Evidence for a Dissociative $S_N1(P)$ Mechanism of Phosphoryl Transfer by Rabbit Muscle Pyruvate Kinase

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Summary Pyruvate kinase from rabbit muscle catalyses the randomisation of ^{18}O between P_β-$^{18}O_2$ and P_γ–O–P_β in adenosine 5′[$\alpha\beta$-^{18}O,$\beta^{18}O_2$]-triphosphate in the presence of pyruvate and oxalate *and in their absence*, the first order rate constants being $2{\cdot}0 \times 10^3$, $2{\cdot}0 \times 10^2$, and $1{\cdot}1 \times 10^2$ min^{-1}, respectively, which indicates that phosphoryl transfer occurs by a dissociative $S_N1(P)$ mechanism.

THE mechanisms of phosphoryl transfer by kinases are likely to fall into one of four types. The simplest of these is the associative 'in line' transfer of the group, the $S_N2(P)$ mechanism (Figure 1i). The dissociative 'in line' phosphoryl transfer, the $S_N1(P)$ mechanism, involves the formation of the metaphosphate ion (PO_3^-) as an intermediate (Figure 1ii). The third mechanism involves 'adjacent' attack on the phosphoryl residue which must be accompanied by pseudorotation of the pentacovalent intermediate for phosphoryl transfer to occur (Figure 1iii), and the fourth mechanism involves the formation of a phospho-enzyme intermediate (which may occur by an associative or dissociative mechanism, but which are not distinguished here) (Figure 1iv).

Pyruvate kinase (PK) from rabbit muscle catalyses phosphoryl transfer between phosphoenolpyruvate and ADP to give pyruvate and ATP and has a requirement for both mono- and di-valent cations.[1] The equilibrium is in favour of pyruvate and ATP (K_{eq} $6{\cdot}45 \times 10^3$ at pH 7·4).[2] The enzyme also catalyses the keto–enol tautomerisation of pyruvate,[3] and several pieces of evidence indicate that a phospho-enzyme intermediate (Figure 1iv) is not involved

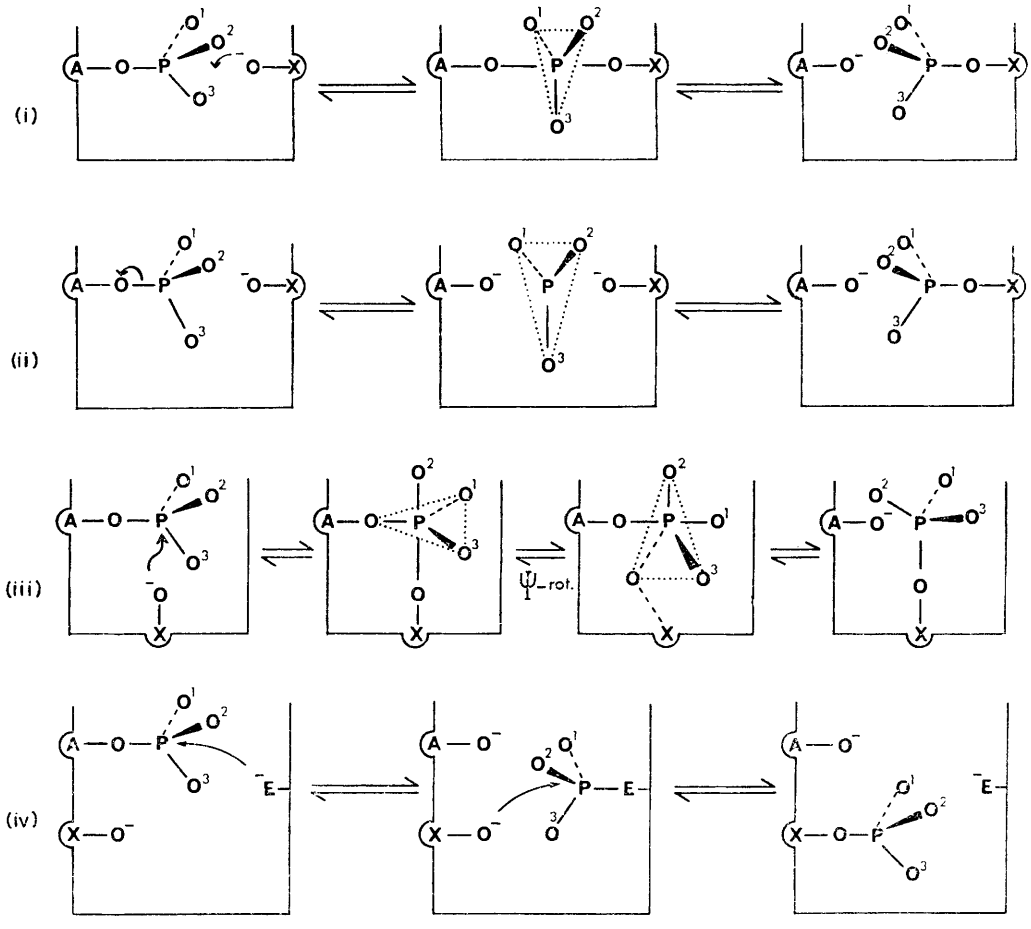
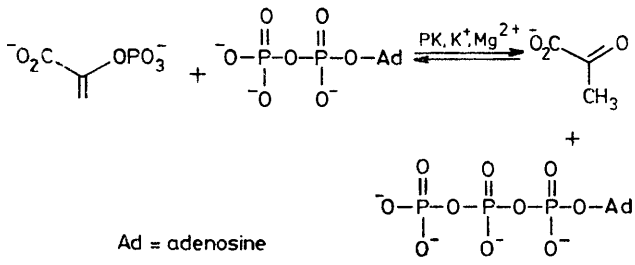


FIGURE 1. Possible mechanisms of enzyme catalysed phorphoryl transfer: (i) the associative 'in line' mechanism: $S_N2(P)$, (ii) the dissociative 'in line' mechanism: $S_N1(P)$, (iii) the adjacent mechanism with pseudorotation, (iv) the double displacement mechanism *via* a phospho-enzyme intermediate.

in the reaction pathway.[4] Of the three remaining mechanisms only the dissociative $S_N1(P)$ mechanism could lead to randomisation of ^{18}O between P_β–$^{18}O_2$ and the P_γ–O–P_β bridge in adenosine 5′[$\alpha\beta$-^{18}O,β-$^{18}O_2$]-triphosphate,[5] in the absence of the second substrate.



Adenosine 5′[$\alpha\beta$-^{18}O,β-$^{18}O_2$]-triphosphate was incubated with pyruvate kinase in the presence of pyruvate and sufficient di(adenosine-5′)pentaphosphate to inhibit possible traces of adenylate kinase.[6] The ^{31}P n.m.r. spectrum of the [$^{18}O_3$]-ATP isolated shows two sets of doublets separated by 0·8 Hz in the region of P_γ (Figure 2i; the departure of the ratio of the two P_γ doublets from 1 : 2 is due to incomplete labelling of P_β–O_2 in adenosine 5′[$\alpha\beta$-^{18}O,β-$^{18}O_2$]-triphosphate).† The experiment was repeated except that pyruvate was replaced by the potent inhibitor, oxalate.[7] The ^{31}P n.m.r. spectrum of the [$^{18}O_3$]-ATP isolated after incubation is shown in Figure 2ii. Again two closely spaced doublets appear for the P_γ resonance separated by 0·8 Hz, indicating again that ^{18}O originally present in P_β–O_2 has been randomly distributed between P_β–O_2 and P_γ–O–P_β. The third experiment was conducted in the same way as the first except that pyruvate was omitted. The ^{31}P n.m.r. spectrum of the [$^{18}O_3$]-ATP isolated (Figure 2iii) again shows that ^{18}O initially present in P_β–O_2 has been randomly distributed between P_β–O_2 and P_γ–O–P_β.‡ As a control experiment a solution containing in addition to all the ingredients of the third experiment, phosphoenolpyruvate at a concentration sufficient to saturate the enzyme, NADH and lactate dehydrogenase, was incubated as before. The phosphoenolpyruvate should prevent the [$^{18}O_3$]-ATP from binding to pyruvate kinase, but not to any other enzyme which may be present as a trace impurity. Lactate dehydrogenase and NADH was added in order to remove any pyruvate that might arise adventitiously. Under these conditions no redistribution of ^{18}O in adenosine 5′[$\alpha\beta$-^{18}O,β-$^{18}O_2$]-triphosphate occurred as can be seen from the ^{31}P n.m.r. spectrum of the [$^{18}O_3$]-ATP isolated (Figure 2iv).

Pyruvate kinase therefore catalyses P_γ–OP_β cleavage of ATP in the presence of pyruvate as expected, in the presence of oxalate a good substrate analogue of the enolate form

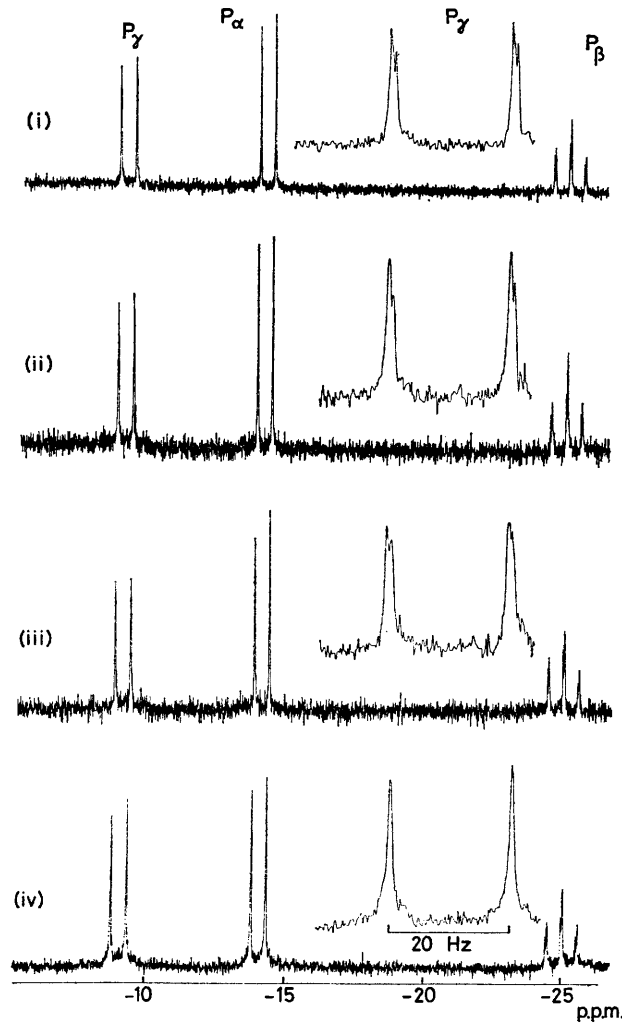


FIGURE 2. The ^{31}P n.m.r. spectra of [$^{18}O_3$]-ATP in diethanolamine hydrochloride buffer (100 mM, 1·6 ml, 50% D_2O, pH 9) containing ethylenediaminetetra-acetic acid (EDTA) (5 mM), after incubation with pyruvate kinase (3 mg) in triethanolamine hydrochloride buffer (2·5 ml, 100 mM, pH 7·6) containing potassium chloride (100 mM), magnesium acetate (15 mM), EDTA (1 mM), adenosine 5′[$\alpha\beta$-^{18}O,β-$^{18}O_2$]-triphosphate (6 mM, randomly but incompletely labelled),† di(adenosine-5′)pentaphosphate (0·4 mM), bovine serum albumin (5 mg), and (i) sodium pyruvate (50 mM), (ii) oxalic acid (5 mM), (iii) nothing further, and (iv) phosphoenolpyruvate (50 mM), NADH (4 mM) and lactate dehydrogenase (0·5 mg) for 80 min. For each spectrum, 10^4 transients were obtained with an offset of 1950 Hz, a bandwidth of 800 Hz, a memory of 8 K, a pulse repetition rate of 5·12 s (except for iv which was 2·56 s) and a pulse width of 15 μs, with broad band proton noise decoupling at 311 K with zero line broadening. The inset is that of the expanded P_γ resonance in each case.‡

† Two samples of adenosine 5′[$\alpha\beta$-^{18}O,β-$^{18}O_2$]-triphosphate were prepared, the first from randomly but incompletely labelled [^{18}O]-phosphate and the second from completely labelled [^{18}O]-phosphate. The first was used in those experiments giving [$^{18}O_3$]-ATP whose spectra are shown in Figure 2. In those experiments where partial ^{18}O-redistribution occurred (see Table) the completely labelled adenosine 5′[$\alpha\beta$-^{18}O,β-$^{18}O_2$]-triphosphate was used. This was done in order to obtain satisfactory resolution of the two sets of P_γ doublets. An isotope shift is only observed when ^{18}O is directly bonded to ^{31}P (G. Lowe and B. S. Sproat, *J.C.S. Chem. Comm.*, 1978, 565; M. Cohn and A. Hu, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 200).

‡ The spectrum in Figure 2i represents the complete *randomisation* of ^{18}O between P_β–O_2 and P_γ–OP_β since the concentrations were such that adenosine 5′[$\alpha\beta$-^{18}O,β-$^{18}O_2$]-triphosphate would be converted into [$^{18}O_3$]-ADP (free) and back to [$^{18}O_3$]-ATP with a half-life of *ca.* 4 min. [$^{18}O_3$]-ADP (free) can clearly achieve torsional symmetrisation of β-phosphoryl group. The similarity of the P_γ resonance in Figure 2i, ii, and iii indicates that complete *randomisation* of ^{18}O between P_β–O_2 and P_γ–OP_β occurs in each of these experiments.

TABLE. ^{18}O-Redistribution in adenosine 5′[$\alpha\beta$-^{18}O,β-$^{18}O_2$]-triphosphate (6 mM)† catalysed by pyruvate kinase in the presence and absence of pyruvate, oxalate, and phosphoenolpyruvate. All solutions contained triethanolamine hydrochloride (100 mM; pH 7·6), potassium chloride (100 mM), magnesium acetate (15 mM), ethylenediaminetetra-acetic acid (1 mM), di(adenosine-5′)pentaphosphate (0·4 mM), and bovine serum albumin and were incubated at 37 °C. k is the first order rate constant for ^{18}O-redistribution at equilibrium under conditions of enzyme saturation (*i.e.* maximum velocity).

Second substrate or inhibitor	Enzyme conc/μM	Substrate or inhibitor conc /mM	Incubation period/min.	^{18}O redistribution %	k/min^{-1}
Pyruvate	5·1	50	80	100	
	0·17	50	27	75	$2·0 \times 10^3$
	0·017	50	21	11	
Oxalate	5·1	5	80	100	
	0·17	5	27	11	$2·0 \times 10^2$
—	5·1	—	80	100	
	0·89	—	200	88	
	0·21	—	130	29	$1·1 \times 10^2$
	0·17	—	27	6	
Phosphoenolpyruvate	5·1	50	80	0	

of pyruvate, *and in the absence* of the second substrate or an analogue of it. It is conceivable that oxalate could become transiently phosphorylated by ATP in the presence of pyruvate kinase with the equilibrium favouring overwhelmingly oxalate and ATP, but the fact that the P_γ–OP_β bond of ATP is cleaved in the absence of the second substrate or an analogue of it, indicates that the dissociative mechanism must operate.

An investigation of the rate of ^{18}O redistribution between P_β–$^{18}O_2$ and P_γ–O–P_β was undertaken and the first order rate constants determined in the presence of pyruvate and oxalate and in their absence are shown in the Table. The 18 fold difference in the rate constant for ^{18}O redistribution in ATP in the presence and absence of pyruvate is most probably associated with the conformational change of the enzyme which is known to occur on binding pyruvate.[8] It seems very unlikely that this small change in rate constant is due to a change in mechanism. The evidence indicates therefore that pyruvate kinase catalyses phosphoryl transfer by a dissociative $S_N1(P)$ mechanism.

This is a contribution from the Oxford Enzyme Group supported by the S.R.C. The authors gratefully acknowledge the award of a S.R.C. grant.

(*Received, 2nd May* 1978; *Com.* 453.)

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