BBA 66155

RELAXATION SPECTRA OF PYRUVATE KINASE

GORDON G. HAMMES AND JON SIMPLICIO
Department of Chemistry, Cornell University, Ithaca, N. Y. 14850 (U.S.A.)

(Received March 31st, 1970)

SUMMARY

Relaxation studies using the temperature jump have been carried out on pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40) isolated from rabbit muscle. Several concentration-independent relaxation effects with associated relaxation times of approx. $1 \cdot 10^{-4}$ sec were observed in the presence of some of the activator divalent metal cations and substrates used. These effects are attributed to isomerizations or conformational changes of the enzyme. The appearance and disappearance of these effects, as observed in both the visible (with a pH indicator) and ultraviolet region, as a function of the temperature range studied (15–36°) indicate that distinct conformational states of the enzyme exist and that the proportion of each state varies with temperature and the liganded state of the protein. Although a large range of enzyme and substrate concentrations were examined relatively few relaxation processes could be detected. This indicates that either a suitable detection system could not be found and/or many of the reactions associated with the enzymatic reaction are too rapid to observe.

INTRODUCTION

Pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) catalyzes the reaction

$$\text{ATP} + \text{pyruvate} \rightleftharpoons \text{ADP} + \text{phosphoenolpyruvate} \quad (1)$$

and is activated by the divalent metal cations Mn^{2+}, Co^{2+} (*cf.* ref. 1 and references therein). Detailed kinetic and binding studies[1,2] with NMR and ESR techniques have suggested that the activator metal ion binds simultaneously to the enzyme and substrates. Furthermore the results of their studies suggest that upon combination of the divalent metal cations and substrates with the protein, a conformational change of the protein occurs. Changes in ultraviolet difference spectra[3–5] have paralleled changes in enzyme activity resulting from the variation of the type of mono- and divalent cations or anions present and of the ionic strength, temperature and pH. The spectral differences observed are believed to occur from an environmental pertur-

bation of the tryptophan or tyrosine residues which is presumably the result of a protein conformational change. Nonlinearity in the Arrhenius plot of the steady state initial velocity of the reverse of Reaction 1 has also been interpreted as kinetic evidence for the reactivity of two conformational states of the enzyme in equilibrium with one another[5].

The work reported here used the temperature-jump technique to investigate the interaction between pyruvate kinase and a variety of divalent metal cations and substrates. Relaxation processes, which could be attributed to conformational changes, were found with enzyme alone, and with enzyme *plus* Mn^{2+}, Mg^{2+}, or phosphoenolpyruvate. The observed relaxation times, except those associated with Co^{2+} binding to the enzyme, are independent of metal concentration over the range studied. The overall evaluation of the concentration-independent relaxation effects observed suggests the existence of multiple conformational equilibria.

EXPERIMENTAL

Materials

Pyruvate kinase was isolated from rabbit muscle by the procedure of TIETZ AND OCHOA[6]. The muscle was obtained frozen from Pel-Freeze, and was thawed and briefly washed under distilled water before use. The preparations were further purified with Sephadex G-25 (refs. 5–7). A Radiometer Type TTT1 Titrator and Titrigraph was used for the pH-stat assay employed[5]. The activities from different preparations varied from the production of approx. 130–180 μmoles H^+ per mg per min at 25° and pH = 7.6, generally centering about 150 μmoles H^+ per mg per min. Preparations were stored at 0° at a concentration of about 40 mg/ml.

ADP and ATP were purchased from P-L Biochemicals, Inc. Only trace impurities were found in the nucleotides by descending chromotography[8]. Phosphoenolpyruvate was purchased from Calbiochem and was used without further purification. Metal chlorides were standardized with EDTA and murexide or Erio T as described by SCHWARZENBACH[9]. All dialysis tubing was boiled in 0.001 M EDTA for 30 min before use. Spectra for determining protein and nucleotide concentrations were recorded at room temperature with a Cary Model 14 recording spectrophotometer.

Kinetic studies

The temperature-jump apparatus previously described[10,11] was used in the present studies. A 10 kV pulse was rapidly passed through a test solution containing protein, substrates and 0.10 M KCl* and caused a temperature rise of approx. 7.5° in less than 15 μsec. Relaxation effects could be observed from 15 μsec to 0.5 sec before cooling of the thermostatted solution became important. All nucleotides and enzyme solutions were prepared immediately before use. The concentrations were determined spectrally: for ADP and ATP an extinction coefficient of $1.54 \cdot 10^4$ $cm^{-1} \cdot M^{-1}$ at pH 7.0 was used and for pyruvate kinase an extinction coefficient of 0.54 cm^2/mg was

* The ultraviolet difference spectra[3,4] indicate that K^+ causes a spectral change in the absorption spectrum of the native protein. Furthermore MELCHIOR[12] found that K^+ functions as an activator of pyruvate kinase in the presence of Mg^{2+} and ADP. Since temperature-jump solutions require about 0.1 M ionic strength for conduction purposes, KCl was used in all solutions and its effect was assumed to be a constant factor in affecting the observed relaxation effects.

TABLE I

SUMMARY OF EXPERIMENTAL OBSERVATIONS

The pyruvate kinase concentration was approx. 0.1 mM (0.08–0.19 mM). The enzyme alone exhibited a relaxation effect (with an associated relaxation time of 0.070 msec) only at 15° with phenol red as a pH indicator. All concentrations are based on the total amount of material added. N designates no observable effect from 15 μsec to 500 msec with a sensitivity of about $5 \cdot 10^{-4}$ absorbance units. The uncertainty in the relaxation times is ±20% or less. The entries in the table indicate the various combinations of reactants used. For example the second entry indicates that experiments were done with Mn^{2+} starting at a concentration of 0.0 mM, then jumping to the concentration range 0.20–3.4 mM, with no ADP added until the last addition of Mn^{2+} (3.4 mM) was reached. Then ADP was added in the presence of the last metal concentration over the concentration range of 0.10–6.9 mM.

Metal	*Metal concn. (mM)*	*ADP (mM)*	*Other substrates*	*Other substrate concn. (mM)*	*Relaxation effect, τ(msec)*
Temp., 25°					
Mg^{2+}	0.0, 0.55–9.4	0.0, 3.3			N*
Mn^{2+}	0.0, 0.2–3.4	0.0, 0.10–6.9			N*
Mg^{2+}	0.0, 0.24–5.5				N**
Mg^{2+}	0.10–1.7				0.135**
Mg^{2+}	0.0, 0.55–5.2	0.0, 0.55			N***
Mn^{2+}	0.35–10.0				0.072**
Mn^{2+}	0.0, 0.24–0.90				N***
Mn^{2+}	0.0, 0.40–1.6	0.0, 0.77–0.90	ATP	0.0, 0.30–1.4	N***
Mn^{2+}	1.60	0.90	ATP	0.0, 1.40	0.083***
			Phosphoenolpyruvate	0.0, 1.0–6.0	
Ni^{2+}	0.10–3.6				N**
Co^{2+}	0.50–3.4				N**
Temp., 36°					
Mn^{2+}	0.0, 0.50–3.4	0.0, 0.14–3.3			N*, **
Temp., 15°					
Mn^{2+}	0.97–2.8				0.070*
Mn^{2+}	5.2				N*
Mn^{2+}	5.2		EDTA	6.0	~0.070*
None		0.0, 0.14–4.1			0.076*
Mn^{2+}	0.49–3.4	0.0, 0.17–2.0			N**
Mn^{2+}	5.6–8.0		Pyruvate	0.0, 0.43	N*
None			Pyruvate	0.067–0.57	0.045*
None			Pyruvate	1.7	N*
			ATP	0.0, 0.47–4.5	
Mg^{2+}	0.25–6.2	0.0, 0.08–1.5	Phosphoenolpyruvate	0.0, 0.2–1.1	N**
Ca^{2+}	0.07–4.8	0.0, 0.5–3.0	Phosphoenolpyruvate	0.0, 0.5–3.0	N**
Ca^{2+}	0.18–9.2		Phosphoenolpyruvate	0.0, 0.50	0.058*
Ca^{2+}	9.2		ATP	1.30	‡
			Phosphoenolpyruvate	3.0	
Co^{2+}	0.17				0.050*
Co^{2+}	0.50–15				1.06–0.34*
Co^{2+}	12		Phosphoenolpyruvate	0.0, 0.5–3.2	0.55*
Co^{2+}	12.0	0.50–4.6	Phosphoenolpyruvate	3.2	0.44–0.22*
Ni^{2+}	0.17				0.050*
Ni^{2+}	1.2–13	0.0, 0.32–1.9	Phosphoenolpyruvate	0.0, 0.50–2.5	~6.0*, §§
Mg^{2+}	2.5–6.2		Pyruvate	0,0. 0.07–4.4	§
			ATP	0.0, 0.4–2.0	
Mg^{2+}	0.0, 0.25–6.4		Pyruvate	0.0, 0.2–4.2	N***, †
			Tris	10	
			ATP	0.0, 0.23–4.9	

* Experimental observations at 560 nm in the presence of 20 mM phenol red.
** Experimental observations at 295–297 nm.
*** Experimental observations at 260–265 nm.
† Possible small relaxation effect with $\tau \sim 0.050$ msec.
§ Small relaxation effect with $\tau \sim 0.5$ msec.
§§ These effects were superimposed on a slower relaxation effect with $\tau \sim 0.1$ sec.

used[13] with a molecular weight of 237 000 (ref. 14). Stability constants for metal–nucleotide complexes were taken from the literature (*cf.* refs. 8, 15 and 16), as were the thermodynamic constants for binding involving the protein[1,2,17,18].

All solutions were adjusted with 0.1 M KOH to a pH of 7.6 at 25°. Experiments in the visible region incorporated 20 μM phenol red as an indicator to observe pH changes upon perturbing the solutions. No buffers were used with experiments in the ultraviolet or visible regions. After several temperature jumps (approx. 30) some preparations showed a slight turbidity. Centrifuging these solutions at 0° and 11 000 $\times$ *g* for 15 min resulted in clear solutions of nearly the same activity and absorption spectrum. The relaxation effects, when present, were unaffected within the experimental reproducibility after centrifuging. Assays run after a complete series of temperature-jump experiments usually indicated minor loss of activity although some preparations showed losses of up to 25%. Portions of the work were repeated with different samples of pyruvate kinase and gave the same relaxation effects with minor amplitude variations.

RESULTS AND DISCUSSION

A summary of the data is given in Table I. The reported relaxation times have been calculated from plots of the logarithm of the change in transmission, which is small enough in all these experiments to be directly proportional to the change in absorbance *versus* time. The values reported are the average from a minimum of three determinations for each set of experimental conditions. The concentration of phenol red was varied 6-fold and did not cause a change in the relaxation time of 70 μsec observed with pyruvate kinase at 15°; this makes it unlikely that an enzyme–indicator interaction is being observed.

Although data were obtained over as wide a range of conditions as possible, relatively few relaxation processes were observed and almost none of these were concentration dependent. For example a temperature jump from 17.5 to 25°, as observed at 560 mμ by using phenol red as a pH indicator, has failed to detect any accompanying pH change even in the presence of Mg^{2+}, Mn^{2+}, ADP, ATP and various concentration combinations of metals and nucleotides, whether under saturated or equilibrium conditions. This is most likely due to a buffering of the solution by the protein. In the ultraviolet region at 25° no effect is observed at either 295–297 or 260 mμ (the nucleotide peak) with pyruvate kinase in the presence of either ADP or ATP. This is not too surprising considering the absence of an ultraviolet difference spectra[4] with these nucleotides. The relatively small changes in the ultraviolet spectrum of pyruvate kinase[5] for a 7.5° temperature jump is probably the reason for the absence of observable relaxation effects. The addition of Mg^{2+} and Mn^{2+} to pyruvate kinase resulted in a relaxation effect at 25° in the ultraviolet with relaxation times of 135 and 72 μsec, respectively. Both effects were concentration independent within experimental error. Since these effects are observed when the enzyme is virtually saturated with metal ion, the mechanism undoubtedly involves an equilibrium between two or more conformational states, such as $EM \rightleftharpoons E + M$. At non-saturating concentrations of metal the relaxation time should be concentration dependent, but the amplitude of the relaxation process is too small to observe under these conditions. Since the relaxation process associated with the bimolecular reaction is not observed,

a lower bound for the second order rate constant of about 10^7–10^8 $M^{-1} \cdot sec^{-1}$ can be estimated. The relaxation effects in the presence of Mn^{2+} with pyruvate kinase in the ultraviolet at 25° disappear when the solutions are temperature-jumped to 15 or 36°. The extremes of temperature apparently stabilize one conformational state of the enzyme–metal complex.

Although Co^{2+} can also serve as an activator[1] for pyruvate kinase, with a binding constant of 0.25 mM, no relaxation effects were noted with Co^{2+} or with Ni^{2+} in the ultraviolet at 25°, with the same conditions employed for the Mn^{2+} and Mg^{2+} experiments. The metal concentration dependent effects for Co^{2+} as observed at 15° using phenol red as the indicator are quite similar to those found for Co^{2+}–imidazole complex formation[19]. This suggests that the Co^{2+} is binding to such residues on the pyruvate kinase. The relaxation times for Ni^{2+} under the comparable conditions used for Co^{2+} revealed two relaxation effects. The slow effect could not be measured accurately due to cooling of the thermostatted cell but seemed to have a relaxation time $\gtrsim$ 0.1 sec. A faster relaxation effect, superimposed upon the slower one, had an associated relaxation time of approx. 6 msec. The lack of concentration dependence for this latter relaxation time, which is probably associated with a Ni^{2+}–imidazole complex formation, could possibly have been obscured by the slower relaxation effect, which had such a large amplitude that it was difficult to find the exact final position of the oscilloscope trace at the end of the faster effect. The mechanistic basis for the second slower effect has not been ascertained.

MILDVAN AND COHN[2] have interpreted their ESR and EPR data in terms of the existence of a ternary complex where the metal is simultaneously bound to the protein and nucleotide. Solutions containing the ternary complexes of pyruvate kinase + Mn^{2+} with either ADP, phosphoenolpyruvate, ATP or pyruvate exhibited no observable relaxation effects in the ultraviolet region.

At 25° in the presence of phenol red neither Mg^{2+} or Mn^{2+} exhibited any relaxation effects with enzyme either under saturated or unsaturated conditions. However, upon temperature-jumping the metal–enzyme solutions to 15° the solutions of the metal activators caused the relaxation effects, present with enzyme alone at 15°, to disappear. The addition of Mn^{2+} to enzyme solutions at 15° (observed in the visible with phenol red indicator) caused the amplitude of the relaxation effect to diminish but the relaxation time remained unchanged. The addition of excess EDTA which complexed all the Mn^{2+} present caused a recurrence of the relaxation effect. The addition of Mn^{2+} to the enzyme at 15° caused a decrease in the amplitude of the effect; the addition of ADP has no effect on either the amplitude or relaxation effect present with just enzyme and phenol red. On the other hand, the addition of pyruvate causes a disappearance of the relaxation process of the enzyme as observed with phenol red. Equilibrium dialysis experiments[18] indicate a dissociation constant for pyruvate of about 0.25–0.8 mM, the binding being independent of the presence of other substrates. A mechanistic explanation for these results is that at 15° two (or more) conformational states of the enzyme exist in comparable amounts and the binding of metal ion shifts the equilibria so as to predominately stabilize a single conformational state.

The failure of Ca^{2+} to elicit an effect with the protein in the ultraviolet is not surprising since no ultraviolet difference spectra had been found previously.[4]

The results reported here suggest multiple conformational states of the enzyme exist and that temperature, metal ion activators and substrates can markedly affect

the proportions of the various states. The relaxation times associated with the isomerizations or conformational changes are similar in magnitude to those observed with other enzymes[20,21]. Moreover, these results show that the interconversions of conformational states occur sufficiently rapidly so that they may be of importance in the catalytic process, and that metal ion activators modify the conformational equilibria in a specific manner.

ACKNOWLEDGEMENT

This work was supported by a grant from the National Institutes of Health (GM 13292).

REFERENCES

1 A. S. MILDVAN AND M. COHN, *J. Biol. Chem.*, 240 (1965) 238.
2 A. S. MILDVAN AND M. COHN, *J. Biol. Chem.*, 241 (1966) 1178.
3 R. H. WILSON, H. J. EVANS AND R. R. BECKER, *J. Biol. Chem.*, 242 (1967) 3825.
4 C. H. SUELTER, R. SINGLETON, F. J. KAYNE, S. ARRINGTON, J. GLASS AND A. S. MILDVAN, *Biochemistry*, 5 (1966) 131.
5 F. J. KAYNE AND C. H. SUELTER, *J. Am. Chem. Soc.*, 87 (1965) 897.
6 A. TIETZ AND S. OCHOA, *Arch. Biochem. Biophys.*, 78 (1958) 477.
7 F. J. KAYNE AND C. H. SUELTER, *Biochemistry*, 7 (1968) 1678.
8 G. G. HAMMES AND J. K. HURST, *Biochemistry*, 8 (1969) 1083.
9 G. SCHWARZENBACH, *Complexometric Titrations*, Interscience Publishers, Inc., New York, 1957, p. 62.
10 T. C. FRENCH AND G. G. HAMMES, *Methods in Enzymology*, 16 (1969) 3.
11 E. FAEDER, Ph.D. Dissertation, Cornell University, 1970.
12 J. B. MELCHIOR, *Biochemistry*, 4 (1965) 1518.
13 T. BÜCHER AND G. PFLEIDERER, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. I, Academic Press, Inc., New York, 1955, p. 435.
14 R. C. WARNER, *Arch. Biochem. Biophys.*, 78 (1958) 494.
15 M. M. T. KHAN AND A. E. MARTELL, *J. Am. Chem. Soc.*, 89 (1967) 5585.
16 M. M. T. KHAN AND A. E. MARTELL, *J. Am. Chem. Soc.*, 88 (1966) 668.
17 L. A. KERSON, D. GARFINKEL AND A. S. MILDVAN, *J. Biol. Chem.*, 242 (1967) 2124.
18 A. M. REYNARD, L. F. HASS, D. D. JACOBSEN AND P. D. BOYER, *J. Biol. Chem.*, 236 (1961) 2277.
19 G. G. HAMMES AND J. I. STEINFELD, *J. Am. Chem. Soc.*, 84, (1962) 4639.
20 G. G. HAMMES, *Advan. Protein Chem.*, 23 (1968) 1.
21 G. G. HAMMES, *Accounts Chem. Res.*, 1 (1968) 321.