SOME MECHANISMS OF CLEAVAGE OF ADENOSINE TRIPHOSPHATE AND 1,3-DIPHOSPHOGLYCERIC ACID*

by

MILDRED COHN**

Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Mo. (U.S.A.)

INTRODUCTION

In a previous communication from this laboratory[1], it was demonstrated that the mechanisms of enzymic reactions involving phosphate may be elucidated by studying such reactions with ^{18}O as exemplified by determining the bond cleavage of glucose-1-phosphate catalyzed by phosphatases and by muscle and sucrose phosphorylases. In these studies it was found that in the hydrolytic reactions, the O–P bond of glucose-1-phosphate was cleaved, in contrast to the phosphorylase reactions where the C–O bond was cleaved. Further work by KOSHLAND and co-workers on alkaline phosphatase[2] and other specific phosphatases[3,4] again demonstrated the cleavage of the O–P bond in these hydrolytic reactions. Thus the phosphatases may be considered as phosphoryl-transferring enzymes and the phosphorylases as glucosyl-transferring enzymes.

The present study was undertaken to investigate the generality of the dependence of bond cleavage on the type of reaction irrespective of substrate and enzyme. The cleavage point of two phosphate compounds in different types of enzymic reactions has been investigated; with ATP***, the hydrolysis catalyzed by the soluble Mg^{++}-activated ATPase of muscle and the phosphate transfers catalyzed by hexokinase, phosphoglycerate kinase and adenylate kinase. Two reactions of 1,3 diphosphoglyceric acid have been investigated, the reversible phosphorylation coupled to the oxidation of glyceraldehyde-3-phosphate catalyzed by the dehydrogenase and the transfer of phosphate to ADP catalyzed by the phosphokinase. BOYER and his co-workers[5,6,7] have also used this approach to study a wide variety of reactions including those of phosphokinases and phosphorylases.

* This work was supported in part by a grant from the National Science Foundation.

** Part of this work was done during the tenure of an Established Investigatorship of the American Heart Association.

*** The following abbreviations will be used in this paper: ATP = adenosine triphosphate. ATPase = adenosine triphosphatase. ADP = adenosine diphosphate. DPN = diphosphopyridine nucleotide. DPNH = reduced diphosphopyridine nucleotide. 3-PGA = 3 phosphoglyceric acid. TRIS = tris[hydroxymethyl] aminomethane. TCA = trichloroacetic acid. GDH = glyceraldehyde-3-phosphate dehydrogenase.

References p. 99.

MATERIALS AND METHODS

Enzymes

The yeast hexokinase preparation[8] containing 8.9 mg protein per ml, approximately 20% hexokinase, was kindly supplied by Dr. F. E. HUNTER. The intestinal phosphatase was a commercial preparation obtained from Armour Laboratories.

The muscle extract containing ATPase activity was obtained from rabbit skeletal muscle. The muscle was ground in a meat grinder, frozen and stored at —20° overnight. The ground muscle was then partially thawed and homogenized in the Waring Blendor with 3 volumes of ice-cold water for six minutes at 4°. The homogenate was centrifuged in the cold for 10 minutes at 13,000 r.p.m. in a Servall centrifuge (SS-1). The supernate was poured through cheesecloth and then dialyzed against 1% KCl for about 20 hours at 4°. This extract had no ATPase activity in the presence of added Ca^{++} and a small amount of activity without added Mg^{++}.

Crystalline aldolase was prepared from rabbit skeletal muscle by the method of TAYLOR, GREEN AND CORI[9] and crystalline glyceraldehyde-3-phosphate dehydrogenase (GDH) from rabbit skeletal muscle by the method of CORI, SLEIN AND CORI[10]. Crystalline phosphoglycerate kinase from yeast[11] was kindly supplied by Dr. W. VISHNIAC.

Substrates. DPN and DPNH approximately 90% pure were obtained from the Sigma Chemical Co. Disodium ATP was obtained from the Pabst Co. Inorganic orthophosphate labeled with ^{18}O was prepared as described previously[1].

Analysis of phosphate compounds

Inorganic orthophosphate was analysed by the method of FISKE AND SUBBAROW[12]. Labile phosphate of ADP and ATP were determined by analysis of the inorganic phosphate formed after hydrolysis for 10 minutes in 1 *N* HCl at 100°. Total phosphate was analyzed by the method of LE PAGE[13]. Glucose-6-phosphate was determined by enzymic assay with glucose-6-phosphate dehydrogenase[14] and fructose-6-phosphate was determined by the fructose method[15].

Isotopic analyses. Radioactivity measurements for ^{32}P were done on dried samples with an end-window Geiger counter. The ^{18}O determinations on inorganic phosphate were done by dehydration of KH_2PO_4[16] in all experiments except the ATPase experiment in which the determination was carried out by reduction of $(Ba)_3(PO_4)_2$ with carbon[17]. The ^{18}O concentration of the phosphate in hexose phosphate was determined on the inorganic phosphate obtained by hydrolysis with alkaline phosphatase as described for other organic phosphates[18]. The ^{18}O concentration of the labile phosphates of ADP and ATP were determined on the inorganic phosphate obtained by acid hydrolysis[17].

EXPERIMENTAL AND RESULTS

Hexokinase reaction

The reaction was carried out in a medium of $H_2{}^{18}O$ (concentration 1.80 atom per cent excess) in a total volume of 5.0 ml and contained: Tris buffer 0.3 *M*, pH 8.1; glucose 0.2 *M*; $MgCl_2$ 0.02 *M*; ATP 0.09 *M*; yeast hexokinase 0.3 ml (8.9 mg protein per ml, approximately 20% hexokinase). After one hour of incubation, the reaction was 93% complete as determined by analysis of 10-minute hydrolyzable phosphate. At the end of two hours, 0.1 ml of the reaction mixture was withdrawn for analysis of the ^{18}O content of the medium and the reaction was stopped by the addition of 1.5 ml of ice-cold 20% TCA. After centrifugation, the precipitate was discarded.

The products of the reaction in the TCA supernate were separated by barium fractionation. An excess of 25% barium acetate was added at pH 8.2 and the barium salt of ADP was allowed to precipitate for 30 minutes at 4°. The glucose-6-phosphate was precipitated from the supernate and washings of the ADP precipitate by the addition of ethyl alcohol to a final concentration of 66%. This procedure did not yield clean separations; each fraction thus obtained was therefore redissolved and fractionated in the same manner again. In the final sample of glucose-6-phosphate the yield was 46% and of the total phosphate, 3% analysed as hydrolyzable phosphate, 70% as glucose-6-phosphate and 20% as fructose-6-phosphate, indicating the

References p. 99.

presence of phosphohexose isomerase in the hexokinase preparation. The hexose phosphates were hydrolyzed enzymically and the phosphate was analyzed for ^{18}O.

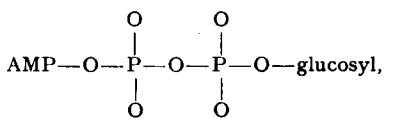
TABLE I

HEXOKINASE REACTION IN $H_2{}^{18}O$ (1.80 ATOM % EXCESS)

(conditions as in text)

	^{18}O concentration atom per cent excess	
	Found	*Calculated for 1 ^{18}O in phosphate*
Phosphate of glucose-6-phosphate	0.019	0.450
Terminal phosphate of ADP	0.015	0.450

Contamination of the ADP fraction by glucose-6-phosphate was irrelevant since only the terminal phosphate of ADP was obtained for ^{18}O analysis by hydrolysis in 1 *N* HCl for 10 minutes at 100°. The results of the ^{18}O analyses are given in Table I and within experimental error both glucose-6-phosphate and ADP contained no excess ^{18}O. It follows that the cleavage in the transfer of phosphate from ATP to glucose occurs between oxygen and the terminal P of ATP. Had the bond between oxygen and the penultimate P of ATP been cleaved, one oxygen from $H_2{}^{18}O$ would have appeared in the terminal phosphate of ADP. The possibility of a diester intermediate of the type suggested by BALDWIN[19] for the creatine kinase reaction and discussed by WHITTAM *et al.*[20] for the adenylate kinase reaction is also excluded by the results. Hydration of the intermediate, in this case



would necessitate the introduction of 1 oxygen from $H_2{}^{18}O$ either in ADP or glucose-6-phosphate.

ATPase of muscle extract

The results of two experiments on the hydrolysis of ATP in $H_2{}^{18}O$ are recorded in Table II. In experiment 1, the reaction mixture contained in a total volume of 3.0 ml: Tris buffer 0.05 *M*, pH 7.2, $MgCl_2$ 0.05 *M*, ATP 0.005 *M* and 0.6 ml muscle extract. The control reaction mixture contained 0.0025 *M* inorganic phosphate and no ATP. In experiment 2, the reaction mixture was the same except that the buffer was bicarbonate at pH 7.6. The control in this experiment contained 0.01 *M* inorganic phosphate. The reaction proceeded for 30 minutes at 37°. Both labile phosphates were hydrolysed, indicating the presence of adenylate kinase in the muscle extract. At the end of 30 minutes, the reaction was complete and 1 ml of 20% ice-cold TCA was added. The inorganic phosphate was separated from adenylic acid and the ^{18}O concentration of the inorganic phosphate was determined.

The results indicate that cleavage occurred between O and the terminal P of ATP in the hydrolysis since ^{18}O was found in the inorganic phosphate formed. It will

References p. 99.

be noted from Table II that some ^{18}O was found in the control inorganic phosphate, and somewhat more than 1 oxygen after correction for the control was introduced into the inorganic phosphate formed in the hydrolysis of ATP. This effect, though considerably smaller in magnitude, may be related to the observations of CLARKE AND KOSHLAND[3] on ATPase of lobster muscle. The small exchange of inorganic phosphate may be due to some residual metabolism although no lactic acid formation could be detected with this muscle extract without added glycogen.

TABLE II

HYDROLYSIS OF ATP BY MUSCLE EXTRACT

Experiment number	*Substrate*	*^{18}O concentration atom per cent excess*		*Number of O atoms introduced from H_2O*
		H_2O	*Inorganic phosphate*	
1	Inorganic phosphate	0.86	0.053	0.24
	ATP	0.87	0.303	1.38
2	Inorganic phosphate	0.91	0.072	0.32
	ATP	0.93	0.350	1.51

Glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase

The GDH reaction was studied under two different sets of conditions (1) equilibration of hexose diphosphate, DPN, inorganic phosphate (^{18}O) and their products catalyzed by aldolase and GDH and (2) exchange equilibration between inorganic phosphate ($^{32}P_i{}^{18}O$) and ATP in the presence of 3-PGA and DPNH catalyzed by 3-PGA kinase and GDH.

Experiment 1. The reaction mixture contained in a total volume of 3 ml: hexose diphosphate 0.021 *M*, inorganic phosphate (2.39 atom per cent excess ^{18}O) 0.02 *M*, pH 7.4, DPN $1 \cdot 10^{-4}$ *M*, cysteine 0.003 *M*, crystalline aldolase 1 mg, crystalline GDH 1 mg. Equilibrium was established in less than one half-minute as determined by the measurement of DPNH formation by absorption at 340 mμ on a Beckmann spectrophotometer. The reaction mixture was kept for half an hour at room temperature to attain isotopic equilibrium. The reaction was stopped by the addition of 1 ml ice-cold 20% TCA and the inorganic phosphate was isolated and its ^{18}O concentration determined. The results shown in Table I indicate that no dilution of ^{18}O occurred, indicating that in the formation of 1,3-diphosphoglyceric acid, a C–O bond is made and the same bond is ruptured in the reverse direction.

Experiment 2. The reaction mixture contained in a total volume of 4 ml: 3-PGA 0.0065 *M*, ATP 0.0065 *M*, inorganic phosphate (^{32}P 125,000 c.p.m., ^{18}O 2.60 atom per cent excess) 0.12 *M*, pH 7.4, DPNH 0.0006 *M*, $MgCl_2$ 0.006 *M*, crystalline 3-PGA kinase 0.178 mg, GDH, 3 times recrystallized from versene, 8 mg.

The high concentration of GDH, 2 mg per ml of reaction mixture was employed in order to obtain rapid equilibration of inorganic phosphate and ATP. This high concentration of the enzyme revealed contamination with adenylate kinase activity as evidenced by the exchange of the two labile phosphate groups of ATP with inorganic phosphate ^{32}P. Independent enzymic assay with hexokinase confirmed the presence of adenylate kinase in this preparation of crystalline GDH.

References p. 99.

Aliquots of the reaction mixture were withdrawn at 0, 5, 30 and 120 minutes for radioactivity measurements of inorganic phosphate and ATP. The inorganic phosphate and ATP were separated as described previously[17]; both labile phosphate groups of ATP were isolated together. After 5 minutes, the exchange between inorganic phosphate and ATP was 86% of the equilibrium value, after 30 minutes 97%. The inorganic phosphate and labile phosphates of ATP were isolated after 120 minutes and analysed for ^{18}O.

TABLE III

GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AND PHOSPHOGLYCERATE KINASE

(conditions described in text)

Experiment number	*Enzyme*	*^{18}O concentration atom per cent excess*					
		Inorganic phosphate			*ATP*		
		Initial	*Final*		*Initial*	*Final*	
						Found $\times \frac{4}{8}$	*Calculated*
1	GDH	2.39	2.43				
			Found	*Calculated*			
2	GDH + 3PGA kinase + adenylate kinase	2.60	1.14	1.24	0	1.31	1.24

The calculated values given in Table III are based on the equilibration of the 4 oxygens of inorganic phosphate with the 6 oxygens of the labile phosphate groups of ATP and 2 oxygens of the carboxyl group of 3-PGA. The introduction of oxygen from the medium in any of the reactions involved in the equilibrium would lead to lower concentrations of ^{18}O. If the bond between oxygen and the penultimate P of ATP rather than the terminal P had cleaved in the transfer from ATP to 3-PGA, one oxygen in the ADP formed would have been replaced by normal oxygen from the water and subsequently the ATP-^{18}O would have been similarly diluted by the adenylate kinase reaction. The same consequences would result if the transfer of phosphate from ATP in the adenylate kinase reaction itself had involved the cleavage of the bond between oxygen and the penultimate P of ATP. In the overall equilibrium, the oxygens become randomized in inorganic phosphate, so that if normal oxygen from water is introduced in any reaction in the sequence, eventually all the ^{18}O would be replaced by normal oxygen. The agreement between the calculated and experimentally observed values leads to the conclusion that in both the phosphoglycerate and adenylate kinase reactions, the bond between oxygen and the terminal phosphorus of ATP are cleaved. If attention is now focused on diphosphoglyceric acid it follows that the O–P bond is cleaved in the PGA kinase reaction whereas in the GDH reaction, the C–O bond of 1,3 diphosphoglyceric acid is cleaved. HARRISON, BOYER AND FALCONE[5] arrived at the same mechanism for GDH and phosphoglycerate kinase by establishing the presence of ^{18}O in the carboxyl group of 3-PGA formed from ^{18}O-labeled inorganic phosphate after the sequential reactions of GDH and 3-PGA kinase.

References p. 99.

DISCUSSION

The reactions investigated in this study fall into two groups. The first group, which involves a rupture of the O–P bond, includes the phosphokinases—hexokinase, phosphoglycerate kinase, adenylate kinase and the phosphatase, ATPase. Other phosphokinase and phosphatase reactions which have been studied conform to the same mechanism including pyruvate kinase[5], intestinal phosphatase[1,2], prostatic phosphatase[1], myosin ATPase[3], and, 5′ nucleotidase[4]. Thus the hydrolytic and kinase reactions may be considered phosphoryl-transferring enzyme reactions which occur by a nucleophilic attack on the phosphorus either by the enzyme or the acceptor. The plausibility of this mechanism for the kinase type of reaction in which a phosphate bond is both formed and broken and the analogy to non-enzymic phosphate transfers has been fully discussed by KOSHLAND[21].

The second group which involves the formation and rupture of a C–O bond includes GDH as found in this study and by HARRISON, BOYER AND FALCONE[5], muscle and sucrose phosphorylases[1] and citrulline phosphorylase[7]. In these reactions it is an acyl or glucosyl bond which is both formed and broken and the reactions may be considered transfers of groups other than phosphate in contrast to the phosphatases and phosphokinases.

Analogous to the two types of cleavage which glucose-1-phosphate can undergo, as a glucosyl donor to form a glycosidic linkage or as a phosphate donor, 1-3 diphosphoglyceric acid can also be cleaved in two ways, between the C and O as an acyl donor to form an acyl-enzyme with GDH, or between O and P as a phosphate donor to form ATP with ADP. It will be noted that it is in the formation of organic phosphates from inorganic phosphate that a C–O bond is formed. When 1-3 diphosphoglyceric acid is formed from ^{18}O-labeled inorganic phosphate, the oxygen in the bridge between carbon and phosphorus will be labeled with ^{18}O. When this reaction is followed by a transfer of PO_3 by the kinase reaction, the 3-PGA formed contains ^{18}O in the carboxyl group. STULBERG AND BOYER[7] found that the CO_2 formed by the reaction of citrulline and ^{18}O-labeled inorganic phosphate, contained ^{18}O and suggested a carboxyphosphate intermediate; subsequently JONES, SPECTOR AND LIPMANN[22] did indeed establish carbamyl phosphate as the intermediate in this reaction. Carbamyl phosphate like glucose-1-phosphate and 1-3 diphosphoglyceric acid can undergo both types of cleavage, on the one hand as a carbamyl donor to ornithine and on the other hand as a phosphate donor to ADP.

There are other reactions which involve the conversion of inorganic phosphate to organic phosphate which are formally similar to the reactions discussed above but no phosphorylated intermediates have yet been isolated. In the formation of ATP from inorganic phosphate coupled to the substrate level oxidation of α-ketoglutarate to succinate in mitochondria, it was observed[23] that the oxygen of inorganic phosphate appeared in the carboxyl group of succinic acid. When the purified enzymes became available, the phosphorylation of succinyl-CoA was investigated[24] with ^{18}O-labelled inorganic phosphate and it was found again that the oxygen of inorganic phosphate appeared in the carboxyl group of succinate; in the reverse direction, ^{18}O-labelled succinate in the presence of ATP and CoA gave rise to ^{18}O in the inorganic phosphate. It would be tempting to suggest succinyl phosphate as the intermediate in this reaction by analogy with 1,3 diphosphoglyceric acid and carbamyl phosphate.

References p. 99.

However, from an extensive study of the phosphorylation of succinyl-CoA, KAUFMAN[25] concludes that succinyl phosphate is not the intermediate and suggests that an enzyme-bound CoA phosphate is the intermediate. Should CoA phosphate prove to be the primary product of the phosphorylation of succinyl-CoA, we are confronted with an unusual and unlikely mechanism involving the simultaneous formation of a C–O bond in succinate with one O of the inorganic phosphate and of an S–P bond in CoA-phosphate with the residual PO_3 of inorganic phosphate. On the basis of the ^{18}O studies in the glutamine synthetase reaction[6] where the oxygen of the carboxyl group is found in inorganic phosphate upon reaction of glutamic acid with ATP and ammonia, again an acyl phosphate intermediate might be anticipated but no direct evidence for such an intermediate has been found.

In general it may be stated that in all reactions studied in which inorganic phosphate reacts with an acyl compound and ADP, the oxygen of the inorganic phosphate is found in the carboxyl group of the acid formed. Only in those reactions where two separate enzymic steps (1) phosphorolysis and (2) a transfer of phosphate have been identified, has the intermediate formation of acyl phosphates been established, namely 1-3 diphosphoglyceric acid and carbamyl phosphate. The phosphorolysis reaction for these acyl compounds is similar in mechanism to the phosphorolysis of glucosyl compounds in the muscle and sucrose phosphorylase reactions.

ACKNOWLEDGEMENTS

The author wishes to acknowledge the collaboration of Dr. J. HARTING in studying the ^{32}P equilibration between inorganic phosphate and ATP with glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase. The author also wishes to thank Dr. I. DOSTROVSKY for a gift of $H_2{}^{18}O$ used in some of the experiments.

SUMMARY

The cleavage of ATP has been investigated with ^{18}O in several phosphokinase reactions, namely yeast hexokinase, phosphoglycerate kinase, and adenylate kinase, and in the hydrolysis of ATP catalyzed by a Mg^{++}-activated ATPase in muscle extract. In all these reactions, the bond was cleaved between oxygen and the terminal P of ATP. The cleavage of 1,3-diphosphoglyceric acid was investigated in the glyceraldehyde-3-phosphate dehydrogenase reaction and the phosphoglycerate kinase reaction. In the first reaction, the C–O bond was cleaved and in the second reaction the O–P bond was cleaved. The mechanism of these reactions is discussed in relation to the behavior of 1,3 diphosphoglyceric acid as an acyl or phosphate donor. Comparison with other reactions reveals a general pattern in analogous reactions.

ADDENDUM*

It was pointed out to the author by Dr. W. BARTLEY that the results of the ATPase experiments were ambiguous due to the fact that both labile phosphates had been hydrolyzed. It is not possible to distinguish between the alternatives: (1) all the inorganic phosphate contains 1 ^{18}O atom, and (2) half the inorganic phosphate contains no ^{18}O and half contains 2 ^{18}O atoms. To eliminate the second alternative, the experiment was repeated and only 50% of the ATP was hydrolyzed; the inorganic phosphate contained 1 atom ^{18}O and the ADP contained no ^{18}O, thus establishing unequivocally that cleavage had occurred between the terminal P and the bridge O.

* Added in proof.

References p. 99.

REFERENCES

1 M. COHN, *J. Biol. Chem.*, 180 (1949) 771.
2 S. S. STEIN AND D. E. KOSHLAND, Jr., *Arch. Biochem.*, 39 (1952) 229.
3 E. CLARKE AND D. E. KOSHLAND, Jr., *Nature*, 171 (1953) 1223.
4 D. E. KOSHLAND, Jr., *Commun. 3ème Congr. Intern. Biochimie*, (1955) p. 38.
5 W. H. HARRISON, P. D. BOYER AND A. B. FALCONE, *J. Biol. Chem.*, 215 (1955) 303.
6 P. D. BOYER, O. J. KOEPPE, W. W. LUCHSINGER AND A. B. FALCONE, *Federation Proc.*, 14 (1955) 185.
7 M. P. STULBERG AND P. D. BOYER, *J. Am. Chem. Soc.*, 76 (1954) 5569.
8 D. H. BROWN, unpublished method.
9 J. TAYLOR, A. GREEN AND G. T. CORI, *J. Biol. Chem.*, 73 (1948) 591.
10 G. T. CORI, M. W. SLEIN AND C. F. CORI, *J. Biol. Chem.*, 173 (1948) 605.
11 T. BÜCHER, *Biochem. Biophys. Acta*, 1 (1947) 292.
12 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
13 G. A. LE PAGE in W. W. UMBREIT, R. H. BURRIS AND J. B. STAUFFER, *Manometric Techniques and Tissue Metabolism*, Burgess Pub. Co., Minneapolis, 1949, p. 190.
14 A. KORNBERG, *J. Biol. Chem.*, 182 (1950) 805.
15 J. H. ROE, J. H. EPSTEIN AND N. P. GOLDSTEIN, *J. Biol. Chem.*, 178 (1949) 839.
16 M. COHN, *J. Biol. Chim.*, 201 (1953) 735.
17 M. COHN AND G. R. DRYSDALE, *J. Biol. Chem.*, 216 (1955).
18 D. LIPKIN, P. T. TALBERT AND M. COHN, *J. Am. Chem. Soc.*, 76 (1954) 2871.
19 E. BALDWIN, *Dynamic Aspects of Biochemistry*, Cambridge University Press, Cambridge, 1947, p. 156.
20 R. WHITTAM, W. BARTLEY AND G. WEBER, *Biochem. J.*, 59 (1955) 590.
21 D. E. KOSHLAND, Jr. in W. D. MCELROY AND B. GLASS, *The Mechanism of Enzyme Action*, Johns Hopkins Press, Baltimore, 1954, p. 630.
22 M. E. JONES, L. SPECTOR AND F. LIPMANN, *J. Am. Chem. Soc.*, 77 (1955) 819.
23 M. COHN, in W. D. MCELROY AND B. GLASS, *Phosphorus Metabolism*, Johns Hopkins Press, Baltimore, Vol. I, 1951, p. 374.
24 G. R. DRYSDALE AND M. COHN, unpublished experiments.
25 S. KAUFMAN, *J. Biol. Chem.*, 216 (1955) 153.

Received November 5th, 1955