The regulatory properties of rabbit muscle pyruvate kinase

The effect of pH

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(Received 15 January 1981/Accepted 13 February 1981)

The regulatory behaviour of rabbit muscle pyruvate kinase has been studied as a function of pH. The initial velocity of the enzyme-catalysed reaction as a function of ADP concentration was analysed with the exponential model for a regulatory enzyme. The analysis of the exponential model parameters as functions of pH provided pK values of 6.6 and 8.08 for the free enzyme in its fully ADP-bound conformation. By contrast, the binding of ADP to the ADP-free conformation of the free enzyme did not involve groups that ionize within the pH range (6.2–8.5) of these experiments. The results suggest that homotropic allosteric interactions actually alter the mode of ADP binding. The pK values of 6.63 and 9.00 determined from the analysis of V as a function of pH are readily interpreted in terms of a direct phosphoryl-transfer mechanism in which the β -phosphoryl group of ADP (pK 6.63) acts as the nucleophile and a lysine ϵ -amino group (pK 9.0) acts as the proton donor in the pyruvate kinase reaction.

The glycolytic enzyme pyruvate kinase (ATP: pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) as isolated from a wide variety of sources displays regulatory properties which may be broadly classified into two types. Thus the enzyme from mammalian liver (L-type), erythrocytes (R-type), the M_2 isoenzyme and the enzyme from yeast catalyse a reaction between ADP and phosphoenolpyruvate that shows a sigmoidal relationship between the initial velocity and the concentration of phosphoenolpyruvate. These forms of the enzyme are allosterically activated by fructose 1,6-bisphosphate and are inhibited by ATP (Hall & Cottam, 1978). In contrast, the enzyme from skeletal muscle (M_1 type) displays a hyperbolic relationship between the initial velocity and phosphoenolpyruvate concentration but sigmoidal relationships between the initial velocity and the concentrations of ADP and Mg^{2+} . In addition, the enzyme is only weakly activated by fructose 1,6-bisphosphate and is allosterically inhibited by phenylalanine (Phillips & Ainsworth, 1977; Carminatti *et al.*, 1971; S. Ainsworth & R. B. Gregory, unpublished work).

Investigations of the effect of pH on the regulatory behaviour of pyruvate kinase have been

reported for the L-type isoenzyme (Rozengurt *et al.*, 1969) and the yeast enzyme (Wieker & Hess, 1971). These enzymes are allosterically activated by H^+ . Evidence that the muscle pyruvate kinase displays pH-dependent regulatory properties has been given by Phillips & Ainsworth (1977). Activation of the enzyme by fructose 1,6-bisphosphate becomes evident above pH 6.2 and is maximal around pH 7.4. However, no detailed analysis of the kinetic parameters of the muscle enzyme reaction as a function of pH has been reported. This paper reports a more detailed study of the kinetics of the skeletal muscle pyruvate kinase as a function of pH. The pK values of groups that are involved in ADP binding and catalysis have been determined, and the nature of the groups that may be implicated within the active site is discussed in the light of evidence from chemical-modification studies.

Materials and methods

Materials

Phosphoenolpyruvate (lot 15308) cyclohexylammonium salt, NADH (lot 15142) sodium salt, ADP (lot 15016) free acid and rabbit muscle pyruvate kinase (lot 15744) crystalline suspension in 3.2M $(NH_4)_2SO_4$, were supplied by Boehringer. Lactate dehydrogenase (L-1254, type XI) lyophilized salt-free powder and *N*-ethylmorpholine

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(E-0252) were obtained from Sigma. Tetrapropylammonium hydroxide was supplied by Eastman-Kodak. All other reagents were either Analar or reagent grade as supplied by BDH or Fisons.

Preparation of substrates and buffers

pH studies were conducted in triethanolamine hydrochloride/tetrapropylammonium hydroxide or cacodylic acid/tetrapropylammonium hydroxide buffer systems. Stock solutions of ADP were adjusted to pH 7.0 with 10% (w/v) tetrapropylammonium hydroxide. The purity and concentration of the substrates were determined enzymically and by direct-absorption measurements as described by Ainsworth & Macfarlane (1973). In addition the purity of ADP was checked by t.l.c. (Randerath, 1962). Examination of the chromatograms in u.v. light did not reveal any impurities.

Preparation of pyruvate kinase

When pyruvate kinase is desalted in the manner described by Ainsworth & Macfarlane (1973), deviations from linearity in the progress curve are observed when assayed under the conditions employed in this study. Similar deviations have been reported by Phillips & Ainsworth (1977). The non-linearity of the progress curves makes the estimation of the initial rates extremely difficult. Because of this, conditions were sought to remove or minimize this problem. When the enzyme suspension was diluted to a concentration of 0.1 mg/ml with distilled water and used immediately, linear progress curves were obtained. As the enzyme preparation was allowed to age at 4°C, it slowly lost activity and after 1–2 h the progress curves became increasingly non-linear. Pyruvate kinase was therefore routinely prepared by diluting to a concentration of between 0.1 and 0.25 mg/ml with 0.1 M dithiothreitol in distilled water. The presence of low concentrations of NH_4^+ did not inhibit the enzyme. The enzyme stock was discarded after about 45 min.

Enzyme assays

Reaction mixtures were prepared on the assumption that the true substrates of pyruvate kinase 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 over the range of pH values employed in this study are given in Table 1. The actual concentrations employed are indicated on the figures. Pyruvate kinase activity was measured by using the continuous lactate-dehydrogenase-coupled assay (Bücher & Pfeleiderer, 1955). Initial velocities were determined at 25°C in a solution (1 ml) containing, in addition to the indicated substrates,

Table 1. Apparent dissociation constants for the binding of Mg^{2+} to ADP and phosphoenolpyruvate

pH	Apparent dissociation constant (M) for:	
	ADP ($\times 10^3$)	Phosphoenolpyruvate ($\times 10^2$)
6.2	1.9862	1.9927
6.4	1.5043	1.7042
6.6	1.1779	1.5222
6.8	0.9622	1.4074
7.0	0.8219	1.3349
7.2	0.7318	1.2892
7.4	0.6742	1.2604
7.6	0.6375	1.2422
7.8	0.6143	1.2307
8.2	0.5903	1.2189
8.5	0.5824	1.2150

0.15 μmol of NADH and an excess (12 units) of lactate dehydrogenase. The initial velocities reported in the figures are values corrected to an enzyme specific activity of 300 units/mg, and are expressed as μmol of NADH oxidized/min per mg of protein at 25°C. The specific activity of the enzyme was estimated as described by Phillips & Ainsworth (1977).

Theory

The initial velocities of the pyruvate kinase reaction, v , were analysed as functions of the ADP concentration, A , by using the exponential model for a regulatory enzyme (Ainsworth, 1977). The exponential model equation is given by:

$$v = Vp_A = \frac{VA\alpha_0 \exp kp_A}{1 + A\alpha_0 \exp kp_A} \quad (1)$$

where p_A is the fractional saturation of the enzyme by ADP and V is the maximum velocity. α_0 is the association binding constant that describes the binding of ADP to the fully unbound enzyme and k is the interaction free energy in units of RT . Values of V , α_0 and k were determined by the program of Kinderlerer & Ainsworth (1978). The further analysis of these parameters as functions of pH employed the procedure of Gregory & Ainsworth (1981). This method makes use of the two limiting hyperbolae predicted by eqn. (1):

$$\lim_{p_A \rightarrow 0} v = \frac{VA\alpha_0}{1 + A\alpha_0} \quad (2)$$

$$\lim_{p_A \rightarrow 1} v = \frac{VA\alpha_1}{1 + A\alpha_1} \quad (3)$$

where $\alpha_1 = \alpha_0 \exp k$.

It is assumed that the activity of each enzyme form is determined by the ionizations of two groups

which are operationally linked to the active site, the active form of the enzyme being the half-ionized species (Michaelis & Davidsohn, 1911; Walker & Schmidt, 1944; Dixon & Webb, 1964).

α_0 , α_1 and V are given as functions of pH by the following equations:

$$\alpha_0 = \frac{\bar{\alpha}_0 \Phi_E^0 \Phi_A}{\Phi_{EA}} \quad (4)$$

$$\alpha_1 = \frac{\bar{\alpha}_1 \Phi_E^1 \Phi_A}{\Phi_{EA}} \quad (5)$$

and

$$V = \bar{V} \Phi_{EA} \quad (6)$$

where $\bar{V}$ is the specific maximum velocity of the half-ionized enzyme and $\bar{\alpha}_0$ and $\bar{\alpha}_1$ are the specific association constants of the half-ionized species that describe the binding of ADP to the enzyme in conformations characteristic of the unbound and fully bound form, respectively. Φ_E^0 , Φ_E^1 , and Φ_{EA} are the fractional concentrations of the half-ionized enzyme forms; in this connection, note that it is assumed that the maximum velocity does not change with saturation by ADP, at constant pH, and therefore that substrate-bound active sites have a single conformation described by one set of ionization constants. We also have:

$$\Phi_E^0 = \left[\frac{H}{K_{E_0,1}} + 1 + \frac{K_{E_0,2}}{H} \right]^{-1} \quad (7)$$

$$\Phi_E^1 = \left[\frac{H}{K_{E_1,1}} + 1 + \frac{K_{E_1,2}}{H} \right]^{-1} \quad (8)$$

and

$$\Phi_{EA} = \left[\frac{H}{K_{EA,1}} + 1 + \frac{K_{EA,2}}{H} \right]^{-1} \quad (9)$$

The constants $K_{x,1}$ and $K_{x,2}$ are ionization constants. The subscript x refers to the particular enzyme species; EA denotes the enzyme-substrate complex and E_0 and E_1 denote the free enzyme in the ADP-free and fully bound conformations, respectively. Φ_A is the fractional concentration of the substrate species that binds to the enzyme. The pK values of the enzyme-substrate complex can be determined directly from an analysis of V as a function of pH according to eqns. (6) and (9). The pK values of the free enzyme in its extreme conformational states are determined by combining equations (4) and (5) with equation (6):

$$F_0 = \frac{V \alpha_0}{\Phi_A} = \bar{V} \bar{\alpha}_0 \Phi_E^0 \quad (10)$$

and

$$F_1 = \frac{V \alpha_1}{\Phi_A} = \bar{V} \bar{\alpha}_1 \Phi_E^1 \quad (11)$$

F_0 and F_1 are proportional to the fraction of the ligand-free and ligand-bound conformations of the enzyme in the half-ionized state. Finally, it should be noted that the interaction constant k as a function of pH is given by:

$$\exp k = \frac{\bar{\alpha}_1}{\bar{\alpha}_0} \cdot \frac{\Phi_E^1}{\Phi_E^0} \quad (12)$$

(Gregory & Ainsworth, 1981).

The pK values of the enzyme-substrate complex and the free enzyme species can be determined with the program (PHFIT) of Gregory & Kinderlerer (1980).

Results

Fig. 1 shows the dependence of the initial velocity of the reaction catalysed by pyruvate kinase on the concentration of free ADP at a number of different fixed pH values covering the range $6.2 \leq \text{pH} \leq 8.5$. It is apparent from the curves at pH 6.2, 6.6 and 7.0 that the two buffer systems employed to cover the range give very similar results. The function $v = f(\text{ADP})$ was analysed at each pH value with the program of Kinderlerer & Ainsworth (1978). The values of the exponential model parameters V , α_0 and k so obtained are given in Table 2. As the pH is raised the function $v = f(\text{ADP})$ becomes increasingly sigmoidal as indicated by the value of k which takes its maximum value at pH 7.4, while the maximum velocity increases to a maximum at pH 7.8, in agreement with the findings of Phillips & Ainsworth (1977). As the pH is increased further, the curves become less sigmoidal and the maximum velocity decreases.

The exponential model parameters were analysed as functions of pH as described in the Theory section (Gregory & Ainsworth, 1981). In calculating the values of F_0 and F_1 (eqns. 10 and 11) it is necessary to account for the ionization of ADP. ADP interacts with protons and metal ions to give a complex distribution of variously ionized and metal-complexed species. The nucleotide substrate has been shown to bind to the enzyme in its Mg^{2+} -free form (Ainsworth & Macfarlane, 1973; Phillips & Ainsworth, 1977) and in the present studies the binding of Mg^{2+} ions by ADP has been compensated. However, the Mg^{2+} -free ADP itself consists of three species; ADP^{3-} , ADPK^{2-} and ADPH^{2-} , whose relative proportions vary with pH. Consequently, the free ADP concentrations are expressed in terms of the ADP^{3-} species:

$$\text{ADP}^{3-} = \text{ADP}_{\text{free}} \cdot \Phi_A =$$

$$\text{ADP}_{\text{free}} \left[1 + \frac{K^+}{K_1} + \frac{H^+}{K_2} \right] \quad (13)$$

Fig. 1. Effect of pH on the initial velocity of the reaction catalysed by pyruvate kinase as a function of ADP concentration

Solutions contained 0.1 M-buffer, 0.1 M- K^+ , 0.1 M- Mg^{2+} and 0.2 mM-phosphoenolpyruvate at the indicated pH values. The curves are calculated from the exponential model parameters given in Table 2. The units of v are given in the text. T, C, see the legend to Table 2.

Fig. 2. Dependence of V (Δ), F_0 (O), F_1 ($\square$) and k (∇) on pH

Open symbols represent data obtained in triethanolamine hydrochloride/tetrapropylammonium hydroxide buffer; closed symbols represent data obtained in cacodylate/tetrapropylammonium hydroxide buffer. The curves are calculated from the parameters given by the program PHFIT according to eqns. (6), (10), (11) and (12).

Here, K_1 is the K^+ ion dissociation constant and K_2 is the ionization constant for ADP, which take the values 0.181 M and 1.4×10^{-7} M, respectively (Phillips, 1966). The adjustment of F_0 and F_1 in this way assumes that ADP^{3-} is the true substrate of the pyruvate kinase reaction.

The values of Φ_A , F_0 and F_1 are shown in Table 2. Fig. 2 shows the dependence of V , F_0 , F_1 and k on pH. The values of F_0 are nearly constant within the error of the estimates (mean $F_0 = 61.95 \pm 13.07$) with the exception of the values at pH 8.2 and 8.5 which are increased. This behaviour cannot be explained on the basis of an active half-ionized enzyme and may reflect variations with pH in the fractional saturation of the enzyme by other ligands. This is particularly likely with Mg^{2+} which was maintained at a low concentration. This was necessary, firstly, so as to reduce the concentration of the Mg -ADP complex, which is a dead-end inhibitor of the pyruvate kinase reaction, but more importantly because at high concentrations of Mg^{2+} , the function $v = f(ADP)$ reverts to a non-inflected form (Phillips & Ainsworth, 1977; S. Ainsworth & R. B. Gregory, unpublished work).

The pK values obtained from an analysis of V , F_1 and $\exp k$ as functions of pH with PHFIT are given in Table 3. F_1 and $\exp k$ pass through maxima at pH values of 7.34 ± 0.17 and 7.24 ± 0.24 , respectively,

Table 2. *Exponential model parameters for the effect of pH on the function $v = f(\text{ADP})$*

V , α_0 and k were determined by the program of Kinderlerer & Ainsworth (1978). Φ_A , F_0 and F_1 were calculated according to eqns. (13), (10) and (11), respectively. The buffer systems triethanolamine hydrochloride/tetrapropylammonium hydroxide and cacodylic acid/tetrapropylammonium hydroxide are indicated by T and C, respectively.

pH	Buffer	V (units of v)	α_0 (mM^{-1})	k	Φ_A	F_0 (units of $v \cdot \text{mM}^{-1}$)	F_1
6.2	C	38.18 ± 1.62	0.261 ± 0.012	1.34 ± 0.18	0.165	60.37	231.5
6.2	T	37.05 ± 13.6	0.318 ± 0.062	1.09 ± 0.96	0.165	71.39	211.91
6.4	C	67.00 ± 4.50	0.223 ± 0.016	1.54 ± 0.27	0.227	65.68	308.07
6.6	C	87.03 ± 3.90	0.199 ± 0.014	1.95 ± 0.23	0.299	57.97	409.54
6.6	T	89.40 ± 3.42	0.159 ± 0.010	2.23 ± 0.19	0.299	47.58	441.63
6.8	C	109.08 ± 3.66	0.179 ± 0.014	2.32 ± 0.21	0.373	52.42	530.98
7.0	C	115.42 ± 2.07	0.200 ± 0.009	2.25 ± 0.12	0.440	52.33	494.52
7.0	T	125.77 ± 4.37	0.182 ± 0.014	2.30 ± 0.21	0.440	51.89	517.56
7.2	T	128.61 ± 3.22	0.190 ± 0.013	2.44 ± 0.17	0.500	48.95	564.14
7.4	T	136.97 ± 2.72	0.231 ± 0.014	2.23 ± 0.14	0.544	58.12	538.46
7.6	T	152.29 ± 4.14	0.235 ± 0.022	2.29 ± 0.22	0.577	61.99	608.50
7.8	T	152.00 ± 2.88	0.239 ± 0.015	2.33 ± 0.15	0.600	60.53	625.42
8.2	T	152.58 ± 9.61	0.333 ± 0.034	1.35 ± 0.35	0.626	81.14	312.39
8.5	T	125.40 ± 9.71	0.491 ± 0.043	0.52 ± 0.41	0.635	96.97	163.74

Table 3. *pK values determined by the program PHFIT (Gregory & Kinderlerer, 1980)*

Enzyme species (x)	$\text{pK}_{x,1}$	$\text{pK}_{x,2}$
EA	6.63 ± 0.05	9.00 ± 0.12
E_1^a	6.60 ± 0.11	8.08 ± 0.13
E_1^b	6.74 ± 0.16	7.75 ± 0.18

^a The pK values are derived by analysis of eqn. (11).

^b The pK values are derived by analysis of eqn. (12) but relate to the enzyme species E_1 because $\Phi_E^0 = 1$.

and are described by similar pK values. Eqns. (11) and (12) show that this result can only be obtained if Φ_E^0 is independent of pH, a conclusion supported by the constancy of F_0 (eqn. 10 and Fig. 2).

Discussion

Essential groups of the active site

The use of group-specific reagents has made possible the determination of several amino acid side chains that are essential for the activity of pyruvate kinase. The muscle enzyme has long been known to react with sulphhydryl reagents (Boyer, 1962), but in addition, chemical-modification studies implicate histidine, lysine and arginine residues in the active site (Johnson & Deal, 1970; Hollenberg *et al.*, 1971; Dann & Britton, 1974; Bloxham & Chalkley, 1976; Berghauer, 1977). With this information and the results from substrate-protection studies it is possible to assign the pK values determined in the present study to specific groups. The assignment is necessarily tentative because it has been shown that

the pK values of groups in the active site can be perturbed by as much as 4 pH units from their values observed in solution (Schmidt & Westheimer, 1971; Holbrook & Shore, 1975).

Binding of ADP

The results suggest that the binding of ADP to the unbound and fully bound enzyme conformations involve different groups within the active site. The binding of ADP to the ADP-free conformation of the enzyme does not involve groups that ionize within the pH range employed in these studies. By contrast, the binding of ADP to the fully bound conformation of the enzyme and the appearance of co-operativity involve ionizable groups with pK values of about 6.6 and 8.0. In this connection, it is interesting that Stammers & Muirhead (1975) observed differences in the binding of ADP to the enzyme at pH 6.0 and pH 8.0. Jacobson & Black (1971) have observed the alkylation of two sulphhydryl groups by iodoacetamide and report that an 80% loss of activity occurs on modifying a group with a $\text{pK} \approx 7.8$. However, the enzyme is protected from this inactivation by the presence of $\text{Mg}^{2+} > \text{phosphoenolpyruvate} > \text{K}^+ > \text{ADP}$ and this order of effectiveness suggests that the sulphhydryl group is situated in the phosphoenolpyruvate subsite rather than being directly involved in binding ADP.

The pK value of 6.6 corresponds closely with the pK value for the ionization of phosphoenolpyruvate. The possibility that the ionization of enzyme-bound phosphoenolpyruvate promotes the subsequent co-operative binding of ADP is an interesting one. Indeed, recent kinetic studies at pH 7.4 have shown that the binding of ADP

becomes increasingly co-operative as the concentration of phosphoenolpyruvate is raised (S. Ainsworth & R. B. Gregory, unpublished work). This interpretation also implies that the two extreme conformations of the enzyme display different kinetic mechanisms, the reaction of the fully-bound enzyme conformation proceeding with the ordered addition of phosphoenolpyruvate before ADP, while that of the unbound enzyme conformation proceeds with the random addition of substrates. Similar ordered mechanisms have been observed with pyruvate kinase from yeast (Macfarlane & Ainsworth, 1972) and pig liver (Macfarlane & Ainsworth, 1974).

The binding of ADP to the enzyme is clearly complex. It appears that the allosteric interactions actually alter the mode of ADP binding. This conclusion, in turn, suggests that considerable conformational changes are induced at the active site by the binding of ADP at sites on neighbouring subunits. This behaviour may be contrasted with that displayed by aspartate transcarbamylase, in which the pK values of groups involved in binding aspartate are shifted as a result of allosteric interactions (Gregory & Ainsworth, 1981).

Chemical mechanism of catalysis

The mechanism that has been proposed for muscle pyruvate kinase involves direct phosphoryl transfer in a quaternary complex (Boyer, 1962; Mildvan & Cohn, 1965, 1966; Mildvan *et al.*, 1967). The reaction is assumed to proceed with a nucleo-

philic attack by the β -phosphate group of ADP on the phosphoryl group that is to be transferred. A proton is then transferred from a base on the enzyme, converting enolpyruvate to pyruvate (Mildvan, 1970). The pK values determined from the analysis of V as a function of pH are readily interpreted in terms of this direct phosphoryl transfer mechanism. The pK value of 6.63 determined here may be assigned to the ionization of the β -phosphoryl group of enzyme-bound ADP, which acts as the nucleophile in the displacement reaction. The pK value of 9.00 is consistent with the ionization of a lysine ϵ -amino group on the enzyme. Chemical modification studies with pyridoxal 5'-phosphate (Johnson & Deal, 1970) and trinitrobenzenesulphonate (Hollenberg *et al.*, 1971) indicate the presence of an essential lysine residue within the active site. The decrease in the maximum velocity as the pH is increased beyond pH 9.0 suggests that the protonated lysine ϵ -amino group acts as the proton donor in the muscle pyruvate kinase reaction. A reaction scheme that incorporates these features is shown in Scheme 1.

An SRC grant to R. B. G. is gratefully acknowledged.

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Scheme 1. Possible mechanism of muscle pyruvate kinase reaction

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