The Mechanism of Hydrolysis of Adenosine Di- and Tri-phosphate Catalysed by Potato Apyrase

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Under suitable conditions, phosphatases generally catalyse phosphate-transfer reactions (Axelrod, 1956). When these enzymes catalyse the hydrolysis of compounds with an oxygen bridge it has been experimentally demonstrated with ^{18}O that cleavage occurs between P and O, as would be anticipated from the fact that they are phosphoryl-transferring enzymes. By this criterion, adenosine triphosphatases, which have been shown to catalyse the cleavage of the bond between O and the terminal P (Clarke & Koshland, 1953; Cohn, 1956), may be classified as phosphoryl-transferring enzymes.

In the present work, adenosine diphosphate (ADP) and adenosine triphosphate (ATP) have been hydrolysed in H_2^{18}O with a potato-apyrase preparation to determine which bond is cleaved and consequently whether the enzyme may act as a phosphoryl-transferring enzyme or as an adenyl- and adenosine diphosphoryl-transferring enzyme. The effect of calcium ions on the reactions has also been investigated.

EXPERIMENTAL

Materials. ATP in the form of the crystalline disodium salt was obtained from Pabst Co. and contained less than 1% of orthophosphate. ADP sodium salt was obtained from Sigma Chemical Co. and contained approximately 10% of orthophosphate. The charcoal (Norit SX 30 Special) used for the adsorption of adenosine phosphates was ob-

tained from Harrington Bros., London. H_2^{18}O of 1.7 and 3.1 atom % excess was supplied by the Atomic Energy Research Establishment, Harwell. The potato-apyrase preparation, which was kindly supplied by Dr W. Bartley, had been prepared by the method of Lee & Eiler (1951), and had been stored for several months at -18° (Whittam, Bartley & Weber, 1955). All other chemicals were of AnalaR standard.

Experimental procedures. In the experiments in the rates of hydrolysis, the appropriate substrate was incubated with the enzyme preparation in the presence and absence of Ca^{2+} ions under conditions shown in Fig. 1. Samples of the reaction mixture were removed at various times and the reaction was stopped by the addition of enough 7.5% (w/v) trichloroacetic acid (TCA) to yield a final concentration of 5%. After centrifuging, the supernatant was analysed for liberated orthophosphate.

The hydrolysis of ADP was compared under the same conditions (1) with no additions, (2) in 0.0025M-ethylene-diaminetetra-acetate (EDTA) and (3) with 0.005M- CaCl_2 . The rates were approximately the same with EDTA and no additions and considerably higher with CaCl_2 .

In the series of hydrolysis experiments in H_2^{18}O , the reaction components were added in a manner designed to minimize dilution of ^{18}O . Each tube contained 0.2 ml. of M-sodium succinate buffer made up in H_2^{18}O ; all other components were added as solids, except 25 μl . of KOH solution used for neutralization and the amounts of enzyme preparation indicated in Table 1. Enough H_2^{18}O was added in each tube to bring the final volume to 4 ml. Each hydrolysis was allowed to proceed for a sufficient time to yield enough orthophosphate for ^{18}O analysis but not to exceed

Table 1. *Hydrolysis of ATP and ADP in H_2^{18}O*

Reaction mixtures nos. 1-4 had a total volume of 4 ml.; no. 5, 2 ml. All contained sodium succinate buffer, pH 6.5 (0.05M), and enzyme preparation, 0.1 ml./ml. of reaction mixture except no. 2, which had 0.05 ml./ml. of reaction mixture. Temp., 40° .

No.	Substrate	Time (min.)	Hydrolysis of terminal P (%)	^{18}O concn. (atom % excess)		
				H_2O recovered from reaction mixture	Inorganic phosphate	Calc. O/P _{terminal} *
1	ATP (0.017M)	40	70.5	1.13	0.305	0.283
2	ATP (0.016M) + CaCl_2 (~0.013M)	10	62.0	1.17	0.315	0.293
3	ADP (0.011M)	240	73	2.63	0.667	0.665
4	ADP (0.011M) + CaCl_2 (~0.012M)	180	> 90	2.60	0.669	0.650
5	KH_2PO_4	240	—	2.53	0.0	—

* The calculated value is equal to one-quarter of the atom % excess in the water of the reaction mixture.

1 mole of orthophosphate/mole of substrate. The duration of each hydrolysis and the amount of orthophosphate liberated is listed in Table 1. At the end of the experiment, 0.1 ml. samples of each reaction mixture were withdrawn and reserved for ^{18}O analysis of the water in the medium.

In the hydrolysis of ATP (Exps. 1 and 2, Table 1), the reaction was stopped by the addition of 1.33 ml. of 20% (w/v) TCA. After centrifuging, the supernatants were analysed for orthophosphate and 10 min.-hydrolysable phosphate to determine the amount of hydrolysis. Charcoal (1.2 g.) which had been acid-washed (Lipkin, Talbert & Cohn, 1954) and tested for its capacity to adsorb nucleotides, was added to each supernatant to remove the nucleotides. The inorganic orthophosphate was precipitated as the barium salt and subsequently isolated as the monopotassium salt for ^{18}O analysis as described by Cohn (1953).

In the experiment with higher concentrations of H_2^{18}O (nos. 3, 4 and 5, Table 1), the reaction was stopped by heating in a stoppered tube in a boiling-water bath for 2 min. The enriched water was recovered by vacuum distillation at room temperature. The residue was suspended in 2 ml. of 5% (w/v) TCA and centrifuged. The precipitate was washed with 1 ml. of 5% (w/v) TCA and the washing was added to the supernatant. The inorganic orthophosphate was precipitated as the magnesium ammonium salt and subsequently as the monopotassium salt (Cohn, 1953).

Analytical methods. Inorganic orthophosphate was analysed by the Fiske-Subbarow method (1925). The water and orthophosphate were analysed in the form of CO_2 for ^{18}O as described by Cohn (1953). The isotope ratios were determined on a mass spectrometer of the type described by Graham, Harkness & Thode (1947).

RESULTS

Effect of calcium ions on the rate of hydrolysis

As shown in Fig. 1, under the conditions specified, the rate of ATP hydrolysis is increased six- to seven-fold by CaCl_2 and that of ADP approximately threefold. Other investigators, e.g. Krishnan (1949), have found similar stimulatory effects of calcium. Nguyen-Van Thoai, Roche & Tran-Thi (1954) prepared a purified form of the enzyme and found no effect of Ca^{2+} , Mg^{2+} or Mn^{2+} ions over a concentration range of 10^{-2} to 10^{-4}M . One interpretation of the discrepancy is that the potato-apyrase preparation of Lee & Eiler (1951) used in the present investigation might be a mixture of enzymes, including one stimulated by Ca^{2+} ions, which has been removed in the purification procedure of Nguyen-Van Thoai *et al.* (1954). However, both preparations exhibit the same relationship to substrates, namely that no ADP is hydrolysed until all the ATP has been converted into ADP (cf. Whittam *et al.* 1955). The negative effect of bivalent ions found by Nguyen-Van Thoai *et al.* (1954) with the purified enzyme may be misleading in view of the fact that the assay system contained approximately 0.002M -ATP and 0.1M -histidine buffer; the latter may be responsible for binding all the calcium in the reaction mixture. The enzyme preparation

was assayed for its hydrolytic activity with adenylic acid (AMP) as substrate. With 0.1 ml. of enzyme preparation/ml. of reaction mixture and in the presence of 0.005M - CaCl_2 and 0.025M -AMP, 7 and 30% hydrolysis occurred in 1 and 4 hr. respectively.

Hydrolysis in H_2^{18}O

From the ^{18}O content of the orthophosphates liberated in the hydrolyses as shown in Table 1, it is apparent that one oxygen of the four was incorporated from the medium. The cleavage of ATP and ADP with or without Ca^{2+} ions always occurs between O and the terminal P. Whether the potato-apyrase preparation is a single enzyme or a mixture of enzymes, the cleavage of the same bond is catalysed. The orthophosphate formed in experiments 3 and 4, which proceeded for 4 and 3 hr. respectively, was probably in part liberated from adenylic acid; the experiments thus indicate that the phosphatase contaminating the preparation catalyses the cleavage of the bond between O and P in adenylic acid. The control with KH_2PO_4 (Expt. 5) indicates that the preparation does not catalyse any significant exchange of orthophosphate and water.

Fig. 1. Rates of hydrolysis of ADP and ATP with and without CaCl_2 . The reaction mixtures contained, in a total volume of 2 ml., 0.1 ml. of enzyme preparation and final concentrations of sodium succinate buffer, pH 6.5, 0.05M ; ATP (0.008M) or ADP (0.006M); CaCl_2 (0.01M). Temp., 40° . Orthophosphate values at 2 hr. are somewhat high owing to hydrolysis of AMP.

DISCUSSION

Many hydrolytic enzymes, including the phosphatases, catalyse group-transfer reactions so that hydrolysis may be considered a particular case of the general reaction when water is the acceptor. A number of non-hydrolytic enzymes has been described which catalyse adenyl transfer rather than phosphoryl transfer (1) from ADP, polynucleotide phosphorylase (Grunberg-Manago & Ochoa, 1955); (2) from ATP, acetate activation (Berg, 1955), pantoate activation (Maas & Novelli, 1953), amino acid activation (Hoagland, 1954) and others. The most direct method for ascertaining which group is being transferred in a hydrolytic reaction is the determination of the site of bond cleavage by hydrolysis in $H_2^{18}O$ (for review, see Axelrod, 1956). In the hydrolysis of the terminal phosphate of ATP or ADP, a P-O bond is broken when cleavage occurs on either side of the oxygen. However, the two alternative types of enzymic catalysis are basically different, in mechanism and in function; in one a phosphoryl group is transferred and in the other an adenosine diphosphoryl or adenyl group is transferred. The enzymes from muscle catalysing the hydrolysis of ATP have been shown to effect the cleavage of the bond between the terminal P and O (Clarke & Koshland, 1953; Cohn, 1956); ATP may be considered as a donor of phosphoryl groups in these reactions.

ATP may be either a donor of phosphoryl groups, as in kinase reactions, or a donor of adenyl groups, as in the acetate-activation reaction. In the former group of reactions, the bond between the terminal P and O is ruptured (Harrison, Boyer & Falcione, 1955; Cohn, 1956) and in the latter, the bond between the P of the adenyl moiety and O is cleaved (Boyer, Koeppe & Luchsinger, 1956). There are no known reactions in which ATP acts as a donor of adenosine diphosphoryl groups.

There are few reactions in which ADP acts as a donor. In the adenylate kinase reaction, ADP is a donor of phosphoryl groups as indicated by ^{18}O experiments (Cohn, 1956). In the polynucleotide phosphorylase reaction (Grunberg-Manago & Ochoa, 1955), ADP appears to act as a donor of adenyl groups, although the detailed mechanism of this reaction has not yet been elucidated.

From the present study it is clear that the potato-apyrase preparation which catalyses the hydrolysis of ATP and ADP can catalyse solely the transfer of phosphoryl groups from these substrates. Furthermore the catalysis by Ca^{2+} ions does not change the mechanism of the reaction with regard to the site of bond cleavage.

SUMMARY

1. The hydrolysis of ATP and ADP catalysed by a potato-apyrase preparation has been investigated in $H_2^{18}O$. It was found that the cleavage occurs between O and the terminal P of either substrate both in the presence and absence of calcium ions.

2. The implications of these findings for the mechanisms of these reactions is discussed.

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