The regulatory properties of yeast pyruvate kinase

Effect of pH

Julian KINDERLERER, Stanley AINSWORTH, Claudia N. MORRIS and Nicholas RHODES

Department of Biochemistry, University of Sheffield, Western Bank, Sheffield S10 2TN, U.K.

The kinetics of pyruvate kinase from *Saccharomyces cerevisiae* were studied at 25 °C as a function of the concentrations of the substrates ADP, phosphoenolpyruvate and Mg^{2+} and the effector H^+ in the pH range 5–6.6. The enzyme was activated by 100 mM- K^+ and 32 mM- NH_4^+ throughout. It was found that the data could be described by the exponential model for a regulatory enzyme. On that basis, it was concluded that the binding of H^+ is positively interactive and that the protonated enzyme is catalytically inactive. It was also found that H^+ interacts positively with phosphoenolpyruvate but negatively with both ADP and Mg^{2+} .

INTRODUCTION

The glycolytic enzyme pyruvate kinase (EC 2.7.1.40) from *Saccharomyces cerevisiae* displays sigmoidal relationships between the initial velocity of pyruvate formation and the concentration of any one of its substrates when the enzyme is saturated by the activating univalent cations K^+ and NH_4^+ at pH 6.2 (Morris *et al.*, 1984; 1986). Wieker & Hess (1971) have shown, however, in studies of the enzyme from *Saccharomyces carlsbergensis*, that the sigmoidicity of the phosphoenolpyruvate response is modified on changing the pH of the reaction solutions, as are also the affinity of the enzyme for this substrate and the maximum velocity of reaction. The present paper reports the results of a systematic examination of the effect on the initial velocity of reaction of varying the concentrations of ADP, phosphoenolpyruvate and Mg^{2+} at five pH values in the range pH 5–6.6. The results are described 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 K^+ and NH_4^+ remains constant throughout (Morris *et al.*, 1986).

EXPERIMENTAL METHODS AND ANALYSIS OF RESULTS

Materials, methods and the analysis of results were as described by Morris *et al.* (1984, 1986) except as follows. A new preparation of enzyme was made as described by Morris *et al.* (1984). The protein migrated as one major staining band on polyacrylamide-gel electrophoresis and was found to have a specific activity of 306 units ($\mu\text{mol/min}$)/mg. Reaction mixtures (1 ml) contained, in addition to substrates at concentrations indicated in the Figures, 100 μmol of K^+ and 100 μmol of Mes titrated to the required pH by HCl or tetrapropylammonium hydroxide. (Mes was obtained from BDH Chemicals, Poole, Dorset, U.K.) The reaction mixtures were incubated for 10 min at 25 °C before the addition of 0.15 μmol of NADH and an excess (14 units) of lactate dehydrogenase. Reaction was then started by adding 5 μl of a suitably diluted pyruvate kinase solution. Note, in particular, that addition of the standard volume (5 μl) of coupling enzyme to the assay establishes a concentration

of 32 mM- NH_4^+ in the reaction solution (Morris *et al.*, 1986). The initial velocities reported in the Figures are means of duplicate measurements, corrected to an enzyme specific activity of 306 units/mg of protein at 25 °C, and are expressed as μmol of NADH oxidized/min per mg of protein at 25 °C.

RESULTS

The data for two pH values 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 1.9% for the complete data set.

Table 1 gives the constants of a fit to 533 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 H^+ . The distribution of error associated with the estimate has been sampled by calculating the error parameters that arise when sub-sets of the data are fitted by its constants. For sub-sets defined by the experimental pH blocs, it is evident that there is a tendency for both the RSS and MD% errors (Morris *et al.*, 1984) to increase with pH to maximum values at pH 6.2, falling to lower values at pH 6.6. We have no explanation for this apparently systematic trend.

The optimized fits to the individual pH blocs are also given in Table 1. 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 sub-sets. No clear trend seems to be present.

Figs. 1–6 display the points for two pH blocs with curves calculated by using the constants of the corresponding optimized fits. The data points lie rather closer to these curves than they do to curves calculated from the overall fit, but they are displayed in this way to compensate for the systematic errors that accompany the making up of separate batches of enzyme assays.

DISCUSSION

The work of Wieker & Hess (1971), referred to in the Introduction, has established three effects that attend a change in the pH of the pyruvate kinase-catalysed

Fig. 1. Effect of [ADP] on the initial velocity of pyruvate formation at several fixed values of $[Mg^{2+}]$ with [phosphoenolpyruvate] = 5 mM at pH 5.0

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 in the presence of 0.1 M-K⁺ and 32 mM-NH₄⁺ 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-ligand exponential model as described by Morris *et al.* (1986), except that the effector D now represents H⁺, with optimized values of the constants given in Table 1 relevant to the pH of the reaction solution. Mg^{2+} concentrations were: ■, 1.0 mM; ●, 1.5 mM; ▲, 2.0 mM; □, 2.5 mM; ○, 3.0 mM; △, 4.0 mM; ▽, 5.0 mM.

reaction from pH 6.5 to pH 5.0: first, a 3-fold increase in affinity of the enzyme for phosphoenolpyruvate; secondly, a 3-fold decrease in the maximum velocity of reaction; thirdly, a decrease from 2.7 to 1.7 in the slope of the Hill plot constructed as a function of phosphoenolpyruvate concentration. The first two of these effects have parallels in Table 1. The value of k_{BD} shows that the dissociation constant of phosphoenolpyruvate decreases 1.4-fold on saturation by H⁺, and, secondly, the value of $y = 0.027$ shows that enzyme activity is almost completely lost on binding H⁺. The third property predicted by Wieker & Hess (1971) finds no obvious parallel, for our data show that $v = f([\text{phosphoenolpyruvate}])$ is obviously sigmoidal at all pH values. This is confirmed by calculations of Hill slopes, reported in Table 2, which show clearly that decreasing pH actually leads to a small increase in Hill slope, as might be expected from the positive value of k_{BD} (note that quasi-saturating concentrations of ADP and Mg^{2+} were employed in these calculations). Furthermore, the large decrease in Hill slope with decreasing pH, predicted by Wieker & Hess (1971), is not obtained when the calculations are repeated with uncomplexed substrate concentrations corresponding to the total concentrations of ADP (10 mM) and Mg^{2+} (50 mM) that were specified by these workers. Because of this discrepancy, we have repeated the experiments of Wieker & Hess (1971) at pH

Fig. 2. Effect of [phosphoenolpyruvate] on the initial velocity of pyruvate formation at several fixed values of [ADP] with $[Mg^{2+}] = 5$ mM at pH 5.0

ADP concentrations were: ■, 0.4 mM; ●, 0.6 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. 1. This Figure also contains additional velocity determinations (not included in the analysis) at ADP concentrations of ▽, 0.8 mM; ▽, 2.0 mM.

Fig. 3. Effect of $[Mg^{2+}]$ on the initial velocity of pyruvate formation at several fixed values of [phosphoenolpyruvate] with [ADP] = 2.4 mM at pH 5.0

Phosphoenolpyruvate concentrations were: ■, 2.0 mM; ●, 2.5 mM; ▲, 3.0 mM; □, 3.5 mM; ○, 4.0 mM; △, 5.0 mM. Other conditions and calculations were as described in the legend to Fig. 1.

Fig. 4. Effect of [ADP] on the initial velocity of pyruvate formation at several fixed values of $[Mg^{2+}]$ with [phosphoenolpyruvate] = 5 mM at pH 6.6

Mg^{2+} concentrations were: ■, 0.75 mM; ●, 1.00 mM; ▲, 1.25 mM; □, 1.5 mM; ○, 1.75 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 mM at pH 6.6

Phosphoenolpyruvate concentrations were: ■, 1.5 mM; ●, 2.0 mM; ▲, 2.5 mM; □, 3.0 mM; ○, 3.5 mM; △, 4.0 mM; ▽, 5.0 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 at pH 6.6

ADP concentrations were: ■, 0.2 mM; ●, 0.4 mM; ▲, 0.6 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. 1.

values of 5.0 and 6.6 and can confirm their report that the relationship $v = f(\text{total phosphoenolpyruvate})$ is distinctly more sigmoidal at the higher pH. The velocity profiles showed maxima as expected (Ainsworth & Phillips, 1976), which, though broad, prevent the accurate definition of $V_{\max}$; nevertheless, the observed reduction of Hill slope from 2.8 at pH 6.6 to 1.3 at pH 5 is too large an effect to be upset by error in the calculation of $\log[v/(V_{\max} - v)]$. By contrast, a selection of the data displayed in Fig. 2 have been re-measured but with the addition of extra velocity determinations to extend the range of the Figure to lower phosphoenolpyruvate concentrations: again, the results confirmed the original conclusion that the sigmoidicity of $v = f(\text{phosphoenolpyruvate})$ is not diminished at pH 5 under our conditions.

The principal difference between the conditions of Wieker & Hess (1971) and those employed here is the much larger concentration of free Mg^{2+} present in the assay solutions, respectively 40–42 mM as compared with a maximum of 5 mM. It must be concluded, therefore, that a real discontinuity in behaviour is generated by the 8-fold increase in free Mg^{2+} concentration. A similar discontinuity was observed in the response of pyruvate kinase to a large increase in the concentration of the activator, fructose 1,6-bisphosphate (Morris *et al.*, 1986). We do not have an explanation for either effect, and clearly further investigation is required.

We return now to other aspects of behaviour revealed by the constants given in Table 1. First, it is evident that the non-D constants are very close to those given by Morris *et al.* (1984, 1986) and hence will sustain the interpretation that was previously given. The similarity is to be expected because the earlier experiments were

Table 1. Values of the four-ligand exponential model constants for the initial velocities of the yeast pyruvate kinase-catalysed reaction as a function of pH

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

$$v/V_G = p_A \cdot p_B \cdot p_C \cdot [1 + (v-1)p_D]$$

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

to 533 datum points and optimized fits to each data bloc defined by pH. 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 the rounding-off would considerably increase the values of the error parameters. The Table also gives for comparison the result of a fit by the three-substrate exponential model to data obtained at pH 6.2 by Morris *et al.* (1984). Temperature, 25 °C; A = ADP; B = phosphoenolpyruvate; C = Mg²⁺; D = H⁺ (the effector); [K⁺] = 100 mM; [NH₄⁺] = 32 mM.

Constant	Overall fits to 533 points	Optimized fits to data blocs						Mean of optimized fits	S.E.M.
		pH 6.2 (Morris <i>et al.</i> , 1984)	pH 5.0 (113 points) (Figs. 1-3)	pH 5.5 (114 points)	pH 5.8 (127 points)	pH 6.2 (71 points)	pH 6.6 (108 points) (Figs. 4-6)		
$\ln[\alpha_0(\text{mM}^{-1})]$	1.121	0.571	1.116	1.132	1.034	0.950	1.068	1.060	0.032
$\ln[\beta_0(\text{mM}^{-1})]$	-2.068	-2.292	-2.012	-2.088	-2.013	-2.095	-2.192	-2.080	0.033
$\ln[\gamma_0(\text{mM}^{-1})]$	-2.736	-2.735	-2.848	-2.748	-2.822	-2.797	-2.725	-2.788	0.023
$\ln[\delta_0(\text{mM}^{-1})]$	5.278	—	5.076	5.264	5.221	5.057	5.518	5.227	0.083
k_{AA}	0.403	0.224	0.387	0.394	0.354	0.388	0.740	0.452	0.072
k_{BB}	1.739	2.300	1.779	1.751	1.780	1.952	2.249	1.902	0.094
k_{CC}	2.698	2.714	2.326	2.750	2.725	3.014	2.863	2.734	0.114
k_{DD}	1.831	—	1.671	1.812	1.852	1.846	1.839	1.804	0.034
k_{AB}	-0.585	-0.234	-0.575	-0.630	-0.518	-0.599	-0.737	-0.612	0.036
k_{AC}	0.501	0.877	0.367	0.516	0.560	0.604	0.628	0.535	0.046
k_{BC}	0.947	0.684	0.760	0.947	0.877	0.777	0.673	0.807	0.048
k_{AD}	-0.610	—	-0.717	-0.603	-0.647	-0.607	-0.632	-0.641	0.021
k_{BD}	0.350	—	0.370	0.350	0.352	0.363	0.355	0.358	0.004
k_{CD}	-0.564	—	-0.428	-0.529	-0.626	-0.626	-0.556	-0.553	0.037
V_G (μmol/min per mg)	496.0	515.1	508.6	489.2	502.1	469.3	479.6	489.8	7.17
y	0.027	—	0.090	0.027	0.023	0.000	0.033	0.033	0.015
RSS	8.40	—	2.66 (5.87)	3.96 (4.52)	6.40 (8.55)	7.92 (12.34)	7.77 (10.25)	14.66	—
MD%	25.48	—	11.09 (23.56)	19.10 (20.42)	25.65 (25.95)	27.13 (31.32)	25.84 (27.34)	30.46	—
Error product	214.13	—	29.49 (138.3)	75.71 (92.30)	164.20 (221.7)	214.8 (386.5)	200.7 (280.3)	446.8	—

Table 2. Hill slopes, $d\{\ln[v/(V_{\max} - v)]\}/d(\ln B)$, calculated at $v = 0.5V_{\max}$. (Ainsworth, 1986), by using the constants of the overall fit given in Table 1

$V_{\max} = v$ with $B = 10^7$ M, the given A , C and D and $V_G = 1$. $V_G = 1$ when $p_A = p_B = p_C = 1$, $p_D = 0$. A = ADP; B = phosphoenolpyruvate; C = Mg²⁺; D = H⁺. (Note that the last two entries correspond to total concentrations of ADP and Mg²⁺ of 10 mM and 50 mM respectively.)

Concentrations of uncomplexed substrate at $v = 0.5V_{\max}$				Fractional saturations at $v = 0.5V_{\max}$				$V_{\max}$ ($B = 10^7$ M)	Hill slope at $v = 0.5V_{\max}$ (n_H)
A (mM)	B (mM)	C (mM)	D [μM (pH)]	p_A	p_B	p_C	p_D		
2	1.6887	5	10 (5.0)	0.862	0.441	0.815	0.781	0.149	2.15
2	1.9918	5	3.155 (5.5)	0.896	0.457	0.874	0.313	0.499	2.03
2	2.2460	5	1.581 (5.8)	0.905	0.490	0.893	0.142	0.682	1.96
2	2.3686	5	0.6293 (6.2)	0.909	0.501	0.901	0.053	0.779	1.92
2	2.4102	5	0.2505 (6.6)	0.911	0.505	0.903	0.021	0.814	1.91
1.75	1.2078	41.74	10 (5.0)	0.864	0.363	0.982	0.748	0.167	1.83
0.47	1.7121	40.43	0.2505 (6.6)	0.705	0.443	0.988	0.022	0.604	1.86

conducted at pH 6.2, where the enzyme is only 11% saturated by H^+ ions in the absence of other ligands ($\delta_0^{-1} = 0.0051$ mM); the non-D constants that were provided should therefore be barely affected by H^+ and be comparable with the non-D constants given here.

The D constants in Table 1 show that the binding of H^+ is positively interactive (k_{DD}), as is the interaction of H^+ with phosphoenolpyruvate (k_{BD}). On the other hand, H^+ interacts negatively with both ADP (k_{AD}) and Mg^{2+} (k_{CD}). The limiting affinity of pyruvate kinase for H^+ given by δ_0^{-1} corresponds to a pK of 5.29. The value of $y = 0.027$ suggests very strongly that H^+ binds within the active site of pyruvate kinase in a position such as to destroy its catalytic action, and it would appear from the value of its pK that the basic group involved may be a histidine residue (Mildvan & Cohn, 1965, 1966). Finally the exponential model, by hypothesis, requires that all the ligands bind independently; within this context, the positive value of k_{BD} and the negative value of k_{CD} suggest that H^+ binds in a position that enhances the binding of the phosphoenolpyruvate through its negatively charged carboxylate group (Nowak & Mildvan, 1972) while repelling the positive charged Mg^{2+} ion. The

negative interaction between H^+ and ADP may also be caused by the repulsion of Mg^{2+} , for it has been suggested that the nucleotide depends on Mg^{2+} for one of its links to the enzyme structure (Mildvan, 1970; Gupta & Mildvan, 1977).

REFERENCES

- Ainsworth, S. (1986) *Biochem. J.* **234**, 717–726
 Ainsworth, S. & Phillips, F. C. (1976) *Int. J. Biochem.* **7**, 625–632
 Ainsworth, S., Kinderlerer, J. & Gregory, R. B. (1983) *Biochem. J.* **209**, 401–411
 Gupta, R. K. & Mildvan, A. S. (1977) *J. Biol. Chem.* **252**, 5967–5976
 Mildvan, A. S. (1970) *Enzymes* 3rd Ed. **2**, 445–536
 Mildvan, A. S. & Cohn, M. (1965) *J. Biol. Chem.* **240**, 238–246
 Mildvan, A. S. & Cohn, M. (1966) *J. Biol. Chem.* **241**, 1178–1193
 Morris, C. N., Ainsworth, S. & Kinderlerer, J. (1984) *Biochem. J.* **217**, 641–647
 Morris, C. N., Ainsworth, S. & Kinderlerer, J. (1986) *Biochem. J.* **234**, 691–698
 Nowak, T. & Mildvan, A. S. (1972) *Biochemistry* **11**, 2819–2828
 Wieker, H.-J. & Hess, B. (1971) *Biochemistry* **10**, 1243–1248

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