The regulatory properties of yeast pyruvate kinase

Effects of NH_4^+ and K^+ concentrations

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The kinetics of pyruvate kinase from *Saccharomyces cerevisiae* were studied at 25 °C and pH 6.2 as a function of the concentrations of ADP, phosphoenolpyruvate, Mg^{2+} and either NH_4^+ or K^+ . The data were analysed by the exponential model for four substrates, obtained by extension of the model described by Ainsworth, Kinderlerer & Gregory [(1983) *Biochem. J.* **209**, 401–411]. On that basis, it was concluded that NH_4^+ binding is almost non-interactive but leads to the appearance of positive interaction in the velocity response to increase in its concentration because of positive interactions with phosphoenolpyruvate and Mg^{2+} . The data obtained with K^+ lead to the same conclusions and differ only in suggesting that NH_4^+ is bound more strongly to the enzyme than is K^+ . Both data sets are used as the basis for a discussion of the substrate interactions of pyruvate kinase and it appears therefrom that the heterotropic interactions accord with what is known of the events that take place at the active site during catalysis. The paper also reports a determination of the dissociation constants for the NH_4^+ complexes with ADP and phosphoenolpyruvate and an examination of the simultaneous activation of pyruvate kinase by K^+ and NH_4^+ ions.

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 and the enzyme is saturated by the activating univalent cations K^+ and NH_4^+ (Morris *et al.*, 1984, 1986). 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 either NH_4^+ or K^+ . The results are interpreted by extending the exponential model as described by Ainsworth *et al.* (1983) with the assumption that the univalent cation can be treated as a fourth substrate because of its obligatory requirement by the enzyme (Ainsworth, 1977a). It is also assumed in the analysis that the fractional saturation by H^+ is constant.

The paper also reports a determination of dissociation constants for the equilibria of ADP and phosphoenolpyruvate with NH_4^+ and an examination of the activation of pyruvate kinase by K^+ and NH_4^+ acting together.

EXPERIMENTAL METHODS AND ANALYSIS OF RESULTS

Enzyme assays

Materials were as described by Kinderlerer *et al.* (1986) except for the use of pig muscle lactate dehydrogenase, supplied in glycerol (Boehringer Corp., Lewes, East Sussex, U.K.), as the coupling enzyme. The methods were also unchanged except that the pH of assay solutions was 6.2 throughout and that pyruvate kinase was desalted before use on a Sephadex G-25 column when NH_4^+ was absent. Note that desalted pyruvate kinase is less stable

than that employed previously and that stock solutions had to be re-made hourly rather than 2-hourly. The results were analysed by extending the equations and methods described by Morris *et al.* (1984) to four substrates.

Determination of NH_4^+ binding constants

8-Hydroxyquinoline and Hepes were obtained from BDH Chemicals, Poole, Dorset, U.K. ADP was employed as the free acid, and phosphoenolpyruvate as the tricyclohexylammonium salt; NH_4^+ concentrations were established by standardization of solutions of the hydroxide. Assay solutions, in duplicate, were made up in 10 mM-Hepes buffer adjusted to pH 8 by tetrapropylammonium hydroxide.

RESULTS

Determination of the dissociation constants for the NH_4^+ complexes of ADP and phosphoenolpyruvate

The dissociation constants of the NH_4^+ -substrate complexes were determined by the spectroscopic method of Burton (1959) based on the formation of a coloured 1:1 complex of 8-hydroxyquinoline with Mg^{2+} . Fig. 1 shows the absorbance at 360 nm of 0.5 mM-8-hydroxyquinoline solutions measured as a function of Mg^{2+} concentration of 25 °C and pH 8.0. Fig. 1 also shows how this relationship is changed when the solution contains either ADP (2 mM) or phosphoenolpyruvate (5 mM) both in the presence and in the absence of NH_4^+ (150 mM).

The straight line defining the right-hand limit of the hatched area in Fig. 1 is the absorbance of a solution of the Mg^{2+} -8-hydroxyquinoline complex, with the stated equivalent Mg^{2+} concentration, as calculated from the molar absorption coefficient of $1800 \text{ M}^{-1} \text{ cm}^{-1}$ given by Manchester (1980).

On any horizontal line drawn on the Figure, the distance A–B represents the concentration of Mg^{2+} –

Fig. 1. Effect of NH_4^+ and either ADP or phosphoenolpyruvate on complex-formation between Mg^{2+} and 8-hydroxyquinoline at 25 °C and pH 8.0

△ and ▲, No additions; ●, ADP (2 mM), NH_4^+ (150 mM); ■, ADP (2 mM); ○, phosphoenolpyruvate (5 mM), NH_4^+ (150 mM); □, phosphoenolpyruvate (5 mM).

Table 1. Dissociation constants of NH_4^+ complexes of ADP and phosphoenolpyruvate

Calculations are based on measures of B–C, shown in Fig. 1, and eqns. (1)–(4). The dissociation constants for the K^+ complexes are given for comparison. Temperature, 25 °C, pH 8.0. References: ^aPhillips *et al.* (1966); ^bSmith & Alberty (1956); ^cWold & Ballou (1957); ^dBurton (1959); ^emeasured in the presence of Na^+ (15 mM), Manchester (1980).

	Constant (mM)					
	K_1	K_1'	K_2	K_{H^+}	$K_{\text{NH}_4^+}$	K_{K^+}
ADP	0.378 (0.610 ^d)	0.738	157.5	1.4×10^{-4a}	147.0	181.0 ^b
Phosphoenolpyruvate	2.05 (4.35 ^e)	5.07	101.8	4.4×10^{-4c}	100.0	83.0 ^c

hydroxyquinolate, B–C the concentration of free Mg^{2+} and C–D the concentration of Mg^{2+} –substrate complex, SMg^{2+} . Hence, when C–D is scaled to equal half the added substrate concentration, S_0 , B–C is equal to the dissociation equilibrium constant (real or apparent) of the SMg^{2+} complex (Burton, 1959). B–C can therefore be defined either as:

$$K_1 = \frac{[\text{Mg}^{2+}] \cdot [\text{S}]}{[\text{SMg}^{2+}]} \quad (1)$$

or as:

$$K_1' = \frac{[\text{Mg}^{2+}] \cdot [\text{S} + \text{SNH}_4^+]}{[\text{SMg}^{2+}]} \quad (2)$$

depending on whether NH_4^+ is absent or present in solution. Re-arrangement of the two equations then provides:

$$K_2 = \frac{[\text{NH}_4^+] \cdot [\text{S}]}{[\text{SNH}_4^+]} = \frac{K_1 \cdot [\text{NH}_4^+]}{K_1' - K_1} \quad (3)$$

K_2 is also an apparent constant that depends on pH. By

assuming a competition between H^+ and NH_4^+ for the substrate binding site, we obtain:

$$K_{\text{NH}_4^+} = K_2 \cdot \left(1 + \frac{[\text{H}^+]}{K_{\text{H}^+}}\right)^{-1} \quad (4)$$

The calculations for ADP and phosphoenolpyruvate are completed in Table 1. For comparison, the Table also includes previously determined values of K_1 and the dissociation constants for the K^+ complexes with the substrates. It can be seen that the corresponding values of the constants obtained here are very similar. The values of $K_{\text{NH}_4^+}$ were used to calculate the equilibrium distribution of substrates species and Mg^{2+} in the assay solutions described in Figs. 3–8 (Macfarlane & Ainsworth, 1972).

Simultaneous activation of pyruvate kinase by NH_4^+ and K^+

Morris *et al.* (1984) employed 100 mM- K^+ and 32 mM- NH_4^+ as activating univalent cations, and the

Fig. 2. Simultaneous activation of pyruvate kinase by NH_4^+ and K^+

Temperature, 25 °C; pH 6.2; [ADP] = 2 mM; [phosphoenolpyruvate] = 5 mM; $[\text{Mg}^{2+}]$ = 2 mM. The datum points are fitted by curves calculated according to eqns. (6), (7) and (10) with values of the constants given in Table 2. (a) K^+ concentrations were: ■, 0 mM; ●, 25 mM; ▲, 50 mM; □, 75 mM; ○, 100 mM. (b) NH_4^+ concentrations were: ■, 0 mM; ●, 5 mM; ▲, 10 mM; □, 15 mM; ○, 25 mM; △, 32 mM.

Table 2. Simultaneous activation of pyruvate kinase by NH_4^+ and K^+

The Table gives the fit to eqns. (6), (7) and (10). Temperature, 25 °C; pH 6.2; [ADP] = 2 mM; [phosphoenolpyruvate] = 5 mM; $[\text{Mg}^{2+}]$ = 2 mM.

$\ln[K_0 \text{ (mM}^{-1}\text{)]}$	$\ln[N_0 \text{ (mM}^{-1}\text{)]}$	k_{KK}	k_{NN}	k_{KN}	V_{G}	$K_0^{-1} \text{ (mM)}$	$N_0^{-1} \text{ (mM)}$
-5.680	-4.368	1.908	2.270	1.362	240.4	293	78.9
RSS = 10.02; MD% = 25.24; error product = 254.95							

'cocktail' was continued in use when the effects of fructose biphosphate and pH on the pyruvate kinase-catalysed reaction were examined (Morris *et al.*, 1986; Kinderlerer *et al.*, 1986). Fig. 2 shows the approach to maximum activation as the concentration of one ion is varied at increasing fixed concentrations of the other. Inspection of the Figure shows that the two ions facilitate one another's binding, the ion at constant concentration moving the relationship $v = f(X)_Y$ to higher X values.

We attempted to fit the data shown in Fig. 2 by the equations:

$$v = V_{\text{G}} (p_{\text{K}} + p_{\text{N}}) \quad (5)$$

$$\ln K_i = \ln K_0 + k_{\text{KK}} \cdot p_{\text{K}} + k_{\text{NK}} \cdot p_{\text{N}} \quad (6)$$

$$\ln N_i = \ln N_0 + k_{\text{KN}} \cdot p_{\text{K}} + k_{\text{NN}} \cdot p_{\text{N}} \quad (7)$$

where K stands for K^+ and N for NH_4^+ . The result obtained by optimization gave V_{G} a value of approximately half the highest recorded velocity appearing in Fig. 2 and a sum $p_{\text{K}} + p_{\text{N}} = 1.79$ when $[\text{K}^+] = 100 \text{ mM}$ and $[\text{NH}_4^+] = 32 \text{ mM}$. This result is inconsistent with the obvious hypothesis that K^+ and NH_4^+ compete for a

single site and hence that $v \leq V_{\text{G}}$. We therefore assumed that the enzyme is divided into three compartments, E, EK and EN. EN is not considered as part of the total enzyme determining p_{K} because it does not have K bound and is incapable of binding K. Hence by defining:

$$p_{\text{K}} = \frac{EK}{E + EK}; \quad p_{\text{N}} = \frac{EN}{E + EN} \quad (8)$$

and V_{X} as the maximum velocity observed for unit concentration of enzyme when X is the activator:

$$v = V_{\text{K}} \cdot \left(\frac{E + EK}{E + EK + EN} \right) \cdot p_{\text{K}} + V_{\text{N}} \cdot \left(\frac{E + EN}{E + EK + EN} \right) \cdot p_{\text{N}} \quad (9)$$

Table 5 shows that V_{K} and V_{N} are equal to within 2%; hence by assuming their equality eqn. (9) becomes:

$$v = V_{\text{G}} \cdot (p_{\text{K}} + p_{\text{N}} - 2p_{\text{K}} \cdot p_{\text{N}}) / (1 - p_{\text{K}} \cdot p_{\text{N}}) \quad (10)$$

The fit to eqns. (6), (7) and (10) is represented in Table 2 and the curves are illustrated in Fig. 2.

Table 3. Values of the four-substrate exponential-model constants for the initial velocities of the yeast pyruvate kinase-catalysed reaction illustrated in Figs. 3-8

The Table gives the overall fit by the equations (Morris *et al.*, 1986):

$$v/V_G = p_A \cdot p_B \cdot p_C \cdot p_D$$

$$\ln w_i = \ln w_0 + k_{wW} \cdot p_W + k_{xW} \cdot p_X + k_{yW} \cdot p_Y + k_{zW} \cdot p_Z$$

to 305 datum points and optimized fits to each data bloc defined by NH_4^+ 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 = NH_4^+ .

Constant	Overall fit to all data (305 points)	Optimized fits to data blocs				Mean of optimized fits	S.E.M.
		$[\text{NH}_4^+] = 25 \text{ mM}$ (61 points) (Figs. 3-5)	$[\text{NH}_4^+] = 32.5 \text{ mM}$ (61 points)	$[\text{NH}_4^+] = 40 \text{ mM}$ (61 points)	$[\text{NH}_4^+] = 50 \text{ mM}$ (61 points)	$[\text{NH}_4^+] = 75 \text{ mM}$ (61 points) (Figs. 6-8)	
$\ln[x_0 \text{ (mM}^{-1})]$	1.308	1.109	1.232	1.296	1.152	1.773	1.313
$\ln[\beta_0 \text{ (mM}^{-1})]$	-2.028	-2.043	-2.088	-2.078	-2.189	-1.615	-2.003
$\ln[\gamma_0 \text{ (mM}^{-1})]$	-3.439	-3.580	-3.377	-3.358	-3.260	-3.311	-3.378
$\ln[\delta_0 \text{ (mM}^{-1})]$	-4.385	-4.242	-4.228	-4.283	-4.216	-4.437	-4.281
k_{AA}	0.517	0.451	0.488	0.518	0.462	0.779	0.540
k_{BB}	1.744	1.750	1.765	1.711	1.677	1.779	1.737
k_{CC}	1.614	1.573	1.490	1.582	1.555	1.375	1.515
k_{DD}	-0.226	-0.180	-0.186	-0.191	-0.110	-0.157	-0.165
k_{AB}	-0.444	-0.455	-0.478	-0.460	-0.329	-0.721	-0.489
k_{AC}	1.334	1.527	1.315	1.262	1.161	1.188	1.291
k_{BC}	0.886	0.622	0.700	0.743	0.843	1.035	0.788
k_{AD}	-1.188	-1.051	-1.061	-1.105	-1.183	-1.424	-1.165
k_{BD}	0.570	0.700	0.667	0.579	0.426	0.526	0.580
k_{CD}	1.906	1.873	1.873	1.881	1.968	1.935	1.906
$V_G \text{ (}\mu\text{mol/min per mg)}$	443.5	474.0	468.0	467.9	479.7	446.2	467.2
RSS	8.88	3.55 (7.31)	3.60 (5.81)	4.10 (8.68)	6.05 (7.50)	7.56 (13.27)	9.12
MD%	13.40	15.78 (15.55)	8.96 (10.43)	14.38 (15.73)	9.77 (10.44)	7.78 (13.79)	14.73
Error product	119.05	56.14 (113.64)	32.26 (60.56)	58.99 (136.56)	59.13 (78.25)	58.84 (183.05)	134.35
							5.68
							—
							—
							—

Kinetics of NH_4^+ -activated pyruvate kinase

Table 3 gives the constants of a fit to 305 data points $v = f(A, B, C, D)$, where A stands for ADP, B for phosphoenolpyruvate (both in their Mg^{2+} -free forms), C for free Mg^{2+} and D for NH_4^+ . The distribution of error associated with this estimate has been sampled by calculating the error parameters that arise when sub-sets of the data defined by the NH_4^+ concentration are fitted by its constants. Table 3 shows that the sub-set error does not correlate with the NH_4^+ concentration. The optimized fits to the individual NH_4^+ blocs are also given in Table 3. The merit of each constant in the context of this sub-division of the data can be judged by the range of values that each covers in representing the several subsets.

The data for two NH_4^+ concentrations illustrated in Figs. 3–8 are means of duplicate measurements of the initial velocity with curves calculated by using the corresponding optimized fits (Kinderlerer *et al.*, 1986). The mean percentage deviation from the average of each paired measurement is 1.8% for the complete NH_4^+ data set. In general, the fit to the lower-velocity points is better than we have observed elsewhere, an improvement that is reflected by the lower values of the error parameter MD%.

Kinetics of K^+ -activated pyruvate kinase

Table 4 gives the constants of a fit to 304 data points $v = f(A, B, C, D)$, where D now stands for K^+ . Table 4 shows that the sub-set error is close to that of the set, except for $\text{K}^+ = 125 \text{ mM}$, where the error is much lower.

The data for two K^+ concentrations illustrated in Figs. 9–14 are the means of duplicate measurements of the initial velocity with curves calculated by using the corresponding optimized fits (Kinderlerer *et al.*, 1986). The mean percentage deviation from the average of each paired measurement is 3.4% for the complete K^+ data set: this value is higher than that observed previously in comparable experiments where NH_4^+ was also present in the reaction solutions (Morris *et al.*, 1984, 1986; Kinderlerer *et al.*, 1986), and reflects the lower stability of desalted pyruvate kinase. The general features of the curve shapes are very similar to those observed when NH_4^+ was used as the activating univalent cation, but differ from them in the much greater error that is present at low velocities, an observation that is reflected by the relative values of the error parameters RSS and MD% found for the two data sets. Here again is an indication that NH_4^+ is more effective than K^+ in preserving the stability of pyruvate kinase solutions.

DISCUSSION

K^+/NH_4^+ activation

The description of the K^+/NH_4^+ activation of pyruvate kinase represented by the fit to eqns. (6), (7) and (10), given in Table 2, demonstrates that the apparent dissociation constant of the NH_4^+ -enzyme complex is smaller than that for the K^+ -enzyme complex. This observation agrees with results obtained by Hunsley & Suelter (1969), as does the positive homotropic interaction shown in the relationships $v = f(X)_Y$ (Fig. 2). The greater value of k_{NN} when compared with k_{KK} is also in agreement with Hunsley & Suelter (1969). The positive value of the heterotropic constant k_{KN} is also to be

Fig. 3. Effect of [ADP] on the initial velocity of pyruvate formation at several fixed values of $[\text{Mg}^{2+}]$ with [phosphoenolpyruvate] = 5 mM and $[\text{NH}_4^+] = 25 \text{ mM}$

In Figs. 3–8 initial velocities, v (represented by symbols), are expressed as μmol of NADH oxidized/min per mg of protein at 25°C and pH 6.2, with other conditions as described in the Experimental methods and analysis of results section. The datum points are fitted by curves calculated in accordance with the four-substrate exponential model with optimized values of the constants given in Table 3 relevant to the NH_4^+ concentration. Mg^{2+} concentrations were: ■, 1.0 mM; ●, 1.25 mM; ▲, 1.5 mM; □, 1.75 mM; ○, 2.0 mM.

Fig. 4. Effect of [phosphoenolpyruvate] on the initial velocity of pyruvate formation at several fixed values of [ADP] with $[\text{Mg}^{2+}] = 2 \text{ mM}$ and $[\text{NH}_4^+] = 25 \text{ mM}$

ADP concentrations were: ■, 0.4 mM; ●, 0.8 mM; ▲, 1.2 mM; □, 1.6 mM; ○, 2.0 mM. Other conditions and calculations were as described in the legend to Fig. 3.

Fig. 5. Effect of $[Mg^{2+}]$ on the initial velocity of pyruvate formation at several fixed values of $[phosphoenolpyruvate]$ with $[ADP] = 2 \text{ mM}$ and $[NH_4^+] = 25 \text{ mM}$

Phosphoenolpyruvate concentrations were: ■, 2.0 mM; ●, 2.5 mM; ▲, 3.0 mM; □, 4.0 mM; ○, 5.0 mM. Other conditions and calculations were as described in the legend to Fig. 3.

Fig. 7. Effect of $[phosphoenolpyruvate]$ on the initial velocity of pyruvate formation at several fixed values of $[ADP]$ with $[Mg^{2+}] = 2 \text{ mM}$ and $[NH_4^+] = 75 \text{ mM}$

ADP concentrations were: ■, 0.4 mM; ●, 0.6 mM; ▲, 0.8 mM; □, 1.2 mM; ○, 2.0 mM. Other conditions and calculations were as described in the legend to Fig. 3.

Fig. 6. Effect of $[ADP]$ on the initial velocity of pyruvate formation at several fixed values of $[Mg^{2+}]$ with $[phosphoenolpyruvate] = 5 \text{ mM}$ and $[NH_4^+] = 75 \text{ mM}$

Mg^{2+} concentrations were: ■, 0.75 mM; ●, 1.0 mM; ▲, 1.25 mM; □, 1.5 mM; ○, 2.0 mM. Other conditions and calculations were as described in the legend to Fig. 3.

Fig. 8. Effect of $[Mg^{2+}]$ on the initial velocity of pyruvate formation at several fixed values of $[phosphoenolpyruvate]$ with $[ADP] = 2 \text{ mM}$ and $[NH_4^+] = 75 \text{ mM}$

Phosphoenolpyruvate concentrations were: ■, 1.5 mM; ●, 2.0 mM; ▲, 2.5 mM; □, 3.5 mM; ○, 5.0 mM. Other conditions and calculations were as described in the legend to Fig. 3.

Table 4. Values of the four-substrate exponential-model constants for the initial velocities of the yeast pyruvate kinase-catalysed reaction illustrated in Figs. 9–14

The Table gives the overall fit to 304 datum points and optimized fits to each data bloc defined by K^+ concentration. Temperature, 25 °C; pH 6.2; A = ADP; B = phosphoenolpyruvate; C = Mg^{2+} ; D = K^+ . Other details are as described in the legend to Table 3.

Constant	Optimized fits to data blocs					Mean of optimized fits	S.E.M.
	Overall fit to 304 points	$[\text{K}^+] = 75 \text{ mM}$ (61 points) (Figs. 9–11)	$[\text{K}^+] = 10 \text{ mM}$ (61 points)	$[\text{K}^+] = 125 \text{ mM}$ (61 points)	$[\text{K}^+] = 150 \text{ mM}$ (60 points)	$[\text{K}^+] = 175 \text{ mM}$ (61 points) (Figs. 12–14)	
$\ln[\alpha_0] \text{ (mm}^{-1}\text{)}$	1.058	0.830	0.732	1.143	1.346	1.323	0.126
$\ln[\beta_0] \text{ (mm}^{-1}\text{)}$	–2.149	–2.253	–2.215	–2.187	–2.365	–1.889	0.079
$\ln[\gamma_0] \text{ (mm}^{-1}\text{)}$	–3.376	–3.622	–3.415	–3.400	–3.468	–3.541	0.041
$\ln[\delta_0] \text{ (mm}^{-1}\text{)}$	–4.925	–4.925	–4.816	–4.684	–4.752	–4.386	0.041
k_{AA}	0.353	0.255	0.203	0.351	0.462	0.566	0.066
k_{BB}	1.867	2.214	1.935	2.139	1.991	1.815	0.071
k_{CC}	1.395	1.398	1.252	1.259	1.309	1.701	0.083
k_{DD}	–0.343	–0.347	–0.308	–0.274	–0.308	–0.261	0.015
k_{AB}	–0.543	–0.661	–0.587	–0.549	–0.586	–0.507	0.025
k_{AC}	1.274	1.417	1.467	1.282	1.208	1.484	0.054
k_{BC}	0.738	0.889	0.561	0.733	0.841	0.463	0.081
k_{AD}	–1.046	–1.099	–0.862	–1.051	–1.018	–1.200	0.055
k_{BD}	0.517	0.468	0.572	0.503	0.514	0.308	0.045
k_{CD}	1.573	1.545	1.539	1.432	1.623	1.569	0.031
V_{G} ($\mu\text{mol/min per mg}$)	454.4	453.7	462.2	465.10	460.6	452.9	2.40
RSS	7.28	3.51 (5.46)	4.49 (8.25)	4.41 (5.24)	4.02 (8.42)	5.21 (8.35)	—
MD%	24.67	20.59 (29.25)	19.57 (24.80)	16.31 (17.31)	14.15 (25.63)	23.46 (25.29)	—
Error product	180.23	72.32 (159.71)	87.89 (204.51)	71.93 (90.76)	56.82 (215.76)	122.23 (211.22)	—

Fig. 9. Effect of [ADP] on the initial velocity of pyruvate formation at several fixed values of $[Mg^{2+}]$ with [phosphoenolpyruvate] = 5 mM and $[K^+] = 75$ mM

In this and succeeding Figures initial velocities, v (represented by symbols), are expressed as μmol of NADH oxidized/min per mg of protein at 25°C and pH 6.2, with other conditions as described in the Experimental methods and analysis of results section. The datum points are fitted by curves calculated in accordance with the four-substrate exponential model with optimized values of the constants given in Table 4 relevant to the K^+ concentration. Mg^{2+} concentrations were: $\blacksquare$, 1.0 mM; $\bullet$, 1.25 mM; $\blacktriangle$, 1.5 mM; $\square$, 1.75 mM; $\circ$, 2.0 mM.

Fig. 10. Effect of [phosphoenolpyruvate] on the initial velocity of pyruvate formation at several fixed values of [ADP] with $[Mg^{2+}] = 2$ mM and $[K^+] = 75$ mM

ADP concentrations were: $\blacksquare$, 0.4 mM; $\bullet$, 0.8 mM; $\blacktriangle$, 1.2 mM; $\square$, 1.6 mM; $\circ$, 2.0 mM. Other conditions and calculations were as described in the legend to Fig. 9.

Fig. 11. Effect of $[Mg^{2+}]$ on the initial velocity of pyruvate formation at several fixed values of [phosphoenolpyruvate] with $[ADP] = 2$ mM and $[K^+] = 75$ mM

Phosphoenolpyruvate concentrations were: $\blacksquare$, 2.0 mM; $\bullet$, 2.5 mM; $\blacktriangle$, 3.0 mM; $\square$, 4.0 mM; $\circ$, 5.0 mM. Other conditions and calculations were as described in the legend to Fig. 9.

Fig. 12. Effect of [ADP] on the initial velocity of pyruvate formation at several fixed values of $[Mg^{2+}]$ with [phosphoenolpyruvate] = 5 mM and $[K^+] = 175$ mM

Mg^{2+} concentrations were: $\blacksquare$, 0.75 mM; $\bullet$, 1.00 mM; $\blacktriangle$, 1.25 mM; $\square$, 1.50 mM; $\circ$, 2.00 mM. Other conditions and calculations were as described in the legend to Fig. 9.

expected from the similarity of the two ions. Finally, the value of V_G is greater than the highest velocity recorded in Fig. 2 and suggests, on calculation, that $p_K = 0.859$ and $p_N = 0.911$ when $K = 100$ mM and $N = 32$ mM, i.e. that the protein is very nearly half-saturated by each ion under these conditions.

Fig. 13. Effect of [phosphoenolpyruvate] on the initial velocity of pyruvate formation at several fixed values of [ADP] with $[\text{Mg}^{2+}] = 2 \text{ mM}$ and $[\text{K}^+] = 175 \text{ mM}$

ADP concentrations were: ■, 0.4 mM; ●, 0.6 mM; ▲, 0.8 mM; □, 1.2 mM; ○, 2.0 mM. Other conditions and calculations were as described in the legend to Fig. 9.

Fig. 14. Effect of $[\text{Mg}^{2+}]$ on the initial velocity of pyruvate formation at several fixed values of [phosphoenolpyruvate] with $[\text{ADP}] = 2 \text{ mM}$ and $[\text{K}^+] = 175 \text{ mM}$

Phosphoenolpyruvate concentrations were: ■, 1.5 mM; ●, 2.0 mM; ▲, 2.5 mM; □, 3.5 mM; ○, 5.0 mM. Other conditions and calculations were as described in the legend to Fig. 9.

K^+ or NH_4^+ activation

The response of yeast pyruvate kinase to change in the concentrations of its substrates ADP, phosphoenolpyruvate and Mg^{2+} and the obligatory cation, K^+ or NH_4^+ , is described by the exponential-model constants

given in Table 5. Inspection shows a very similar spectrum of values in the two fits; it can therefore be concluded that the univalent cations are all but indistinguishable in their effects on the reaction. This conclusion demonstrates that the joint activation by K^+ (100 mM) and NH_4^+ (32 mM) featured in previous studies is unlikely to have been attended by serious disadvantage (Morris *et al.*, 1986; Kinderlerer *et al.*, 1986).

We propose now to examine how the exponential-model constants given in Table 5 accord with what is known of the reaction mechanism. Mechanistic studies of pyruvate kinase have generally employed the rabbit muscle enzyme and have concentrated upon events at the catalytic site rather than upon the origins of the homotropic interactions evident in the overtly regulatory examples of the enzyme. We shall assume, nevertheless, that the hypotheses that have been made concerning the rabbit muscle enzyme apply equally to the yeast enzyme presently under consideration. In brief, it has emerged that the catalytic site contains binding sites for both the univalent and bivalent cations, that phosphoenolpyruvate is linked to the enzyme by an interaction of its carboxylate group with the univalent cation (Nowak & Mildvan, 1972) and that Mg^{2+} is responsible both for linking the nucleotide to the enzyme and for mediating the transfer of the phospho group between the substrates (Mildvan, 1970). Kinetic studies of the rabbit muscle and fructose biphosphate-activated yeast enzyme were found to be consistent with these proposals (Macfarlane & Ainsworth, 1972; Ainsworth & Macfarlane, 1973). More recently, evidence has accumulated that suggests that the two roles of Mg^{2+} are played by separate ions: one Mg^{2+} bound to the enzyme is responsible for linking ADP, and the second Mg^{2+} , bound to the phosphate residues of the nucleotide, is responsible for transferring the phospho group (Gupta & Mildvan, 1977). Turning now to the exponential-model constants evaluated in Table 5, we have already shown that the values of k_{AB} , k_{AC} and k_{BC} are both comparable with those observed for the rabbit enzyme and consistent with the proposed mechanism; they do not require further discussion here (Ainsworth *et al.*, 1983; Morris *et al.*, 1984). The co-operative binding of M^+ and phosphoenolpyruvate (Nowak & Mildvan, 1972) is reflected in Table 5 by positive values of k_{BD} , and it is interesting that in this critical interaction K^+ and NH_4^+ are almost indistinguishable. The positive values of k_{CD} show that binding of the univalent and bivalent ions is mutually co-operative, with NH_4^+ being more effective than K^+ as an activator of Mg^{2+} binding; both these observations have clear precedents in results reported by Suelter *et al.* (1966). The interactions of the cations are not as straightforward, however, as this simple confirmation of a result might indicate. Thus Suelter *et al.* (1966) have presented evidence that the univalent-cation and bivalent-cation sites are not wholly specific and that both ions can bind at either site. In keeping with this result, the relationships $v = f([\text{M}^+])$ are known to show maxima (Kachmar & Boyer, 1953; Kayne, 1971): typically that for NH_4^+ is found in the region of 35 mM whereas that for K^+ is found above 100 mM. [We have observed a maximum in $v = f([\text{NH}_4^+])$ at 96 mM at pH 6.2 and 25 °C, with 2 mM-ADP, 5 mM-phosphoenolpyruvate and 2 mM- Mg^{2+} . Maxima in $v = f([\text{K}^+])$ can be observed in the present results.] Further, Kayne (1971) has observed that the greater the bivalent ion concentration, the greater the concentration of Ti^+ required to reach the maximum in

Table 5. Comparison of the exponential-model constants that represent the initial velocities of the yeast pyruvate kinase-catalysed reaction measured under different conditions

The fits represented in the five pairs of columns respectively derive from reports by Morris *et al.* (1984), Morris *et al.* (1986), Kinderlerer *et al.* (1986) and the present paper (two columns). 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 ligand X in the absence of other ligands (Ainsworth, 1977b). 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 for Y, and vice versa. Temperature, 25 °C; A = ADP; B = phosphoenolpyruvate; C = Mg^{2+} . In the second and third pairs of columns D represents respectively the effectors fructose bisphosphate and H^+ , whereas in the fourth and fifth pairs of columns D represents respectively K^+ and NH_4^+ and is treated as the fourth substrate. Abbreviations: FBP, fructose 1,6-bisphosphate; PEP, phosphoenolpyruvate.

Conditions...	[K ⁺] = 100 mM [NH ₄ ⁺] = 32 mM pH 6.2 —		[K ⁺] = 100 mM [NH ₄ ⁺] = 32 mM pH 6.2 D = FBP (≤ 24 mM)		[K ⁺] = 100 mM [NH ₄ ⁺] = 32 mM pH 5.0–6.6 D = H ⁺		[K ⁺] = 75–175 mM — pH 6.2 D = K ⁺		— [NH ₄ ⁺] = 25–74 mM pH 6.2 D = NH ₄ ⁺	
Ligand and binding constant	Binding constants X ₀ (mm ^{−1})									
	ln X ₀	X ₀ ^{−1}	ln X ₀	X ₀ ^{−1}	ln X ₀	X ₀ ^{−1}	ln X ₀	X ₀ ^{−1}	ln X ₀	X ₀ ^{−1}
ADP (A); α ₀	0.571	0.565	0.951	0.386	1.121	0.326	1.058	0.347	1.308	0.270
PEP (B); β ₀	−2.292	9.89	−0.928	2.53	−2.068	7.91	−2.149	8.58	−2.028	7.60
Mg ²⁺ (C); γ ₀	−2.735	15.4	−2.720	15.2	−2.736	15.4	−3.376	29.3	−3.439	31.2
(D); δ ₀	—	—	2.258	0.105	5.278	0.00510	−4.925	137.7	−4.385	80.2
	(pK = 5.29)									
	Homotropic interaction constants									
Ligands	k _{xx}	n _H	k _{xx}	n _H	k _{xx}	n _H	k _{xx}	n _H	k _{xx}	n _H
AA	0.224	1.06	1.302	1.48	0.403	1.11	0.353	1.10	0.517	1.15
BB	2.300	2.35	1.432	1.56	1.739	1.77	1.867	1.88	1.744	1.77
CC	2.714	3.11	2.966	3.87	2.698	3.07	1.395	1.54	1.614	1.68
DD	—	—	1.340	1.50	1.831	1.84	−0.343	0.92	−0.226	0.95
	Heterotropic interaction constants									
Ligands	k _{xy}	e ^{−k_{xy}}	k _{xy}	e ^{−k_{xy}}	k _{xy}	e ^{−k_{xy}}	k _{xy}	e ^{−k_{xy}}	k _{xy}	e ^{−k_{xy}}
AB	−0.234	1.26	−0.860	2.36	−0.585	1.80	−0.543	1.72	−0.444	1.56
AC	0.877	0.42	0.263	0.77	0.501	0.61	1.274	0.28	1.334	0.26
BC	0.684	0.50	0.786	0.46	0.947	0.39	0.738	0.48	0.886	0.41
AD	—	—	1.004	0.37	−0.610	1.84	−1.046	2.85	−1.188	3.28
BD	—	—	1.017	0.36	0.350	0.70	0.517	0.60	0.570	0.56
CD	—	—	0.469	0.63	−0.564	1.76	1.573	0.21	1.906	0.15
V _G	515.1		420.9		496.0		454.4		443.5	
y	—		0.966		0.027		—		—	

the relationship $v = f([I^+])$. Nowak & Suelter (1981) therefore ascribe the inhibition, required by the maximum, to a mutual competition in the binding of univalent and bivalent cations. The data described above suggest strongly that the site competed for is the bivalent-cation site. The conclusion of Suelter *et al.* (1966), regrettably based on two experiments with different conditions, that the presence of M^{2+} increases the affinity of the enzyme for M^+ more than M^+ affects the binding of M^{2+} , is nevertheless consistent with a mechanism in which competition at the bivalent-cation site counteracts the synergistic effect of M^+ on M^{2+} binding. The competitive element in this mechanism has not been incorporated in the exponential model (which assumes independent binding), but it is probably implicit in the negative value of k_{AD} . For, having regard to the role that Mg^{2+} plays in linking the nucleotide to the enzyme, it follows that its

replacement by the univalent cation must inhibit nucleotide binding. In addition, Ainsworth & Gregory (1978) have shown for the two-substrate exponential model that a maximum will be found in $v = f(D)_A$ when $k_{AA} \cdot p_A - k_{AD} \cdot p_D > 1$. The identification of the competitive Mg^{2+} site as that on the enzyme rather than that on the nucleotide is supported by the dissociation constants for the equilibria of NH_4^+ and K^+ with ADP. Assuming that the interactions of the ions with the nucleotide are independent of whether ADP is free or enzyme-bound (Gupta & Mildvan, 1977), for neither ion is the dissociation constant low enough at pH 6.2 ($K_K = 1$ M, $K_{NH_4^+} = 0.8$ M) to suggest that sufficient M^+ could be bound to the nucleotide under the conditions of our experiments significantly to reduce the binding of Mg^{2+} . (The fact that the substrate-level equilibria of ADP make the univalent cations antagonize Mg^{2+} binding is an

additional reason for believing that the synergism in M^+ and M^{2+} binding is based on interactions through the enzyme.)

Some further points are as follows. The values of the dissociation constants K_0^{-1} show that NH_4^+ is bound more strongly to the enzyme than is K^+ ; the same result, though with a larger relative difference, has been reported by Suelter *et al.* (1966).

The homotropic constants k_{CC} reported here are much smaller than those found in previous studies (Table 5), where the univalent cations were present throughout at concentrations that gave complete activation; an associated effect is the lower values given here for $\ln \gamma_0$. It is probable that both these differences can be accounted for by the large positive values of k_{CD} , whose effect, in the previous work, would have been subsumed in raised values of both k_{CC} and $\ln \gamma_0$.

The negative value of k_{DD} both for $\text{D} = \text{K}^+$ and $\text{D} = \text{NH}_4^+$ conflicts with the positive values of k_{KK} and k_{NN} , given in Table 2, and the observed sigmoidicity of $v = f(D)_{\text{A,B,C}}$. It should be remembered, however, that the constants employed in eqns. (6), (7) and (10) subsume all the heterotropic interactions that involve A, B and C, as does the relationship $v = f(D)_{\text{A,B,C}}$. Examination of Table 5 shows that k_{AA} , k_{BB} , k_{CC} , k_{AC} , k_{BC} , k_{BD} and k_{CD} are all positive, and in many instances have relatively large values. It seems therefore that the cause of the positive interaction represented by k_{KK} and k_{NN} and observed in $v = f(D)_{\text{A,B,C}}$ must be the heterotropic interactions starting with k_{BD} and k_{CD} . If so, it would be expected that the apparent interaction of $v = f([\text{NH}_4^+])_{\text{A,B,C}}$ would be more positive than that of $v = f([\text{K}^+])_{\text{A,B,C}}$ because k_{DD} is less negative for NH_4^+ than for K^+ whereas k_{BD} , k_{CD} , k_{AC} , k_{BC} , k_{AA} and k_{CC} are all more positive. This prediction can be confirmed by calculations of the slope of Hill plots of v and D points constructed from the overall fits given in Table 5. Thus with $A = B = C = 2 \text{ mM}$, the maximum Hill slope for the NH_4^+ relationship occurs at $v/V_{\text{max}} = 0.13$ and $[\text{NH}_4^+] = 28.5 \text{ mM}$, and has a value of 2.52; with the same substrate concentrations the maximum Hill slope for K^+ is at $v/V_{\text{max}} = 0.19$ and $[\text{K}^+] = 120.8 \text{ mM}$, and equals 1.87. The relative magnitude of the Hill slopes for the two ions is in agreement with results obtained by Hunsley & Suelter (1969) and with values of k_{NN} and k_{KK} for the simultaneous activation of pyruvate kinase by NH_4^+ and K^+ ions. The true negative value of k_{DD} contrasts markedly with the positive values recorded for all the other homotropic constants, but the reason for this individuality remains unknown.

Finally, it should be noted that the maximum velocity recorded in the presence of NH_4^+ is 2% lower than that observed with K^+ ; for comparison, Kayne (1971) found

with the rabbit muscle enzyme that there was a 19% loss of activity on changing from K^+ to NH_4^+ activation.

It is evident from our previous studies and the above discussion that the heterotropic interactions can be discussed in terms of the events that are assumed to take place at the catalytic site. The apparent success of this correlation would seem to limit the expression of interaction between sites to the homotropic constants. At the present time, the molecular mechanisms of inter-site interaction in pyruvate kinase are not understood, but to identify, even tentatively, specific constants determined by them is to create an opportunity for their experimental investigation.

[A complete record of all the data analysed in this and previous reports (Morris *et al.*, 1984, 1986; Kinderlerer *et al.*, 1986) is preserved in Morris (1985).]

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