The regulatory properties of rabbit muscle pyruvate kinase

The influence of substrate concentrations

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The kinetics of rabbit muscle pyruvate kinase were studied in assays at pH 7.4, where the relationships between the initial velocities of the catalysed reaction and the concentrations of substrates ADP, phosphoenolpyruvate and Mg^{2+} are non-hyperbolic. The data were used to test the applicability of the exponential model for a regulatory enzyme, which has been here extended to describe the behaviour of a three-substrate enzyme. It appears that the data can be represented by the model and as a result permit the conclusion that the substrates influence one another's binding by the same type of charge interactions that are evident in the Michaelis–Menten kinetics of the enzyme observed at pH 6.2. Evidence is also presented indicating that $MgADP$ acts as a dead-end inhibitor of the enzyme at pH 7.4.

The glycolytic enzyme pyruvate kinase (EC 2.7.1.40) is found in mammalian tissues in a variety of isoenzymic forms. Allosteric control of enzyme activity for the L-, R- and M_2 -type isoenzymes has been well characterized (Hall & Cottam, 1978; Seubert & Schoner, 1971; Kayne, 1973). These enzymes display a sigmoidal relationship between the initial velocity of pyruvate formation and the concentration of phosphoenolpyruvate, are allosterically activated by fructose 1,6-bisphosphate and H^+ , and are inhibited by ATP (Hall & Cottam, 1978). It is only recently that similar regulatory properties have been observed with the M_1 -isoenzyme from rabbit skeletal muscle. The activity of this isoenzyme is unaffected by fructose bisphosphate at pH 6.2; however, activation is observed when the pH is raised, and it was found that the rate is maximally enhanced, by 2.55-fold, at pH 7.4. At this pH, the initial velocity is sigmoidally related to the concentrations of both ADP and Mg^{2+} and to the concentration of K^+ when 10 mM-fructose bisphosphate is also present; in contrast, the initial velocity appears to be a hyperbolic function of phosphoenolpyruvate concentration (Phillips & Ainsworth, 1977; Gregory & Ainsworth, 1981). These observations established that the regulatory properties of the M_1 -type isoenzyme are different from those of the L-, R- and M_2 -type isoenzymes. The purpose of the present paper is twofold: first, to present the results of a more detailed investigation of the influence of three substrate concentrations on the initial velocity of reaction catalysed by the rabbit

muscle enzyme; secondly, to investigate whether the exponential model for a regulatory enzyme (Ainsworth, 1977) can be extended to describe the effect of these concentrations on the rate. A subsidiary aim here has been to ascertain whether the two-substrate model (Ainsworth & Gregory, 1978), applied to rate data obtained with one substrate concentration kept constant, preserves information that is consistent with that provided by the more appropriate three-substrate model.

Theory

General description of the exponential model

Initial-rate and product-inhibition studies of the forward reaction of pyruvate kinase are consistent with an equilibrium random-order mechanism (Reynard *et al.*, 1961; Ainsworth & Macfarlane, 1973; Giles *et al.*, 1976). Consequently, the initial velocity of reaction can be represented by:

$$v = V_G \cdot p_A \cdot p_B \cdot p_C \quad (1)$$

where p_A , p_B and p_C represent the fractional saturation of the enzyme by the substrates A (ADP), B (phosphoenolpyruvate) and C (Mg^{2+}) respectively, and V_G is the velocity measured in the presence of saturating concentrations of all three substrates. (K^+ , which is an obligatory activator, is present throughout at a saturating concentration.) Data in the present paper show, however, that each substrate binds non-hyperbolically at pH 7.4 in the presence of fixed concentrations of the other two

substrates. An interpretation of this behaviour is attempted by extending the exponential model for a regulatory enzyme (Ainsworth, 1977; Ainsworth & Gregory, 1978). Accordingly, eqn. (1) becomes:

$$\frac{v}{V_G} = \frac{A \cdot \alpha_{abc}}{1 + A \cdot \alpha_{abc}} \cdot \frac{B \cdot \beta_{abc}}{1 + B \cdot \beta_{abc}} \cdot \frac{C \cdot \gamma_{abc}}{1 + C \cdot \gamma_{abc}} \quad (2)$$

where the association binding constants α_{abc} , β_{abc} and γ_{abc} relate to particular values of the fractional saturation of the enzyme by the three substrates ($p_A = a$, $p_B = b$ and $p_C = c$). With the assumption, which is discussed below, that the substrate-binding sites are allosterically linked:

$$\ln \alpha_{abc} = \ln \alpha_{000} + k_{AA} \cdot p_A + k_{BA} \cdot p_B + k_{CA} \cdot p_C \quad (3)$$

(Ainsworth & Gregory, 1978) and thus varies continuously as the fractional saturations vary between 0 and 1. In this equation, α_{000} represents the binding constant for A {equal to $p_A / [(1 - p_A) \cdot A]$ } measured when $p_A = p_B = p_C = 0$. k_{XY} is an interaction constant that describes the interaction free energy in units of RT . The subscript Y describes the ligand site that experiences the interaction, and the subscript X denotes the ligand whose binding causes a change in the association binding constant for Y. Hence k_{AA} is the homotropic interaction constant that describes the effect of A on the binding of further A molecules, and k_{BA} is the heterotropic interaction constant for the effect of B on the binding of A. Further equations can be written, by analogy with eqn. (3), for the binding constants β_{abc} and γ_{abc} :

$$\ln \beta_{abc} = \ln \beta_{000} + k_{AB} \cdot p_A + k_{BB} \cdot p_B + k_{CB} \cdot p_C \quad (4)$$

$$\ln \gamma_{abc} = \ln \gamma_{000} + k_{AC} \cdot p_A + k_{BC} \cdot p_B + k_{CC} \cdot p_C \quad (5)$$

and reciprocal allosteric linkage requires that $k_{XY} = k_{YX}$. Eqns. (2)–(5) are employed below to fit initial-velocity data measured as a function of the three substrate concentrations, $v = f(A, B, C)$, but it is important to note that the ten constants obtained depend on the assumption that the fractional saturation of the enzyme by H^+ and K^+ remains constant throughout. In order to establish (by analogy) how a failure of this assumption might affect the numerical description of the observed

where V_{AB} is an apparent maximum velocity measured when $p_A = p_B = 1$. Similarly, in eqn. (3), the omission of $k_{CA} p_C$ affects the values of the remaining constants:

$$\ln \alpha_{ab} = \ln \alpha_{00} + k_{AA} \cdot p_A + k_{BA} \cdot p_B \quad (7)$$

so that they too have only an apparent significance.

Allosteric linkage

It is a characteristic of models for regulatory enzymes whose ligand-binding functions can be represented as a ratio of polynomials in the concentration of two ligands that:

$$\left(\frac{dp_A}{d(\ln B)} \right)_A = \left(\frac{dp_B}{d(\ln A)} \right)_B \quad (8)$$

when A and B possess an equal number of binding sites (Wyman, 1964). The exponential model for two ligands also predicts eqn. (8) when (Ainsworth & Gregory, 1978):

$$\begin{aligned} k_{BA} &= \ln \left(\frac{\alpha_{01}}{\alpha_{00}} \right) = \ln \left(\frac{\alpha_{11}}{\alpha_{10}} \right) = k_{AB} = \ln \left(\frac{\beta_{10}}{\beta_{00}} \right) \\ &= \ln \left(\frac{\beta_{11}}{\beta_{01}} \right) \end{aligned} \quad (9)$$

An examination of seven sets of $v = f(A, B)$ data, taken from the literature (Ainsworth *et al.*, 1981b), showed that good fits could be obtained when the truth of these equalities was assumed. Furthermore it was established that the linked constants that were obtained could be interpreted very simply and in terms that agreed exactly with conclusions drawn by the original authors themselves. It is on this basis that the linked eqns. (3), (4) and (5) are proposed for the analysis of $v = f(A, B, C)$ data from pyruvate kinase. Some consequences can now be examined. Thus, if the linkages hold, it can be expected that:

$$\left(\frac{dp_B}{d(\ln A)} \right)_{B,C} = \left(\frac{dp_A}{d(\ln B)} \right)_{A,C} \quad (10)$$

Differentiation of eqns. (3), (4) and (5) gives:

$$\left(\frac{dp_B}{d(\ln A)} \right)_{B,C} = \frac{\phi_A \phi_B (k_{AB} Z_C + k_{AC} k_{CB} \phi_C)}{Z_A Z_B Z_C - Z_A \phi_B \phi_C k_{CB} k_{BC} - Z_B \phi_A \phi_C k_{CA} k_{AC} - Z_C \phi_A \phi_B k_{BA} k_{AB}} \quad (11)$$

behaviour we have also analysed the data $v = f(A, B, C)$ by the two-substrate model, $v = f(X, Y)_Z$, an approach that is inconsistent with the evidence that the saturation of the enzyme by the third substrate does not remain constant when its concentration is fixed (Kinderlerer *et al.*, 1981; Ainsworth *et al.*, 1981a,b). For example, with p_C assumed constant, eqn. (1) becomes:

$$v = V_{AB} \cdot p_A \cdot p_B \quad (6)$$

where $\phi_X = p_X(1 - p_X)$ and $Z_X = (1 - \phi_X k_{XX})$. Hence, by symmetry, it can be seen that eqn. (10) is confirmed and that:

$$(k_{AB} Z_C + k_{AC} k_{CB} \phi_C) \equiv (k_{BA} Z_C + k_{BC} k_{CA} \phi_C) \quad (12)$$

is true for all p_C without the need to invoke assumptions beyond those already made.

Eqn. (12) demonstrates that A will normally influence the binding of B both directly and indirectly through its effect on the binding of C.

However, the latter influence is not to be expected when C is either absent or present at an infinite concentration, for then $p_C \equiv 0$ or 1 respectively and $\phi_C \equiv 0$. Under these circumstances $Z_C \equiv 1$ and interaction is limited to the direct effect. By contrast, when $k_{CC} \geq 4$ with corresponding values of $\phi_C \leq 0.25$, the binding of C passes through a catastrophe (Ainsworth, 1979), during which $Z_C = 0$ and the direct influence of A on the binding of B is then prevented. Eqn. (11) also demonstrates that, when either p_A or p_B equals 0 or 1, $\phi_A \phi_B = 0$, and no interaction between A and B is to be expected; in general, then, saturation by one ligand reduces an n -ligand problem to $n-1$, and it is on these grounds that the influence of K^+ concentration on our data is ignored.

Dead-end inhibition

Phillips & Ainsworth (1977) have shown that the function $v = f[\text{ADP}]$ displays a maximum, which becomes more marked and which moves to lower ADP concentrations as the concentration of Mg^{2+} is increased. Similar findings are reported below. This behaviour cannot be explained by the exponential model for two substrates. The model does predict a maximum in $v = f(A)$, the concentration of C being kept constant (Ainsworth & Gregory, 1978), when:

$$(1 - p_C)^{-1} = (k_{CC} \cdot p_C - k_{AC} \cdot p_A) \quad (13)$$

However, the maxima become less obvious, and eventually disappear as C increases, because the rate equation simultaneously approaches the single-substrate form. In addition, it has been shown that the maxima generated by the two-substrate exponential model usually move to higher values of A as the concentration of C increases (Gregory, 1980). Although the corresponding predictions have not yet been established for the three-substrate case, we see no good reason why the essential behaviour patterns should not be retained. If so, the maxima observed in $v = f[\text{ADP}]$ require a different explanation, and one moreover that will permit the correction of raw data so as to allow an overall analysis.

A simple hypothesis that predicts a maximum with the required dependence on Mg^{2+} concentration is to suppose that a second distinct nucleotide-binding site exists, specific to MgADP , and that binding at this site non-competitively inhibits the enzyme when A is the varied concentration. An empirical equation representing this hypothesis can be written:

$$v = \frac{V_m \cdot A \cdot \alpha_{ac}}{(1 + A \cdot \alpha_{ac})(1 + D \cdot \alpha_D)} \quad (14)$$

where α_D represents the association binding constant of the MgADP dead-end inhibitor, D. If D is related to the known concentrations of free ADP

and free Mg^{2+} by the apparent dissociation constant, K_1 , eqn. (14) becomes:

$$v = \frac{V_m \cdot A \cdot \alpha_{ac}}{(1 + A \cdot \alpha_{ac})(1 + A \cdot C \cdot \alpha_D / K_1)} \quad (15)$$

Differentiation of eqn. (15) with respect to A provides the relationship between the position of the maximum on the ADP-concentration axis, $A_{\max}$, and the concentration of free Mg^{2+} , C:

$$A_{\max} = \left(\frac{K_1}{\alpha_{ac} \cdot \alpha_D \cdot C} \right)^{\frac{1}{2}} \quad (16)$$

Eqn. (16) predicts that the maxima move to lower values of A as C increases. α_D cannot be determined from eqn. (16) because α_{ac} is a variable that depends on the saturation of the enzyme by A and C. Nevertheless, an estimate of α_D / K_1 has been obtained below, enabling data to be corrected by the equation:

$$v_{\text{corr.}} = v(1 + A \cdot C \cdot \alpha_D / K_1) \quad (17)$$

Data corrected in this way do not display maxima and have been analysed by the exponential model equations.

Experimental

Materials

Phosphoenolpyruvate (cyclohexylammonium salt), NADH (sodium salt), ADP (free acid) and rabbit muscle pyruvate kinase [crystalline suspension in 3.2M-(NH_4) $_2\text{SO}_4$] were supplied by Boehringer Corp., Lewes, East Sussex, U.K. Lactate dehydrogenase (type XI; freeze-dried salt-free powder) and N-ethylmorpholine were obtained from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. Tetrapropylammonium hydroxide was obtained from Eastman-Kodak Co., Rochester, NY, U.S.A. All other reagents were either AnalaR or reagent grade as supplied by BDH Chemicals, Poole, Dorset, U.K., or Fisons Scientific Apparatus, Loughborough, Leics., U.K.

Preparation of materials

Buffer solutions were prepared by titrating N-ethylmorpholine with HCl to the desired pH value. Stock solutions of ADP were adjusted to pH 7.0 with 10% tetrapropylammonium hydroxide. The purity and concentration of the substrates was determined enzymically and by direct absorption measurements as described by Macfarlane (1973) and Ainsworth & Macfarlane (1973). Pyruvate kinase was prepared as described previously (Gregory & Ainsworth, 1981).

Enzyme assays

Reaction mixtures were prepared on the assumption that the true substrates of the pyruvate kinase

reaction are free Mg^{2+} and the Mg^{2+} -free species of ADP and phosphoenolpyruvate (Ainsworth & Macfarlane, 1973; Macfarlane *et al.*, 1974; Phillips & Ainsworth, 1977). The apparent dissociation constants for the binding of Mg^{2+} by ADP and phosphoenolpyruvate have been given by Gregory & Ainsworth (1981).

Pyruvate kinase activity was measured by using the continuous lactate dehydrogenase coupled assay (Bücher & Pfeleiderer, 1955). Initial velocities (in triplicate) were determined at 25°C in a solution (1 ml) containing, in addition to the indicated substrates, 100 μmol of *N*-ethylmorpholine, pH 7.4, 100 μmol of KCl, 0.15 μmol of NADH and an excess (12 units) of lactate dehydrogenase. The initial velocities (v) reported in the Figures are values corrected to an enzyme specific activity of 300 units/mg of protein and are expressed as μmol of NADH oxidized/min per mg of protein at 25°C. The specific activity of the enzyme was determined as described by Phillips & Ainsworth (1977).

Computer analysis of data

The analysis of data was based on the unconstrained version of 'PAPB' (Kinderlerer *et al.*, 1981), a program in which an improvement in fit is sought by a 'one-at-a-time' adjustment of the model constants. This program was employed in its original form and in a variant where V_G was also incremented in the search for a better fit. In addition, a new program 'DESCENT' was employed in which all the constants were incremented simultaneously by amounts determined by a steepest-descent criterion. This program has the facility for forcing a shift in the model constants if no improvement is obtained at any stage, a feature designed to diminish the probability of the solution settling in a local minimum. The success or failure of the optimization in all these programs is determined at each stage by change in the product of an absolute and relative error, $A \times R$, where:

$$A = \sum (v_{\text{calc.}} - v_{\text{obs.}})^2$$

$$R = \sum \left(\frac{v_{\text{calc.}} - v_{\text{obs.}}}{v_{\text{calc.}}} \right)^2$$

(Kinderlerer *et al.*, 1981; Ainsworth *et al.*, 1981a). Tables 2 and 3 report these errors in modified forms, thus $\text{RSS} = \sqrt{A/n}$ and $\text{MD\%} = 100\sqrt{R/n}$, where n is the number of observations. The programs exist in two-substrate and three-substrate forms, and listings can be obtained from the authors. Note that 'DESCENT' has the considerable advantage of not requiring the constraints that were necessary in earlier analytical procedures (Kinderlerer *et al.*, 1981). Tests of both two-substrate and three-substrate 'DESCENT' programs have shown that they provide highly accurate fits to artificial, error-free, data.

Results

The data points illustrated in Fig. 1 are uncorrected, but all the remaining points displayed in Figs. 4–9 have been corrected according to eqn. (17) with a value of $\alpha_D/K_1 = 0.1 \text{ mM}^{-2}$. [Note that the data points in Fig. 1 when replotted as $v = f(C)$ show maxima at the two highest values of A .]

Dead-end inhibition

Fig. 2 illustrates the relationship $A_{\text{max.}}^2 = f(C^{-1})$ for values derived from Fig. 10 of Phillips & Ainsworth (1977) and from the two maxima, shown in Fig. 1, which appear at (A, B, C) co-ordinates of (3, 0.2, 0.5) and (2, 0.2, 1.0). Fig. 2 shows that lines can be drawn through the $A_{\text{max.}}^2$ values, which acceptably meet the requirement for intersection at the origin (eqn. 16) and which imply that α_{ac} must not vary greatly over the range of the Figure.

An approximate value of the correction factor was determined as follows. A two-substrate fit was obtained to the 25 points appearing in Fig. 1 for

Fig. 1. Effect of [ADP] on the initial velocity of pyruvate formation at several fixed values of $[\text{Mg}^{2+}]$ with $[\text{phosphoenolpyruvate}] = 0.2 \text{ mM}$

The data points (represented by letters) are uncorrected for dead-end inhibition by MgADP . The dotted curves are calculated in accordance with eqn. (19) with $\alpha_D/K_1 = 0.1 \text{ mM}^{-2}$ and the two-substrate exponential model (eqn. 6) with the constants given in column 2 of Table 3. In this and succeeding Figures velocities, v , are expressed as μmol of NADH oxidized/min per mg of protein at 25°C at pH 7.4 in the presence of 0.1 M-K^+ , with other conditions as described in the Experimental section. Mg^{2+} concentrations (mM) were: A, 0.05; B, 0.1; C, 0.15; D, 0.2; E, 0.5; F, 1.0.

Fig. 2. Effect of [Mg²⁺] on the position of the maximum in the relationship $v = f[ADP]$
□, Present study, Fig. 1; ○, Phillips & Ainsworth (1977), Fig. 10.

Fig. 3. Comparison of the effects of Mn^{2+} and Mg^{2+} on the pyruvate kinase reaction
[Phosphoenolpyruvate] = 0.05 mM with: ○, 0.1 mM- Mn^{2+} ; △, 0.1 mM- Mg^{2+} . Other conditions were as described in the legend to Fig. 1.

which the product $A \cdot C$ was not greater than 0.5 mM^2 . The constants of this fit are given in Table 3. α_D/K_1 was then determined from the 11 points where $A \cdot C > 0.5 \text{ mM}^2$ by:

$$\frac{\alpha_D}{K_1} = \frac{1}{n} \sum_{i=1}^n \left\{ \left(\frac{v_{\text{calc.}}}{v_{\text{obs.}}} - 1 \right) / A \cdot C \right\}_i \tag{18}$$

where $v_{\text{calc.}}$ was calculated from the constants of the two-substrate fit. Eqn. (18) gives $\alpha_D/K_1 = 0.1 \text{ mM}^{-2}$. All 36 points are illustrated in Fig. 1 superimposed on curves, $v_{\text{plot}} = f(A)$, given by the relationship:

$$v_{\text{plot}} = \frac{v_{\text{calc.}}}{(1 + A \cdot C \cdot \alpha_D/K_1)} \tag{19}$$

The agreement between the observed points and the plotted curves is good, considering the empirical nature of the approach, and we have therefore employed 0.1 mM^{-2} as the correction factor applied to all the points appearing in Figs. 4–9. In support of this choice we can demonstrate a consistency of behaviour between the two sets of maxima illustrated in Fig. 2. This comparison is given in Table 1, and is based on the assumption that α_{ac} is a constant.

Note that the chosen value of α_D/K_1 means that the 25 points used to calculate the exponential model constants for Fig. 1 are less than 5% in error due to dead-end inhibition and that most of the points appearing in the remaining Figures are barely affected at all.

Finally, Fig. 3 illustrates that activation by Mn^{2+} also leads to a maximum in the function $v = f(A)$, and at a concentration where Mg^{2+} does not lead to dead-end inhibition.

Analysis of data

Table 2 gives two-substrate and three-substrate exponential model constants for data points corrected for dead-end inhibition as described in the preceding subsection. Table 3 gives corresponding estimates of the constants when uncorrected data points are employed but when the 11 points with $A \cdot C > 0.5 \text{ mM}^2$ are omitted. Figs. 4–9 illustrate the fits obtained when the plotted curves are calculated from the constants described as estimate 1 in Table 2.

Table 1. Estimation of α_{ac}

Line in Fig. 2	Slope (M^3) (eqn. 16)	α_D/K_1 (mM^{-2})	α_{ac}^{-1} (mM)	Concn. of ADP giving half the maximum observed velocity (mM)
□-□ (present study)	4.2×10^{-9}	0.1	0.42	0.43
○-○ (Phillips & Ainsworth, 1977)	1.8×10^{-9}	0.1	0.18	0.20

Table 2. *Values of two-substrate and three-substrate exponential model constants for the corrected initial velocities of the pyruvate kinase-catalysed reaction illustrated in Figs. 4–9*

The error parameters are defined in the text. Temperature 25°C; pH 7.4, A = ADP; B = phosphoenolpyruvate; C = Mg²⁺. The concentration of K⁺ was 0.1 M.

Constant	$v_{\text{corr.}} = f(A, B)_C$ (data from Figs. 4 and 5)	$v_{\text{corr.}} = f(A, C)_B$ (data from Figs. 6 and 7)	$v_{\text{corr.}} = f(B, C)_A$ (data from Figs. 8 and 9)	$v_{\text{corr.}} = f(A, B, C)$ (data from Figs. 4–9)			
				Estimate 1		Estimate 4	
				Start	Final	Start	Final
$\ln \alpha_{000} \text{ (mM}^{-1}) $	–1.491	–1.260	—	0.481	0.516	0.522	0.475
$\ln \beta_{000} \text{ (mM}^{-1}) $	3.131	—	4.165	3.999	3.488	3.610	3.526
$\ln \gamma_{000} \text{ (mM}^{-1}) $	—	0.276	–1.283	–1.038	–0.572	–0.602	–0.622
k_{AA}	2.152	1.171	—	–0.359	–1.410	–1.785	–1.758
k_{BB}	–0.301	—	0.276	0.513	0.072	–0.534	–0.234
k_{CC}	—	–0.067	2.132	0.308	–0.599	–0.905	–0.893
k_{AB}	–0.936	—	—	–1.339	–1.868	–2.019	–1.996
k_{AC}	—	1.640	—	1.936	2.937	3.228	3.231
k_{BC}	—	—	–0.056	1.180	0.937	1.198	1.172
$V_G \text{ (}\mu\text{M/min per mg)}$	245.756	459.330	123.786	504.980	611.499	590.224	625.987
RSS	1.958	8.199	1.725	10.106	8.572	11.043	8.525
MD%	8.197	8.745	8.858	18.882	16.516	16.907	16.551

Table 3. *Values of two-substrate and three-substrate exponential model constants for the uncorrected initial velocities of the pyruvate kinase-catalysed reaction*

These velocities correspond to the values illustrated in Figs. 4–9, but with the omission of 11 values (defined in Fig. 1) that were determined in assays where $A \cdot C > 0.5 \text{ mM}^2$. The error parameters are defined in the text. Temperature 25°C; pH 7.4. A = ADP; B = phosphoenolpyruvate; C = Mg²⁺. The concentration of K⁺ was 0.1 M.

Constant	$v = f(A, B)_C$	$v = f(A, C)_B$ (data from Fig. 1)	$v = f(B, C)_A$	$v = f(A, B, C)$			
				Estimate 2		Estimate 3	
				Start	Final	Start	Final
$\ln \alpha_{000} \text{ (mM}^{-1}) $	–1.347	–1.288	—	0.516	0.535	0	0.522
$\ln \beta_{000} \text{ (mM}^{-1}) $	3.202	—	4.161	3.488	3.528	0	3.610
$\ln \gamma_{000} \text{ (mM}^{-1}) $	—	0.267	–1.264	–0.572	–0.504	0	–0.602
k_{AA}	2.269	1.285	—	–1.410	–1.418	0	–1.785
k_{BB}	0.597	—	0.281	0.072	0.000	0	–0.534
k_{CC}	—	0.063	2.094	–0.599	–0.545	0	–0.905
k_{AB}	–0.885	—	—	–1.868	–1.768	0	–2.019
k_{AC}	—	1.451	—	2.937	2.954	0	3.228
k_{BC}	—	—	–0.047	0.937	0.902	0	1.198
$V_G \text{ (}\mu\text{M/min per mg)}$	178.631	497.053	121.331	500	507.629	500	590.224
RSS	1.686	7.465	1.721	—	7.976	—	7.965
MD%	6.606	10.072	8.844	—	17.768	—	17.421

Note that the starting point for estimate 1 (Table 2) was in fact a stage reached during the development of the 'DESCENT' program, the original start not having been recorded. All the remaining starting estimates are as shown.

The values of the constants in Tables 2 and 3 are given to a precision consistent with the stated error: thus it should be noted that rounding-off the constants to accuracies more appropriate to their

reproducibility would considerably increase the magnitude of the corresponding errors.

Discussion

Mechanism

The conclusion that rabbit muscle pyruvate kinase has an equilibrium random-order mechanism, referred to above, has been questioned by Dann &

Fig. 4. Effect of $[ADP]$ on the corrected initial velocity of pyruvate formation at several fixed values of $[phosphoenolpyruvate]$ with $[Mg^{2+}] = 0.1 \text{ mM}$

In this and succeeding Figures velocities (represented by letters) are corrected for dead-end inhibition by eqn. (17) with $\alpha_p/K_1 = 0.1 \text{ mM}^{-2}$, and are fitted by dotted curves calculated in accordance with the three-substrate exponential model (eqns. 2–5) with the constants given as estimate 1 in Table 2. Other conditions were as described in the legend to Fig. 1. Phosphoenolpyruvate concentrations (mM) were: A, 0.01; B, 0.025; C, 0.05; D, 0.10; E, 0.15; F, 0.20.

Fig. 5. Effect of $[phosphoenolpyruvate]$ on the corrected initial velocity of pyruvate formation at several fixed values of $[ADP]$ with $[Mg^{2+}] = 0.1 \text{ mM}$

ADP concentrations (mM) are: A, 0.5; B, 0.7; C, 1.0; D, 2.0; E, 3.0; F, 5.0. Other conditions and calculations were as described in the legends to Figs. 1 and 4.

Fig. 6. Effect of $[ADP]$ on the corrected initial velocity of pyruvate formation at several fixed values of $[Mg^{2+}]$ with $[phosphoenolpyruvate] = 0.2 \text{ mM}$

Mg^{2+} concentrations (mM) were: A, 0.05; B, 0.10; C, 0.15; D, 0.20; E, 0.50; F, 1.0. Other conditions and calculations were as described in the legends to Figs. 1 and 4.

Fig. 7. Effect of $[Mg^{2+}]$ on the corrected initial velocity of pyruvate formation at several fixed values of $[ADP]$ with $[phosphoenolpyruvate] = 0.2 \text{ mM}$

ADP concentrations (mM) were: A, 0.5; B, 0.7; C, 1.0; D, 2.0; E, 3.0; F, 5.0. Other conditions and calculations were as described in the legends to Figs. 1 and 4.

Fig. 8. Effect of [phosphoenolpyruvate] on the corrected initial velocity of pyruvate formation at several fixed values of $[Mg^{2+}]$ with $[ADP] = 0.1$ mM

Mg^{2+} concentrations (mM) were: A, 0.1; B, 0.3; C, 0.5; D, 1.0; E, 1.5; F, 2.0. Other conditions and calculations were as described in the legends to Figs. 1 and 4.

Fig. 9. Effect of $[Mg^{2+}]$ on the corrected initial velocity of pyruvate formation at several fixed values of [phosphoenolpyruvate] with $[ADP] = 0.1$ mM

Phosphoenolpyruvate concentrations (mM) were: A, 0.01; B, 0.025; C, 0.05; D, 0.10; E, 0.15; F, 0.20. Other conditions and calculations were as described in the legends to Figs. 1 and 4.

Britton (1978), who have shown in studies at pH 8.5 that, although the mechanism is random order, in one pathway phosphoenolpyruvate adds to the enzyme in a rate-limiting step. If this finding also applies at pH 7.4, it must indicate that eqn. (1) cannot be strictly valid. Accepting that hypothesis, it is nevertheless the case that the observed kinetic behaviour of a branched reaction only departs from that required by an equilibrium random-order mechanism when the rate constants of the reaction have unusual values (King, 1956; Cleland & Wratten, 1969; Endrenyi *et al.*, 1971; Pettersson, 1972). Since that departure was not observed in the previous work, eqn. (1) may still be approximately true and its use may be allowed in the description of the allosteric kinetics of pyruvate kinase (Ainsworth *et al.*, 1981b).

Dead-end inhibition

The observation of dead-end inhibition reported in the present paper and by Phillips & Ainsworth (1977) in studies conducted at pH 7.4 can be contrasted with corresponding studies at pH 6.2 by Ainsworth & Macfarlane (1973), where inhibition was not found. Granted the existence of a second nucleotide-binding site, the probable reason for this discrepancy lies in the change of pH. Because the substrates of pyruvate kinase are treated as free Mg^{2+} and Mg^{2+} -free ADP (Ainsworth & Macfarlane, 1973), the increase in affinity of ADP for Mg^{2+} that occurs with increasing pH (Phillips *et al.*, 1966) also increases the proportion of total nucleotide that is present as MgADP when any given concentration of the free substrate is established. This effect must enhance the likelihood of inhibition, and it may be noted that Jansen & Cleland (1974) have also observed dead-end inhibition of muscle pyruvate kinase by ADP at pH 7. The proposal that inhibition is caused by the metal-ion-bound nucleotide is supported by the experiment in which Mn^{2+} was used instead of Mg^{2+} as the activating bivalent cation. The MnADP complex is characterized by a dissociation constant of 7.30×10^{-5} M, compared with 6.74×10^{-4} M for the MgADP complex, at pH 7.4 (Macfarlane *et al.*, 1974), and for that reason should be a more potent inhibitor of pyruvate kinase. Examination of Fig. 3 shows this to be so.

Two-substrate exponential model constants

The three-substrate exponential model constants demonstrate that all three substrates are interactive both between themselves and each other. Data points $v = f(X, Y)_Z$ cannot therefore be properly represented by the two-substrate model, because p_Z will not remain constant as X and Y are varied. Fits by the two-substrate model were nevertheless attempted in order to establish what justification there might be for its use in circumstances, such as

insufficient data, where the more appropriate model could not be employed. The error parameters of the two-substrate solutions given in Tables 2 and 3 demonstrate at once that the fits to individual data sets are considerably better than the overall fit provided by the three-substrate model, and indeed evidence of systematic error, such as that which can be observed in Figs. 4–9, is absent from corresponding plots that use the two-substrate constants. The observation is not surprising, because the two-substrate solutions employ a total of 18 constants, against the 10 required by the three-substrate model. Nevertheless, the demonstration that good empirical fits can be obtained by the simpler model means that it can be employed in the first instance to examine the internal consistency of non-hyperbolic $v = f(X, Y)_Z$ data points. An additional advantage of the two-substrate solutions is that they indicate the net effect, as observed by eye, of what may be confusing interactions in the three-substrate model. Thus it is evident that A and C display positive homotropic interactions [$v = f(A, B)$ and $v = f(B, C)$] and that much of these effects may be due to heterotropic interaction between each other [$v = f(A, C)$]. These observations accord with the curve shapes and with conclusions that can be drawn from the three-substrate solution. Again, B usually appears to display negative homotropic interaction. The values of the constants indicate, however, that the only certain cause of this behaviour is a negative heterotropic interaction between A and B. As this feature is also found in the three-substrate solution, it must be regarded as fundamental. The homotropic interaction of B is equivocal, both positive and negative values being ascribed to the constant k_{BB} . But, again, this behaviour runs through the three-substrate solution, and there is the indication that the smaller values (having regard to sign) tend to be associated with comparably high values of V_G , the change in one constant compensating for change in the other.

This analysis appears to show that a worthwhile amount of information is retained in the two-substrate solution to a three-substrate problem. The simpler model can therefore be employed as an adjunct, to preserve the advantages noted above, or alone when necessity dictates.

Three-substrate exponential model constants

Table 2 gives two solutions to data $v = f(A, B, C)$, corrected for dead-end inhibition; Table 3 gives a further two solutions to corresponding uncorrected data, except that 11 velocities measured with $A \cdot C > 0.5 \text{ mM}^2$ have been omitted. Estimate 1, except for a considerable decrease in V_G , was made the starting point for estimate 2, based on corrected data. Comparison of the two solutions shows that minor movements in the constants are able to

accommodate not only the marginally changed data base but also a very large change in V_G . This observation shows how insensitive a settled solution is to V_G , and correspondingly indicates the level of confidence that can be placed in its values. Estimate 3 has a completely new start, but, in spite of the initially low V_G value, the final solution is very similar to estimate 1, the principal discrepancy being found in k_{BB} . Estimate 4, with estimate 3 as its starting point, is again very similar, but it will be noted that there is usually a small reversion in the values of the constants towards the directly comparable values of estimate 1. Overall, the four estimates provide a measure of confidence that the outlines of a genuine solution have been achieved. Nonetheless, the insensitivity of the solution to V_G and k_{BB} (and their possible association) clearly indicates that the analytical technique leaves room for improvement in dealing with real data and that the accuracy of the data base is critical. Figs. 4–9 compare curves calculated from estimate 1 with the corrected data that were used to obtain the solution. Although no Figure is free from evidence of systematic error, the curve shapes convincingly reproduce the general character of the data and reinforce the belief that the solution is broadly correct.

We turn now to consider a possible interpretation of the constants that have been evaluated and, for that purpose, use the set provided by estimate 4 on the ground that its value of k_{BB} is closest to the mean of the four values obtained.

First of all, the values of $\ln \alpha$, $\ln \beta$ and $\ln \gamma$ correspond to dissociation constants of 0.622, 0.0294 and 1.863 mM respectively, for ADP, phosphoenolpyruvate and Mg^{2+} , and, again respectively, can be compared with values of 0.809, 0.0797 and 59.2 mM obtained by Ainsworth & Macfarlane (1973) in experiments conducted at pH 6.2. The marked difference, for Mg^{2+} , is consistent with the expected ionic mode of binding, which should be facilitated as the pH rises. The maximum velocity of $626 \mu\text{M}/\text{min per mg}$ corresponds to a value of 617 s^{-1} , assuming that pyruvate kinase has a molecular weight of 237000 (Warner, 1958) with four active sites per molecule (Stammers & Muirhead, 1975). This can be compared with 540 s^{-1} that can be calculated from the value of $1/\phi_0$ given by Ainsworth & Macfarlane (1973), and is consistent with the way in which the enzyme activity was standardized at the two pH values (Phillips & Ainsworth, 1977).

The homotropic interaction constants, k_{XX} , are all negative, as is the heterotropic constant k_{AB} ; it is evident, therefore, that the positive interaction visible to the eye arises from the large positive interaction, k_{AC} , with the more modest contribution from k_{BC} . Ainsworth & Macfarlane (1973) compared the

Table 4. Comparison of values and significance of interaction constants (present study) with dissociation constants of binary and ternary complexes of pyruvate kinase (Ainsworth & Macfarlane, 1973)

$$-1.996 = k_{AB} = k_{BA} = \ln \left(\frac{\alpha_{010}}{\alpha_{000}} \right) \rightarrow \ln \left(\frac{K_A}{K_{BA}} \right) = \ln 0.56 = -0.58$$

$$3.231 = k_{AC} = k_{CA} = \ln \left(\frac{\alpha_{001}}{\alpha_{000}} \right) \rightarrow \ln \left(\frac{K_A}{K_{CA}} \right) = \ln 10.5 = 2.35$$

$$1.172 = k_{BC} = k_{CB} = \ln \left(\frac{\beta_{001}}{\beta_{000}} \right) \rightarrow \ln \left(\frac{K_B}{K_{BC}} \right) = \ln 1.46 = 0.38$$

dissociation constant of a given substrate, say X, when it binds to the free enzyme, K_X , with the constant that describes its binding to a binary complex with Y, K_{YX} . The ratio of these values $K_X/K_{YX} = K_Y/K_{XY} = 1.46$ (for example) indicates that the prior binding of X or Y decreases the dissociation constant of Y or X by a factor of 1.46, i.e. the binding of the second substrate is enhanced. The interaction constants provide a similar measure, and the two systems are compared in Table 4. Examination of the Table reveals a noteworthy consistency of behaviour of the enzyme, which spans the change in pH and the transition from Michaelis-Menten to 'non-hyperbolic' behaviour. The original interpretation of the K_X/K_{YX} values was that they reflected charge attractions and repulsions between the substrates at the active site, thus enhancing or decreasing their relative affinities for the enzyme. The idea of charge interaction can be taken over here, and is consistent with the uniformly negative homotropic interactions that are observed: the homotropic interactions make it clear, however, that the charges must influence one another between the active sites, in addition to their mutual effects within one site.

These rather simple interpretations of the interaction constants should not obscure the more complicated consequences that arise from the indirect path of interaction suggested by eqn. (11). For example, the immediately negative interaction of A and B is counteracted by a positive interaction, $k_{AC}k_{CB}\phi_C$, whose relative efficacy depends on the fractional saturation of the enzyme by C. The physical origins of the indirect path are clearly charge attractions, and it may be that their effects can be observed in Figs. 4 and 5. Thus it appears that the curves in Fig. 4 become more obviously sigmoidal functions of A as B rises, and that the 'premature' flattening of $v = f(B)$, shown in Fig. 5, becomes less marked as A increases.

The actual values of the interaction constants reveal apparent interaction free energies in the range -8.1 to $+5.0$ kJ/mol (-1.94 to $+1.2$ kcal/mol), values that are comparable with those estimated for other co-operative protein systems (Weber, 1975).

This description of the allosteric properties of rabbit muscle pyruvate kinase in terms of the exponential model has established a consistency of behaviour with the equilibrium random-order kinetics observed at pH 6.2. The agreement supports the use of the exponential model as a relatively simple means of rationalizing complex results, and it should be noted that the description requires only two constants more than the eight employed by the Michaelis-Menten treatment.

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