The regulatory properties of yeast pyruvate kinase

Effect of fructose 1,6-bisphosphate

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The kinetics of pyruvate kinase from *Saccharomyces cerevisiae* were studied in assays at pH 6.2 at 25 °C as a function of the concentrations of the substrates ADP, phosphoenolpyruvate and Mg^{2+} and the concentration of the effector fructose 1,6-bisphosphate. The enzyme was activated by 100 mM- K^+ and 32 mM- NH_4^+ throughout. It was found that an increase in the fructose bisphosphate concentration from 24 μM to 1.2 mM brings about a transition from a sigmoidal to a non-inflected form in the relationships $v = f([phosphoenolpyruvate])$ and $v = f([Mg^{2+}])$ together with a large increase in the affinity of these substrates for the enzyme. The binding behaviour of ADP is barely affected by the same change in effector concentration. By contrast, increase in fructose bisphosphate concentration below 24 μM increases the affinity of the enzyme for all its substrates and the sigmoidicity of the corresponding velocity–substrate–concentration relationships. As a result of this change in behaviour it has been found impossible to represent all the data by the exponential model for a regulatory enzyme, and it is suggested (supported by comparisons with previous work) that the failure may reflect a secondary action of the effector upon the enzyme.

INTRODUCTION

The glycolytic enzyme pyruvate kinase (EC 2.7.1.40) from the yeast *Saccharomyces cerevisiae* displays sigmoidal relationships between the initial velocity of pyruvate formation and the concentration of any one of the substrates phosphoenolpyruvate, ADP and Mg^{2+} , when the concentrations of the other two are kept constant at pH 6.2 (Morris *et al.*, 1984). The present paper reports the results of a systematic investigation of the influence of these substrate concentrations on the initial velocity of reaction measured in the presence of different concentrations of the allosteric activator fructose 1,6-bisphosphate. The results are interpreted by extending the exponential model described by Ainsworth *et al.* (1983) to four ligands, the assumption being made that the fractional saturation of the enzyme by univalent cations (H^+ , K^+ and NH_4^+) remains constant throughout.

EXPERIMENTAL METHODS AND ANALYSIS OF RESULTS

Materials and general methods

Materials, methods and the preparation of pyruvate kinase were as described by Morris *et al.* (1984). Fructose 1,6-bisphosphate was employed as the tetracylohexylammonium salt (Boehringer Corp., Lewes, East Sussex, U.K.). Values of the initial velocity of pyruvate formation measured in duplicate as a function of the concentrations of ADP (A), phosphoenolpyruvate (B), fructose 1,6-bisphosphate (D), all in their Mg^{2+} -free forms, and free Mg^{2+} (C), at pH 6.2 and 25 °C, were analysed by fitting the equation:

$$\frac{v}{V_G} = p_A \cdot p_B \cdot p_C \cdot [1 + (y - 1)p_D] \quad (1)$$

together with four equations of the type:

$$\ln w_i = \ln w_0 + k_{WW} \cdot p_W + k_{XW} \cdot p_X + k_{YW} \cdot p_Y + k_{ZW} \cdot p_Z \quad (2)$$

as described by Ainsworth *et al.* (1983) and Morris *et al.* (1984). In eqns. (1) and (2) p_X stands for the fractional saturation of the enzyme by ligand X. V_G represents the initial rate of product formation (v) when the enzyme is completely saturated by substrates, $p_A = p_B = p_C = 1$, in the absence of D. A different rate is measured when the enzyme is also saturated by D and the change is represented by multiplying V_G by the constant y (Ainsworth & Gregory, 1978). w_i is the association constant for the ligand W, equal to $p_W / [(1 - p_W) \cdot W]$, measured in the i th solution defined by the particular values of the fractional saturations obtaining therein. k_{XY} is an interaction free energy in RT units, where X denotes the ligand whose binding causes a change in the association constant for Y. Reciprocal allosteric linkage requires $k_{XY} = k_{YX}$.

Activation by K^+ and NH_4^+ ions

The rabbit muscle lactate dehydrogenase employed in the enzyme assay described by Morris *et al.* (1984) and used here was 3.2 M with respect to $(NH_4)_2SO_4$, with the result that addition of the standard 5 μl portion of coupling enzyme to the assay established a concentration of 32 mM- NH_4^+ in the assay solution, in addition to the 100 mM- K^+ added as the activating univalent cation. The experiments have not been repeated without the addition of NH_4^+ (which was unintended) because of the advantages that the presence of NH_4^+ is seen to confer. Both K^+ and NH_4^+ are effective activators of pyruvate kinase, but each, individually, shows a maximum in the relationship $v = f([M^+])$ when its concentration is increased beyond those employed here (Kachmar &

Boyer, 1953; Hunsley & Suelter, 1969). In addition, with typical conditions for our experiments, the maximum activation with K^+ ions alone is not approached below a concentration of 200 mM. By contrast, extensive experiments with a wide range of A, B and C concentrations and 32 mM- NH_4^+ have shown that a plateau in the K^+ response is always achieved at concentrations below 100 mM and maintained well beyond them. In an accompanying paper (Rhodes *et al.*, 1986) it is reported in detail that this result arises because NH_4^+ and K^+ co-operate synergistically in the univalent-activation of yeast pyruvate kinase and that the enzyme is fully activated at the NH_4^+ and K^+ concentrations present in the enzyme assays. NH_4^+ was also found to increase the stability of pyruvate kinase as judged by a smaller error in duplicate rate measurements and a decreased rate at which enzyme solutions lost activity. For these reasons, it seems advantageous to continue the joint use of K^+ and NH_4^+ as activating cations. A determination of the apparent NH_4^+ -binding constants at pH 6.2 of ADP (808 mM) and phosphoenolpyruvate (242 mM), described by Rhodes *et al.* (1986), shows that 32 mM- NH_4^+ has no significant effect on the substrate-level equilibria of Mg^{2+} : the possible binding of NH_4^+ to fructose biphosphate in our experiments was not examined because the very low concentrations of the effector would render the outcome inconsequential.

RESULTS

The data illustrated in Figs. 1–6 are the means of duplicate measurements of the initial velocity. The mean percentage deviation from the average of each paired measurement is 1.3% for the complete data set, a value that appears acceptably low but gives no indication of the errors that arise in making up the assay solutions: previous estimates suggest that these errors will be much larger (Morris *et al.*, 1984). Table 1 gives a fit to 360 data points, with fructose biphosphate concentrations up to 24 μ M, based on eqns. (1) and (2). The 104 data points represented in Figs. 5 and 6 were fitted by the three-ligand exponential model equations (Ainsworth *et al.*, 1983) on the assumption that fructose biphosphate is saturating at a concentration of 1.2 mM: the constants of this fit are given in Table 2.

We have sampled the distribution of error associated with the overall fit to data with fructose biphosphate concentrations up to 24 μ M by calculating the error parameters that arise when sub-sets of the data are fitted by its constants. The sub-sets chosen for this examination correspond to the several values of fructose biphosphate concentration present in the reactant solutions. Table 1 shows that the sub-set values of the error product are lower than the error product of the set except for [fructose biphosphate] = 0, where the error product is much larger. If the fits to the individual sub-sets are now improved by further optimization starting from the overall fit, then the merit of each constant in the context of this subdivision of the data can be judged by the range of values that each covers in representing the several sub-sets. These are given in Table 1. Examination of the table shows that there is not a marked tendency for the fit to worsen as the fructose biphosphate concentration is increased.

Fig. 1. Effect of [ADP] on the initial velocity of pyruvate formation at several fixed values of [phosphoenolpyruvate] with $[Mg^{2+}] = 2.0$ mM and [fructose 1,6-bisphosphate] = 0.001 μ M (assumed for the purposes of calculation; no effector was added to these solutions)

In this and succeeding Figures, initial velocities, v , (represented by symbols) are expressed as μ mol of NADH oxidized/min per mg of protein at 25 °C in the presence of 0.1 M- K^+ and 32 mM- NH_4^+ at pH 6.2 with other conditions as described in the Experimental methods and analysis of results section. The datum points in Figs. 1–4 are fitted by curves calculated in accordance with the four-ligand exponential model [eqns. (1) and (2)] with optimized values of the constants, given in Table 1, relevant to the fructose 1,6-bisphosphate concentration employed. The datum points in Figs. 5 and 6 are fitted by curves calculated in accordance with the three-substrate exponential model [eqns. (1)–(4) in Morris *et al.* (1984)] with constants given as Fit D1200 in Table 2. Phosphoenolpyruvate concentrations were: ■, 0.75 mM; ●, 1.0 mM; ▲, 1.5 mM; □, 2.5 mM.

Figs. 1–4 display curves calculated by using optimized fits given in Table 1. The data points obviously lie closer to these curves than they do to curves calculated from the overall fit to the 360 data points by amounts that may be judged from the corresponding error parameters. The curves drawn in Figs. 5 and 6 have similarly been calculated from the fit given in Table 2.

Examination of the Figures shows that the fit is less good at low velocities, an observation that is reflected by the relative values of the RSS and MD% error parameters. A similar outcome has been noted in the analysis of data relating to other enzymes (Ainsworth, 1977).

DISCUSSION

The allosteric interactions of yeast pyruvate kinase can be described by the constants given in Table 2. For simplicity of reference, Fit D24 represents $v = f(A, B, C, D)$ data measured with fructose bisphos-

Fig. 2. Effect of $[Mg^{2+}]$ on the initial velocity of pyruvate formation at several fixed values of [phosphoenolpyruvate] with $[ADP] = 2$ mM and [fructose 1,6-bisphosphate] = $0.001 \mu M$ (assumed for the purpose of calculation; no effector was added to these solutions)

Phosphoenolpyruvate concentrations were: ■, 0.75 mM; ●, 1.0 mM; ▲, 1.5 mM; □, 2.5 mM. Other conditions and calculations were as described in the legend to Fig. 1.

Fig. 4. Effect of $[Mg^{2+}]$ on the initial velocity of pyruvate formation at several fixed values of [phosphoenolpyruvate] with $[ADP] = 2$ mM and [fructose 1,6-bisphosphate] = $24 \mu M$

Phosphoenolpyruvate concentrations were: ■, 0.125 mM; ●, 0.25 mM; ▲, 0.375 mM; □, 0.5 mM; ○, 0.75 mM; △, 1.0 mM; ▼, 1.5 mM; ▽, 2.5 mM. Other conditions and calculations were as described in the legend to Fig. 1.

Fig. 3. Effect of $[ADP]$ on the initial velocity of pyruvate formation at several fixed values of [phosphoenolpyruvate] with $[Mg^{2+}] = 2$ mM and [fructose 1,6-bisphosphate] = $24 \mu M$

Phosphoenolpyruvate concentrations were: ■, 0.125 mM; ●, 0.25 mM; ▲, 0.375 mM; □, 0.5 mM; ○, 0.75 mM; △, 1.0 mM; ▼, 1.5 mM; ▽, 2.5 mM. Other conditions and calculations were as described in the legend to Fig. 1.

Fig. 5. Effect of [phosphoenolpyruvate] on the initial velocity of pyruvate formation at several fixed values of $[ADP]$ with $[Mg^{2+}] = 2$ mM and [fructose 1,6-bisphosphate] = $1.2 \mu M$

ADP concentrations were: ■, 0.1 mM; ●, 0.2 mM; ▲, 0.5 mM; □, 1.0 mM; ○, 1.5 mM; △, 2.0 mM. Other conditions and calculations were as described in the legend to Fig. 1.

Fig. 6. Effect of $[Mg^{2+}]$ on the initial velocity of pyruvate formation at several fixed values of [phosphoenolpyruvate] with $[ADP] = 2 \text{ mM}$ and [fructose 1,6-bisphosphate] = 1.2 mM

Phosphoenolpyruvate concentrations were: ■, 0.125 mM ; ●, 0.25 mM ; ▲, 0.375 mM ; □, 0.5 mM ; ○, 0.75 mM ; △, 1.0 mM ; ▼, 1.5 mM ; ▽, 2.0 mM . Other conditions and calculations were as described in the legend to Fig. 1.

phate concentrations up to $24 \mu\text{M}$. Fit D0, included for comparison, represents $v = f(A, B, C)_{D=0}$ data obtained independently by Morris *et al.* (1984). Finally, Fit D1200 represents $v = f(A, B, C)_{D=1.2\text{mM}}$ data measured with saturating concentrations of fructose bisphosphate: this fit was obtained by assuming that when $p_D = 1$ its effect on the binding of the substrates is subsumed in apparent affinity constants defined by adding k_{DW} to $\ln w_0$ (eqn. 2).

Modelling the data

The most obvious feature of the data we present is the marked change in behaviour that accompanies an increase in the concentration of fructose bisphosphate in reactant solutions from $24 \mu\text{M}$ to 1.2 mM (Fits D24 and D1200). The change itself corresponds approximately to a sigmoidal-to-hyperbolic transition in the relationships $v = f([\text{phosphoenolpyruvate}])$ and $v = f([Mg^{2+}])$ together with a large increase in the affinity of these substrates for the enzyme. By contrast, the behaviour of ADP towards the enzyme is barely affected by the change in effector concentrations. A very similar result has been reported by Hunsley & Suelter (1969). A sigmoidal-to-hyperbolic transition in the relationship $v = f([\text{phosphoenolpyruvate}])$ has also been found by Johannes & Hess (1973) in studies where ADP and Mg^{2+} were present at high concentrations in reactant solutions kept at pH 7.0. The exponential model for one substrate and an effector with $\gamma = 1$ cannot produce a sigmoidal-to-hyperbolic transition as p_D takes the values 0 and 1 respectively (Ainsworth & Gregory, 1978), but the restriction is lifted when more than one substrate is involved (Ainsworth, 1986). For example, the typical appearance of an inflected-non-

inflected transition (Monod *et al.*, 1965) can be observed in $v = f(A)_{B,D}$ when the following conditions apply: $A = 0-1$, B (second substrate concentration) = 0.1 , D (effector concentration) fixed at values in the range $0-8$, $k_{AA} = -1$, $k_{BB} = 1.5$, $k_{AB} = 2$, $k_{BD} = 2$, $\gamma = 1$, with all other interaction constants and $\ln w_0$ values equal to zero. The same result can also be demonstrated with $k_{AA} = 0$. On the basis of these simulations, it seemed reasonable to expect that the exponential model would be able to describe all the results presented here, as it had earlier successfully represented the equivalent, though simpler, data recorded for the rabbit muscle enzyme (Gregory *et al.*, 1983). In practice, we have been unable to secure an acceptable fit to the complete data base, although many attempts have been made. On the assumption that significant systematic experimental error is absent, the failure to secure a fit can be taken to indicate either that the exponential model is too parsimonious in its definition of constants or that the model itself is inappropriate. With regard to the first alternative, the exponential model generates an additional 18 constants if the principle of linkage is discarded (Ainsworth & Gregory, 1978). We have tested a model in which two of the extra constants are employed and find a marked improvement in the quality of the fit; unfortunately, there is no objective rule to determine which or how many additional constants should be used, and we regard the result as trivial.

On the second alternative it is natural to consider the MWC model (Monod *et al.*, 1965) because of its archetypal demonstration of the sigmoidal-to-hyperbolic transition in the relationship $v = f(A)_D$. This model was used by Johannes & Hess (1973), but they found it necessary to introduce a third, hybrid, conformational state in order to represent the effect of varying but one substrate concentration. Goodford *et al.* (1977) also found that a three-state MWC model was required to explain the effects of bisphosphoglycerate on the oxygen equilibrium curve of haemoglobin. For the present study, a further level of complication is superimposed by the necessity to account for the effect of two additional substrate concentrations on the reaction velocity. In these circumstances, it is difficult to avoid an arbitrary description of the enzyme mechanism and, whichever is employed, the choice is expressed by a rate equation of great complexity (Bardsley *et al.*, 1980).

It is evident from the foregoing that both of the alternative courses of action that have been considered permit the introduction of so many additional constants that the significance of a successful fit to the data would be placed in doubt (Tanford, 1973). For this reason we propose to accept Fits D24 and D1200 as descriptions of the behaviour of the enzyme at 'low' and 'high' effector concentrations respectively. In support, Table 1 subdivides the D24 data according to the fructose bisphosphate concentration and provides the error parameters when these sub-sets are represented by Fit D24, before and after optimization. It can be seen that there is not a progressive deterioration in the quality of the fit as the fructose bisphosphate concentration is raised to $24 \mu\text{M}$, a conclusion that can also be drawn by examination of Figs. 1-4. It is important to note that the fractional saturation of the enzyme by fructose bisphosphate reaches high values in this range of 'low' effector concentrations. For example, by calculation using the optimized constants of Table 1 with $A = 2 \text{ mM}$,

Table 1. Values of the four-ligand exponential-model constants for the initial velocities of the yeast pyruvate kinase-catalysed reaction measured with fructose biphosphate concentrations up to 24 μM

The Table gives the overall fit to the 360 datum points and optimized fits to each data bloc defined by the fructose 1,6-bisphosphate (FBP) concentration. The error parameters RSS and MD% are as defined by Morris *et al.* (1984). The values of the error parameters in parentheses were obtained before optimization when the constants of the overall fit were applied to the data of the individual blocs. The values of the constants are given to a precision consistent with the stated errors; note that rounding-off would considerably increase the values of the error parameters. Temperature, 25 °C; pH 6.2; A = ADP; B = phosphoenolpyruvate; C = Mg^{2+} ; D = fructose 1,6-bisphosphate [the effector, see eqns. (1) and (2)]; $[\text{K}^+] = 100 \text{ mM}$; $[\text{NH}_4^+] = 32 \text{ mM}$.

Constant	Overall fit to 360 points	Optimized fits to data blocs					Mean of optimized fits	S.E.M.
		[FBP] = 0 (48 points) (Figs. 1 and 2)	[FBP] = 3 μM (60 points)	[FBP] = 6 μM (72 points)	[FBP] = 12 μM (84 points)	[FBP] = 24 μM (96 points) (Figs. 3 and 4)		
$\ln[\alpha_0 (\text{mM}^{-1})]$	0.951	0.654	0.838	0.895	1.169	1.040	0.912	0.087
$\ln[\beta_0 (\text{mM}^{-1})]$	-0.928	-1.277	-1.200	-1.046	-0.976	-0.939	-1.087	0.065
$\ln[\gamma_0 (\text{mM}^{-1})]$	-2.720	-3.214	-2.853	-2.693	-2.565	-2.361	-2.737	0.144
$\ln[\delta_0 (\text{mM}^{-1})]$	2.258	2.298	2.366	2.380	2.342	2.232	2.323	0.027
k_{AA}	1.302	0.947	1.219	1.263	1.156	1.268	1.171	0.059
k_{BB}	1.432	2.042	1.549	1.458	1.416	1.112	1.515	0.151
k_{CC}	2.966	2.798	2.941	2.954	2.693	2.752	2.828	0.052
k_{DD}	1.340	1.372	1.368	1.370	1.540	1.388	1.408	0.033
k_{AB}	-0.860	-0.866	-0.811	-0.860	-0.734	-0.677	-0.789	0.037
k_{AC}	0.263	0.815	0.444	0.235	-0.043	-0.174	0.256	0.176
k_{BC}	0.786	1.034	0.742	0.592	0.712	0.922	0.801	0.079
k_{AD}	1.004	1.037	1.181	1.175	0.895	1.030	1.064	0.053
k_{BD}	1.017	1.049	1.072	1.088	0.789	1.107	1.021	0.059
k_{CD}	0.469	0.501	0.694	0.621	0.870	0.461	0.629	0.073
$V_G (\mu\text{mol/min per mg})$	420.9	394.7	396.0	410.0	418.6	444.1	412.7	9.032
y	0.966	0.999	0.946	0.981	1.022	0.945	0.979	0.015
RSS	11.19	4.89 (16.23)	5.74 (9.12)	6.93 (7.49)	8.48 (11.70)	8.12 (11.13)	15.24	—
MD%	32.18	32.16 (48.60)	28.19 (37.03)	27.45 (28.14)	22.44 (25.69)	21.39 (25.93)	32.36	—
Error product	359.98	157.19 (788.83)	161.952 (337.53)	190.14 (210.89)	190.29 (300.60)	173.59 (288.52)	493.36	—

Table 2. Values of exponential-model constants for the initial velocities of the yeast pyruvate kinase-catalysed reaction as a function of fructose 1,6-bisphosphate concentration

Fit D24 is the overall fit to the 360 datum points (partly illustrated in Figs. 1–4) with fructose bisphosphate concentrations up to 24 μM ; Fit D0 included for comparison is a fit to data collected in the absence of effector (Morris *et al.*, 1984); Fit D1200 is the fit to 104 datum points illustrated in Figs. 5 and 6 with a fructose bisphosphate concentration of 1.2 mM. The error parameters RSS and MD% are as defined by Morris *et al.* (1984). The Hill slope at half-saturation is calculated by the equation $n_H = 4/(4 - k_{XX})$ on the assumption that k_{XX} is applicable to the binding of X in the absence of other ligands (Ainsworth, 1977). The Table also gives dissociation constants for ligand binding (X_0^{-1}) and a multiplication factor ($e^{-k_{XY}}$) that shows the effect of saturation by ligand X on the dissociation constant of Y, and vice versa. Other conditions are as indicated in Table 1 legend. Abbreviations: FBP, fructose 1,6-bisphosphate; PEP, phosphoenolpyruvate.

Conditions...	Fit D24 [FBP] ≤ 24 μM (Figs. 1–4)		Fit D0 [FBP] = 0		Fit D1200 [FBP] = 1.2 mM (Figs. 5 and 6)	
	Binding constants X ₀ (mm ^{−1})					
Ligands	ln X ₀	X ₀ ^{−1}	ln X ₀	X ₀ ^{−1}	ln X ₀	X ₀ ^{−1}
ADP (A)	0.951	0.386	0.571	0.565	1.139	0.320
PEP (B)	−0.928	2.53	−2.292	9.89	1.576	0.207
Mg ²⁺ (C)	−2.720	15.2	−2.735	15.4	−0.730	2.074
FBP (D)	2.258	—	—	—	—	—
Homotropic interaction constants						
Ligands	k _{XX}	n _H	k _{XX}	n _H	k _{XX}	n _H
AA	1.302	1.48	0.224	1.06	1.191	1.420
BB	1.432	1.56	2.300	2.35	−0.514	0.886
CC	2.966	3.87	2.714	3.11	0.277	1.075
DD	1.340	1.50	—	—	—	—
Heterotropic interaction constants						
Ligands	k _{XY}	e ^{−k_{XY}}	k _{XY}	e ^{−k_{XY}}	k _{XY}	e ^{−k_{XY}}
AB	−0.860	2.36	−0.234	1.26	−0.032	1.032
AC	0.263	0.77	0.877	0.42	0.483	0.617
BC	0.786	0.46	0.684	0.50	1.530	0.216
AD	1.004	0.37	—	—	—	—
BD	1.017	0.36	—	—	—	—
CD	0.469	0.63	—	—	—	—
V _G	420.9	—	515.1	—	414.9	—
y	0.966	—	—	—	—	—

$B = 2.5 \text{ mM}$ and $D = 12 \mu\text{M}$, $p_D = 0.802$. This calculation suggests very strongly that an additional effect comes into operation when the fructose bisphosphate concentration is increased to 1.2 mM. We therefore propose to compare our findings with those of previous workers in order to see whether this suggestion has any precedents.

Comparison with previous work

The substrate interactions of yeast pyruvate kinase that are independent of fructose bisphosphate are revealed by Fit D0 and the non-D constants of Fit D24. In spite of some significant differences (at present inexplicable), there is a broad agreement between the two independent estimates such that conclusions about the substrate interactions that were drawn by Morris *et al.* (1984) from Fit D0 would also be drawn from the values of the non-D constants presented in Fit D24. As discussed above, the same conclusions cannot be drawn from the non-D constants of Fit D1200.

The value of $\gamma = 0.966$ and the similarity of the values of V_G given by Fits D24 and D1200 indicate clearly that

fructose bisphosphate acts solely by changing the affinity of the enzyme for its substrates: the enzyme and this effector may therefore be regarded as a K system in the nomenclature of Monod *et al.* (1965). The independence of the maximum velocity from the fructose bisphosphate concentration is already well-established for the yeast enzyme (Haeckel *et al.*, 1968; Hunsley & Suelter, 1969; Johannes & Hess, 1973) and for the enzymes from rabbit muscle (Phillips & Ainsworth, 1977) and liver (Irving & Williams, 1973). The observation permits the conclusion that addition of effector does not decrease the molecular mass of the enzyme with its consequent inactivation (Kuczenski & Suelter, 1970, 1971a,b).

The effects of adding 1.2 mM-fructose bisphosphate on the substrate interactions with yeast pyruvate kinase can be judged by comparing the non-D constants of Fit D24 with those of Fit D1200. Briefly, on adding 1.2 mM effector, the w_0 affinity of ADP increases 1.2-fold whereas the equivalent Hill slope for its binding relationship, calculated from k_{AA} (Ainsworth, 1977), remains almost constant at 1.4. For phosphoenolpyruvate the affinity

increases 12-fold with a decrease in Hill slope from 1.56 to 0.89, whereas for Mg^{2+} a 7-fold increase in affinity is associated with a decrease in Hill slope from 3.87 to 1.08. Hunsley & Suelter (1969) have previously reported the effects of adding 1 mM-fructose biphosphate on the kinetic behaviour of yeast pyruvate kinase. A rigorous comparison with their work is not possible for reasons already given (Morris *et al.*, 1984), to which must be added the complication that Hunsley & Suelter (1969) significantly lowered the concentration of the activating univalent cation on adding 1 mM effector. Nevertheless, Hunsley & Suelter (1969) observed that the affinity of ADP for the enzyme and the corresponding Hill slope of 1 remain unaffected on adding 1 mM effector when NH_4^+ was the activating cation. Similarly, they observed, on adding 1 mM effector, that the affinity of phosphoenolpyruvate was increased 21-fold with a fall in Hill slope from 2.9 to 1, and that the affinity of Mg^{2+} increased 4.4-fold as the Hill slope decreased from 4.7 to 1.6. Hunsley & Suelter (1969) also showed that activation by K^+ produces very similar changes to those outlined above for NH_4^+ activation. It is clear from this comparison that our results are prefigured by those of Hunsley & Suelter (1969).

The effects of 'low' concentrations of fructose biphosphate on the substrate interactions are given by the D constants of Fit D24. Thus the values of the constants k_{AD} , k_{BD} and k_{CD} show that when the enzyme is calculated to be 'saturated' by fructose biphosphate, its affinity for ADP, phosphoenolpyruvate and Mg^{2+} is increased 2.7-fold, 2.8-fold and 1.6-fold respectively. The positive values of k_{AD} , k_{BD} and k_{CD} also require the complementary result, that is that the affinity of the enzyme for fructose biphosphate should be increased by saturation with the substrates. Kuczenski & Suelter (1971a) have demonstrated this effect in fluorimetric studies of fructose biphosphate binding by yeast pyruvate kinase at pH 7.5. In experiments where the effector concentrations were generally less than 1 mM, they showed that the dissociation constant of fructose biphosphate was decreased 2.5-fold when 5 mM-ADP was added to solutions of the enzyme that contained 25 mM- Mg^{2+} . Kuczenski & Suelter (1971a) regarded this result (which is in agreement with our findings) as surprising because of the observed insensitivity of ADP binding to an addition of 1 mM-fructose biphosphate (Hunsley & Suelter 1969), which we referred to above when discussing Fit D1200. This is, therefore, an independent recognition of the possibility that the influence of fructose biphosphate on yeast pyruvate kinase is fundamentally different at high and at low concentrations. In the experiments performed by Kuczenski & Suelter (1971a) the effect of phosphoenolpyruvate on effector binding is complicated. In the presence of 25 mM- Mg^{2+} addition of 10 mM-phosphoenolpyruvate was almost without effect, but when 0.23 M- K^+ was added as well the affinity increased 7-fold, a factor more than double that indicated by k_{BD} .

The dissociation constant of fructose biphosphate, corresponding to $\ln \delta_0$, is 0.105 mM. Values close to this have been given by Kuczenski & Suelter (1971a). The value that would appear at half-saturation in the presence of saturating concentrations of the substrates can be calculated from:

$$\ln \delta_i = \ln \delta_0 + k_{\text{AD}} + k_{\text{BD}} + k_{\text{CD}} + \frac{1}{2}k_{\text{DD}}$$

Fit D24 gives $\ln \delta_i = 5.4$ and a dissociation constant of 0.004 mM. For comparison, Hunsley & Suelter (1969) constructed the effector Hill plot from kinetic measurements made over a range of effector concentrations from 4 μM to 100 μM in the presence of high total concentrations of the substrates; by these means they obtained dissociation constants of 0.02 mM in the presence of 10 mM- K^+ and 0.03 mM with 5 mM- NH_4^+ .

Finally, it can be shown by calculation that the positive values of k_{AD} , k_{BD} and k_{CD} cause an increase in the Hill slopes of all the velocity-substrate-concentration relationships when fructose biphosphate is added to give a concentration up to 24 μM and the substrate saturations are maintained at unchanged values for the comparison (Ainsworth, 1986). This predicted behaviour contrasts markedly with the disappearance of sigmoidicity that occurs when 1.2 mM-fructose biphosphate is added to reactant solutions (Fit D1200 and Figs. 5 and 6).

The value of $k_{\text{DD}} = 1.34$ corresponds to a Hill slope of 1.5. Kuczenski & Suelter (1971a) found the Hill slope for fructose biphosphate binding to be 1.58 when measured at pH 7.5 in the presence of 25 mM- Mg^{2+} . The corresponding value from kinetic studies at pH 6.2 is 2.33 (Hunsley & Suelter, 1969).

On the basis of the comparisons we have been able to make, it seems that there is a broad agreement between this and previous studies when regard is paid to the concentration of fructose biphosphate that is employed in the experiments that are being compared.

Conclusions

We have already argued that the heterotropic interactions of pyruvate kinase originate in charge interactions at the active site and that the homotropic interactions reflect inter-site events (Ainsworth & Macfarlane, 1973; Ainsworth *et al.*, 1983; Morris *et al.*, 1984). Additional support for this view is provided by the effects of pH and the concentrations of univalent cations on the reaction velocity (Rhodes *et al.*, 1986). In this context, low concentrations of fructose biphosphate appear to modulate the enzyme activity while leaving the substrate interactions of both kinds largely the same. By contrast, high effector concentrations seem to exert their major effect on the homotropic interactions of phosphoenolpyruvate and Mg^{2+} , both decreasing their magnitude and causing a compensatory increase in affinity of these substrates for the enzyme. The heterotropic interactions remain relatively unaffected. By extension of the previous arguments, it would therefore appear that high concentrations of effector loosen the structure of pyruvate kinase, simultaneously destroying the basis for inter-site interaction and releasing the constraints that limit substrate affinity in the unmodified enzyme.

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Received 31 May 1985/20 September 1985; accepted 18 November 1985