

Fig. 2. Difference sedimentation velocity plots obtained in studies at 35 000 rev./min and 20 °C of the effect of 6 mM phenylalanine (●) and 4 mM MgADP (■) on the sedimentation coefficient of rabbit muscle pyruvate kinase. Results are plotted in accordance with Eq. (8).

[35] was, therefore, used to check for the existence of a MgADP-induced displacement of the preexisting isomerization towards the larger conformational state of the enzyme (Fig. 2). Whereas the decrease in sedimentation coefficient resulting from preferential binding of phenylalanine to the larger isomer [8,33,34] is manifested as a positive slope of data for that system (●) plotted according to Eq. (8), no such effect is observed with 4 mM MgADP as ligand (■). The cosolute effect on V/K_m for rabbit muscle pyruvate kinase is, therefore, not amenable to satisfactory explanation in terms of a reaction scheme with equilibrium attainment between the various binary and ternary enzyme–substrate and enzyme–product complexes. By default, the present findings thus lend support to earlier contentions [3,24,25] that the concentrations of intermediate species depend upon the kinetic pathway of their formation. Under those circumstances the Michaelis constant becomes a steady-state parameter [44,45] that cannot be accorded the thermodynamic scrutiny attempted above.

Despite inability to rationalize satisfactorily the cosolute effect on V/K_m in terms of thermodynamic non-ideality, such interpretation of the enhanced maximal velocity in the presence of cosolute remains unequivocal. The value of V (or V_M) refers to a situation where the enzyme species

distribution is restricted to the EPQ and EPQ‡ states, irrespective of the pathway of EPQ formation. Consequently, the enhancement of maximal velocity by molecular crowding may be attributed unconditionally to a volume decrease associated with transition of the EPQ ternary complex to its activated EPQ‡ state. In that regard, the observation of comparable enhancements of the V_M/V ratio in experiments with MgADP and phosphoenolpyruvate as variable substrate is a mandatory requirement because, as noted above, the maximal velocity refers to the system under conditions where EPQ and EPQ‡ are effectively the sole enzyme states—regardless of the substrate whose concentration is being varied. Calculations similar to those described above signify that the value of 1.4 l/mol for $(B_{\mathrm{EPQ},M} - B_{\mathrm{EPQ}^{\ddagger},M})$ corresponds to a decrease in subunit radius of only 0.01 nm, i.e. to a 0.3% decrease in radius or a 0.9% decrease in subunit volume. Although these calculations cannot be definitive because of the assumptions involved, they suffice to establish that the volume change for the purported EPQ ⇄ EPQ‡ transition is sufficiently small to be within the realm of experimental feasibility.

4. Concluding remarks

The present use of thermodynamic non-ideality to probe substrate-induced isomerization of rabbit muscle pyruvate kinase has provided evidence for the existence of a conformational change that is characterized by a minor volume decrease and a very small isomerization constant—a transition consistent with conversion of the enzyme–product ternary complex (EPQ) to the activated EPQ‡ state encountered in absolute reaction rate theory [26]. This independent evidence thus confirms earlier indications [3–5] that the rate-determining step may be isomerization of the ternary enzyme–product complex rather than the release of products.

In view of the requirement for an input of 30 kJ/mol for the conversion of MgADP to MgATP, the ease with which the ternary enzyme–phosphoenolpyruvate–MgADP complex is converted to enzyme–pyruvate–MgATP [3–5] must reflect an energy transfer that is accomplished by concomitant distortion of the enzyme three-dimensional structure. The rate-determining step in the production of MgATP and pyruvate is now seen to be the additional conformational change undergone by the ternary enzyme–product complex (EPQ) to its activated state (EPQ‡) in order to effect transfer of the 30 kJ/mol energy quantum solely to the adenonucleotide; and thereby to allow the enzyme to regain its original energy state to meet the requirement of its function as a catalyst.

Acknowledgments

The financial support of this investigation by the Australian Research Council is gratefully acknowledged.

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