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MECHANISM OF PHOSPHORYL TRANSFER BY HEXOKINASE

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SUMMARY: Yeast hexokinase was incubated with [$\gamma^{18}O$]-ATP alone or with lyxose. The recovered ATP was found not to have undergone any significant transfer of ^{18}O from the $\beta\gamma$-bridge to the β-nonbridge position. These results are contrary to mechanisms in which the ATP is reversibly cleaved prior to transfer to give product. During hydrolysis of ATP stimulated by lyxose there was no mixing of the P_i formed with water. When glucose was present positional exchange was observed. However, its rate was consistent with earlier measurements of the partition of the enzyme·products complex between return to substrate and release of products and thus does not signify cleavage of the ATP by mechanisms other than direct phosphoryl transfer to glucose. This agreement indicates that rotational freedom of the βPO_3 of ADP on the enzyme·Glc-6-P·ADP complex is not a limiting factor for scrambling oxygens within the ternary complexes.

Positional exchange of oxygens of ATP provides a unique method (1-5) for detecting the process: $E + ATP + X \rightleftarrows E{\cdot}XP{\cdot}ADP$ where X may be a cosubstrate or a group on the enzyme. In a recent application of this method Lowe and Sproat (5) reported that the exchange of ^{18}O from the β-γ bridge position to the βP nonbridge location was catalyzed by pyruvate kinase in the absence of substrate, pyruvate, and stimulated by oxalate, an analogue of enolpyruvate that is not thought to be phosphorylated. Pyruvate kinase is not known to be phosphorylated in the course of catalysis and especially because, as is now known from studies of Knowles and coworkers (6, 7), the net transfer reaction must satisfy the stereochemistry of inversion. The positional exchange was taken as evidence for $E{\cdot}ADP{\cdot}PO_3^-$, a tightly complexed metaphosphate intermediate in phosphoryl transfer. This conclusion has profound implications for our understanding of the mechanism of all enzyme-catalyzed phosphoryl transfer reactions (8-11) and comes at a time of the elaboration by Satterthwaite and Westheimer (12) of the reality of methyl metaphosphate in solution and the demonstration of its extreme reactivity. A demonstration of the occurrence of positional exchange in other enzymatic-$PO_3^=$ transfer reactions known to go with net inversion is therefore called for.

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Yeast hexokinase seemed particularly promising to investigate in this way because in the absence of glucose it has significant ATPase activity that is stimulated by pseudosubstrates such as xylose and lyxose (13-15) and, in fact, with xylose a known slow phosphorylation of the enzyme by ATP (14, 15) may possibly be indicative of such an activated group as metaphosphate. Again the stereochemistry of the hexokinase reaction, inversion (6, 7), rules against a functional phosphorylated enzyme intermediate.

An additional reason for studying hexokinase in this way is that recent isotope trapping experiments (16) have provided information about the partition of the ternary products complex, E·Glc-6-P·ADP, between return to substrates and formation of products. Knowing this partition, one can better interpret the positional isotope exchange that might be measured in the presence of glucose.

MATERIALS AND METHODS

Yeast hexokinase, obtained from several commercial sources, was found to catalyze the positional exchange of oxygens of ATP even in the absence of sugar substrate. To establish if this activity was due to a contaminating enzyme the hexokinase activity was purified further on a DE-52 succinate column by elution with 5 mM Na succinate. The first peak of activity was used in the present studies. From its position and specific activity, 220 units/mg, the enzyme is designated isozyme PI (17).

ATP labeled uniformly with ^{18}O bound to the γP was prepared as before (1). The distribution of ^{18}O in the original ATP and in the ATP recovered after incubation with hexokinase was determined by either ^{31}P-NMR, Table 1, or mass spectral analysis, Tables 2 and 3. Each atom of ^{18}O attached to ^{31}P causes a 0.2 ppm upfield shift in the ^{31}P-NMR peak (18, 19). Exchange of a $\beta\gamma$-bridge ^{18}O with a nonbridge ^{16}O of the βP of ATP leads to a 0.2 ppm downfield shift in the distribution of peaks representing the $\gamma^{31}P$ of ATP. In the mass spectral analysis ATP is first incubated with acetate and acetyl CoA synthetase to randomize the β- and γ-positions of the ATP by reversible formation of Acetyl-AMP + PP_i. The γ-PO_3 of this interchanged ATP is then selectively hydrolyzed. Its analysis as trimethyl-phosphate represents 50% γPO_3 and 50% $-O_3P_\beta$, excluding the bridge atom. Positional exchange introduces bridge ^{18}O into the original $-^{16}O_3P_\beta$ two chances out of three and therefore causes an increase in the $^{18}O_1$ species at the expense of $^{18}O_0$ (1). No change in the original contribution from γ-PO_3 is expected if there is no exchange with water during reversible phosphoryl transfer.

RESULTS AND DISCUSSION

[$\gamma^{18}O$]-ATP was analyzed by the ^{31}P-NMR procedure of Cohn and Hu (18) before and after incubation with hexokinase in the absence of substrate, Table 1.

Table 1

Absence of Positional Exchange by Enzyme Alone

Enzyme	Relative NMR Peak Heights of $\gamma^{31}P$ of ATP $ATP\gamma PO_4$		
	$^{18}O_2$	$^{18}O_3$	$^{18}O_4$
None	20.0	42.2	38.0
11 Units	20.4	41.7	38.3

Hexokinase (11 units), triethanolamine-HCl, TEA-Cl (50 mM, pH 8.0), [$\gamma^{18}O$]ATP (10 μmoles), EDTA (0.5 mM) and $MgCl_2$ (15 mM) were incubated for 1 hour at 25°. EDTA to 20 mM was then added and the ^{31}P-NMR spectrum compared with the starting ATP. The peak height for 0 and 1 ^{18}O in the γ position was in the noise level and is ignored.

Formation of enzyme-bound ADP capable of returning to the ATP pool would have resulted in a 67% probable loss of the ^{18}O initially present in the βγ bridge position if the ADP had sufficient time to undergo torsional randomization of the three oxygens of the βPO_3 group. This would result in a decrease in the $^{18}O_4$ contribution from 38% to 13%, etc. in the limit that all of the ATP participated. No change in this direction was observed under conditions that, in the presence of glucose, would have used up all of the ATP in only a small fraction, ∿ 1/60, of the time. The method of analysis is certainly sensitive to a 5% change in relative peak height which would have been seen if ATP participated in the scrambling process at 0.3% of the capacity of the enzyme for catalysis with glucose. During the incubation ∿ 2% of the ATP was hydrolyzed so that the maximal scrambling rate is still slower than this reaction of the enzyme.

The failure to observe any positional rearrangement of the βγ-bridge oxygen of ATP might be ascribed to a requirement for a substrate to induce activation of ATP (18). Therefore the effect of lyxose, known to stimulate ATPase activity more than 10-fold, was of interest. Because at saturation with lyxose the ATPase rate is only ∿ 0.07% of V_{max} with glucose, phosphorylation of water could be the rate limiting step and any intermediate dependent on lyxose for its formation should give a significant rate of positional exchange.

As shown in Table 2 the presence of lyxose did not cause a significant change in the ^{18}O pattern of the ATP recovered after about 50% of the ATP was hydrolyzed to ADP + P_i. The bridge position of the starting ATP is about 76% enriched judging from the ^{18}O found in the control and the expectation that the γ-oxygens are labeled randomly in the original synthesis of the ATP from ^{18}O-P_i (1). Complete equilibration would therefore have increased the $^{18}O_1$ species and lowered the $^{18}O_0$ species by ∿ 2/3 x 76% = 50.7%. The predicted ratio of $^{18}O_1/^{18}O_3$ for fully scrambled ATP would be (17 + 50.7)/45.8 = 1.48. Analysis of the P_i formed in the reaction had the ratios $^{18}O_1/^{18}O_2/^{18}O_3$ = 15/37.5/45.8. The fact that this is not significantly different from the pattern of the $-P_\gamma O_3$ is consistent with the lack of scrambling at the β-position because washout of ^{18}O would have required reconstitution of ATP on the enzyme. The dissociation of products must be too rapid for this to occur significantly.

Only when glucose was present was scrambling found at the βP of ATP, Table 3. This could have been due to a special ATP-activation mechanism that requires glucose but does not give rise to glucose-6-P or to return of the ternary products complex, E·ADP·Glc-6-P, to ATP instead of to free products. This latter partition has been shown by a steady state isotope trapping technique (16) to be ∿ 0.5. Table 3 shows an experiment in which 25% of the ATP was consumed. The remaining ATP was analyzed after interchange of its β- and γ-positions and was found to have reached 13% of scrambling equilibrium, that is when 25% of the ATP had

Table 2
Failure of Lyxose to Activate Positional Exchange

Substrate (mM)*	ΔATP (mM)	Relative Peak Heights				
		$^{18}O_0$	$^{18}O_1$	$^{18}O_2$	$^{18}O_3$	$^{18}O_1/^{18}O_3$
Control	0	100	17	37.3	45.8	0.37
Lyxose (20)	-0.54	100	16.4	37.8	45.0	0.36

*[$\gamma^{18}O$]-ATP (1 μmol) in 1 ml was hydrolyzed by enzyme with lyxose to the extent of ∿ 54% in 35 m with 24 units enzyme, $MgCl_2$ (2.5 mM), TEA-Cl (pH 8.0, 50 mM) and EDTA (0.5 mM). The control lacked lyxose and enzyme. The recovered ATP was analyzed for scrambling (1).

Table 3

Scrambling during Glc-6-P Formation

	$^{18}O_1$	$^{18}O_2$	$^{18}O_3$	$^{18}O_1/^{18}O_3$
ATPγ-PO_3	8.15	35.7	56.1	0.14
ATP fully scrambled (predicted)	62.15	35.7	56.1	1.10
ATP reacted with glucose*	13.2	34.5	52.3	0.25

*[$\gamma^{18}O$]-ATP (4 mM), $MgCl_2$ (3 mM), Glc (3 mM), TPN^+ (2 mM) TEA-Cl (pH 8.0, 50 mM), Glc-6-P dehydrogenase (2.5 µg), hexokinase PI (0.04 units). The 1 ml incubation at 25° was allowed to produce 1 µm of TPNH in 22 min.

formed product 13% or about half of 25% had undergone positional exchange, in agreement with the partition expected from the ternary products complex (16). In this experiment Glc-6-P was kept low by its removal by the dehydrogenase and Mg^{2+} was kept below the ATP level in order to avoid recombination of liberated ADP and E·Glc-6-P present in the steady state (19).

The use of the positional exchange technique depends on the ability of the bound ADP of any intermediate complex to undergo the rotational motion of its -βPO_3 group necessary to equilibrate the oxygens (4). The fact that positional exchange occurred at the expected rate when glucose was present indicates that rotation is not limiting in the ternary products complex. If a highly reactive metaphosphate is produced the lifetime of the ADP may be short and rotation may occur only rarely. Therefore it was desirable to use large amounts of enzyme in the experiments without substrate and with lyxose. Assuming no rotational restraint the observation that glucose increased the rate of positional exchange more than 15,000 fold per unit of enzyme with or without lyxose is contrary to the stepwise mechanism of phosphoryl transfer as proposed for pyruvate kinase (5).

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References

1. Midelfort, C.F., and Rose, I.A. (1976) J. Biol. Chem. 251, 5881-5887.
2. Wimmer, M.J., Rose, I.A., Powers, S.G., and Meister, A. (1979) J. Biol. Chem 254, 1854-1859.
3. Wimmer, M.J., and Rose, I.A. (1977) J. Biol. Chem. 252, 6769-6775.
4. Rose, I.A. (1979) Adv. in Enzymol. 50, 361-395.
5. Lowe, G., and Sproat, B.S. (1978) J. Chem. Soc. Perkin I, 1622-1630.
6. Orr, G.A., Simon, J., Jones, S.R., Chin, G.J., and Knowles, J.R. (1978) Proc. Natl. Acad. Sci., U.S.A. 75, 2230-2233.
7. Blätter, W.A., and Knowles, J.R. (1979) J. Am. Chem. Soc. 101, 510-511.
8. Benkovic, S.J., and Schray, K.J. (1971) The Enzymes 8, 201-238.
9. Mildvan, A.S. (1979) Adv. in Enzymol. 49, 103-126.
10. Knowles, J.R., Ann. Rev. Biochem., in press.
11. Jencks, W.P. (1969) in Catalyses in Chemistry and Enzymology, McGraw-Hill, New York, New York, pp. 112-115.
12. Satterthwaite, A.C., and Westheimer, F.H. (1978) J. Am. Chem. Soc. 100, 3197-3203.
13. Dela Fuente, G., Lagunas, R., and Sols, A. (1970) Eur. J. Biochem. 16, 226-233.
14. Dela Fuente, G. (1970) Eur. J. Biochem. 16, 240-243.
15. Cheng, L.Y., Inagami, T., and Colowick, S.P. (1973) Fed. Proc. to Am. Soc. Exp. Biol. 32, 667.
16. Wilkinson, K.D., and Rose, I.A. (1979) J. Biol. Chem. 254, 12,567-12,572.
17. Colowick, S.P. (1973) The Enzymes 9, 1-48.
18. Koshland, D.E. (1958) Proc. Natl. Acad. Sci., U.S.A. 44, 98-104.
19. Kosow, D.P., and Rose, I.A. (1970) J. Biol. Chem. 245, 198-204.