Stereochemistry of the Mammalian Adenylate Cyclase Reaction*

(Received for publication, February 23, 1981, and in revised form, May 7, 1981)

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Adenosine 5'-O-(1-thiotriphosphate), $S_\alpha$-diastereomer, is cyclized by adenylate cyclase from bovine brain to adenosine 3',5'-cyclic phosphorothioate, $P_\alpha$ diastereomer, establishing inversion of configuration for this reaction. This result can most easily be explained by a direct nucleophilic attack of the 3'-OH group on $\alpha$-phosphorus without involving a covalent enzyme intermediate.

Since the discovery of cAMP as second messenger, the isolation and characterization of the enzyme which catalyzes its formation from ATP, adenylate cyclase, has been of considerable interest. Although the influence of hormone, Ca$^{2+}$, and GTP on the activity of mammalian adenylate cyclase has been studied extensively (1), little is known about the mechanism of the cyclization reaction itself. In particular, it is not known whether this reaction involves a covalent enzyme intermediate. A detailed description of the reaction mechanism has not been possible because the enzyme preparations are not homogeneous, and studies which might shed some light on that question would not be unambiguous in their interpretation. This also applies to the possible isolation of such an intermediate. As has been outlined in several reviews (2-4), for phosphoryl and nucleotidyl transfer reactions, this question can be answered by analyzing the stereochemical course of the reaction by using either chiral phosphorothioate analogues of ATP or isotopically labeled ATP. One of the advantages of this stereochemical analysis is that it can be performed on impure enzyme preparations, since it relies on the configurational analysis of the isolated product. A one-step reaction is expected to result in inversion of configuration, whereas a two-step reaction, proceeding via a covalent enzyme intermediate, would result in retention of configuration of the particular phosphorus where the reaction occurs.

In the study reported here, we have employed the diastereomers of ATPaS$^1$ (5) as substrates for adenylate cyclase and have analyzed the absolute configuration of the product, cAMPS (6). The results indicate that the reaction proceeds with inversion of configuration.

**MATERIALS AND METHODS**

Preparation of Adenylate Cyclase—Membranes were prepared by the method of Westcott et al. (7). Typically, 400 ml of membranes were solubilized in buffer containing 20 mM Tris, 250 mM sucrose, 1 mM MgCl$_2$, 1 mM EDTA, 1% Lubrol PX, pH 7.4, at a d$_{173}$/protein ratio of 2.5:1 (w/w). The mixture was stirred for 90 min, centrifuged for 2 h at 10,000 $\times$ g, and the supernatant solution containing the solubilized enzyme was decanted. DEAE-Sepharose was equilibrated in 50 mM Tris, 250 mM sucrose, 1 mM MgCl$_2$, 1 mM EDTA, 1% Lubrol PX, pH 7.4, and mixed with the solubilized enzyme using 2 liters of DEAE and 5 liters of detergent extract. The mixture was stirred for 90 min, the resin was collected on a sintered glass funnel, washed with 6 liters of wash buffer identical to the equilibration buffer with 50 mM KCl added, and poured into a column (8.8 cm x 35 cm). The column was eluted with a 150-600 mM linear KCl gradient with all other buffer components identical to the DEAE-equilibration buffer.

Fractions were collected and assayed for enzyme activity and protein. Fractions containing enzyme activity were pooled, so as to avoid fractions with conductivities greater than that of buffer containing 250 mM KCl. Such fractions would be contaminated with endogenous calmodulin that would interfere with subsequent affinity chromatography using calmodulin Sepharose. Material pooled from the DEAE-column was passed through a BioGel P-4 column equilibrated in 50 mM Tris, 250 mM sucrose, 2 mM CaCl$_2$, 0.1% Lubrol PX, 1 mM diethanol, pH 7.4, to remove KCl. Calmodulin Sepharose was equilibrated in the same buffer used for the BioGel P-4 column and mixed with the material pooled from the P-4 desalting column using 100 ml of resin and 400 ml of the desalted pool. The mixture was rotated slowly end over end for 8 h, poured into a column (2.4 cm x 30 cm) and washed with 200 ml of 50 mM Tris, 250 mM sucrose, 0.1 mM CaCl$_2$, 0.1% Lubrol PX, 1 mM diethanol, pH 7.4, and eluted with the same buffer containing 2 mM EDTA in place of CaCl$_2$. Fractions were assayed for enzyme activity and protein, pooled on the basis of specific activity, frozen in liquid nitrogen, and stored at -70 C. The specific activity of this pool was typically 15 nmol of cAMP·mg protein$^{-1}$·min$^{-1}$, representing a purification of approximately 60-fold over the specific activity of washed membranes. All operations were carried out at 4 C.

Adenylate Cyclase Assay—Adenylate cyclase was assayed by the method of Salomon et al. (8). Assays contained 50 mM Tris·HCl, pH 7.4, 1 mM EDTA, 10 mM MgCl$_2$, 1 mM 2-mercaptoethanol, 0.1% bovine serum album, 5 mM theophylline, 1 mM (e$^-32$)ATP as substrate with specific activity of 100 cpm/pmol, an ATP-regenerating system consisting of 40 mM creatine phosphate, 240 units/ml of creatine phosphokinase, and 1 mM (H$^-32$)cAMP (40 cpm/pmol) to monitor recovery of product. Protein determination was by the method of Peterson (6), with bovine serum albumin as a standard. K$_I$ values for the two diastereomers of ATPaS were determined under assay conditions for the cyclase in the presence of 150 μM guanosine 5'-O-(3-thiotriphosphate) without theophylline at two inhibitor and varying ATP concentrations. Calmodulin-sensitive cyclic phosphodiesterase was a gift of Dr. C. Keller, Seattle. The $S_\alpha$ diastereomer of $[^32]$ATPaS was synthesized from $[^32]$SAMPS by a published procedure (10) with myokinase and pyruvate kinase. The $P_\alpha$ diastereomer was synthesized from $[^32]$ATPaS, which was enriched in the $P_\alpha$ isomer by the use of creatine kinase (6, 11).

The diastereomers of cAMPS were synthesized as described (8). In addition, the $P_\alpha$ diastereomer was prepared by an alternative method (12). 5'-Guanylyl imidodiphosphate was purchased from Sigma; guanosine 5'-O-(3-thiotriphosphate) from Boehringer, Mannheim (Germany), and was purified by chromatography on DEAE-Sepharose.
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Fig. 1. High performance liquid chromatography trace of analysis of \[^{35}S\]cAMPs isolated by DEAE-Sephadex chromatography from cyclase reaction in presence of Mn". Fractions were collected for 0.5 min after having passed through the uv detector, and counted for radioactivity in a liquid scintillation counter. A, AMPS; B, cAMPs, R\(_S\) isomer; C, cAMPs, S\(_S\) isomer.

Thin layer chromatography on cellulose plates with 2-propanol-NH\(_2\)H\(_2\)O (7:3:2, v/v) as solvents was used to monitor the phosphodiesterase reaction. High performance liquid chromatography to identify cAMPs was performed on a Waters Associates chromatograph equipped with a Model 440 absorbance detector operating at 254 nm, using the strong anion exchanger Nucleosil 10 S B (Machery and Nagel, Duren, Germany) and 0.3 M KCl, 0.05 M KH\(_2\)PO\(_4\), pH 4.5 as eluent.

RESULTS

The first experiments were designed to establish whether either of the two isomers of ATPaS was a substrate for brain adenylate cyclase. The assays (total volume, 50 \(\mu\)l) contained 1 mm ATP or ATPaS, 10 mm MnCl\(_2\), 20 mm Tris HCl, pH 7.4, 1 mm EDTA, 15 \(\mu\)m 5'-guanylyl imidodiphosphate, 5 mm theophylline, 1 mm 2-mercaptoethanol, 50 \(\mu\)g of enzyme, and were incubated at 30 °C. The results indicated that only the ATPaS \(S_S\) diastereomer was a substrate. It was cyclized at approximately 15% of the rate of ATP in the presence of Mn" but only at about 1% in the presence of Mg" at 5 mm. When Co" (4 mm) was used as metal ion, the rate of cyclization of ATP in the presence of 5'-guanylyl imidodiphosphate, was only 4% of that seen with Mn", and no activity could be observed in the presence of Cd" (0.08–15 mm). No activity could be seen with either isomer of ATPaS with either Co" or Cd".

For the isolation of products, the total reaction volume was 600 \(\mu\)l and the incubation time was increased to approximately 20 h. The reaction mixture was chromatographed on a column (0.9 x 20 cm) of DEAE-Sephadex with a linear gradient of 500 ml each of water and 0.5 M triethylammonium bicarbonate. With Mn", the recovery of \(^{35}\)S label was 66%, which was distributed over cAMPs (13%), unreacted ATPaS (43%), ADPaaS (22%), and AMPs (16%). This result was obtained in the presence as well as absence of theophylline.

The absolute configuration of the cAMPs produced was analyzed by high performance liquid chromatography, where the \(R_R\) and \(S_S\) diastereomers can be separated (Fig. 1). As markers, a mixture of the chemically synthesized diastereomers (6) as well as unlabeled AMPs was coaxied with the labeled material to be analyzed. All the radioactivity in the product was associated with the \(R_R\) diastereomer of cAMPs.

\(K_m\) values of 20 \(\mu\)m for the \(S_S\) and of 15 \(\mu\)m for the \(R_R\) isomer of ATPaS were obtained. Under identical conditions, the \(K_m\) value for ATP was 80 \(\mu\)m.

DISCUSSION

Mammalian adenylate cyclase is an enzyme of great interest (1). In an attempt to determine whether a covalent enzyme intermediate is involved in the mechanism of the enzymic cyclization, we have employed the \(S_S\) and \(R_R\) diastereomers of \[^{35}S\]ATPaS to determine the stereochemical course of the reaction. In the presence of Mg" as well as Mn", only the \(S_S\) diastereomer was cyclized to cAMPs. With Mn" as metal ion, the rate of cyclization was 10–15% of that of ATP, whereas with Mg" it was only 1%. While there are insufficient data to explain this difference, we propose that it may indicate that different steps in the process may be rate-limiting in the presence of different ions.

To investigate whether the inactivity of the \(R_R\) isomer was the result of nonbinding to the enzyme, the \(K_m\) values of both isomers were determined. They are 15 and 20 \(\mu\)m, respectively, and thus indicate that both diastereomers of ATPaS are bound to the active site of the cyclase with approximately equal affinity, but binding of the \(R_R\) isomer is unproductive. This result indicates that the \(K_m\) value is only a rough measure for the fit of a compound into the active site, which neglects the fine but important alignment of groups necessary for catalysis. It follows that inhibition data obtained with substrate analogues do not necessarily permit insight into the arrangement of the substrate at the active site.

The absolute configuration of the cAMPs isolated from the cyclization reaction in presence of Mn" was analyzed in two steps. First, it could be demonstrated that the product was not degraded by the calmodulin-sensitive cyclic phosphodiesterase. Since only the \(S_S\) isomer of cAMPs is a substrate for this enzyme, this result suggested that the product might have the \(R_R\) configuration. Second, a more rigorous analysis by high performance liquid chromatography showed that all the \(^{35}\)S label was associated with the \(R_R\) isomer (Fig. 1). Thus, the two analyses gave identical results, indicating that the cyclization had occurred with inversion of configuration. This can be the result of any odd number of nucleophilic substitutions. One being the smallest odd number, it is generally agreed that inversion is the result of a one-step in-line nucleophilic substitution reaction (2, 3). It can also be argued that inversion is the result of one in-line and one adjacent nucleophilic substitution reaction. Since the latter is associated with retention of configuration, the net result would also be inversion. However, so far no indication for an adjacent mechanism in an enzymatic reaction has been found, and therefore this interpretation is considered unlikely at this time. Therefore, a covalent enzyme intermediate is not involved in the mechanism of the mammalian adenylate cyclase.

Our results obtained with the mammalian enzyme are essentially identical with those obtained with a bacterial enzyme, also employing the diastereomers of ATPaS but using \(^{31}\)P-NMR for analysis (13). This indicates that although the two proteins are probably different, the structure of the active site must be very similar. To our knowledge, this is the first time that such a comparative study on the stereochemical course of an enzyme reaction has been performed for a mammalian and a bacterial enzyme.

The use of phosphorothioate analogues of ATP in studying the stereochemical course of enzymatic reactions has relied previously on the assumption that substitution of oxygen by sulfur at the reactive phosphate will not change the stereochemistry of the reaction. This assumption has been shown to be true for the adenylate cyclase reaction, since the bacterial enzyme catalyzes the reverse reaction from cyclic \([^{30}\)O]dAMP also with inversion of configuration (14). Stereochemical results obtained with isotopically labeled ATP derivatives for

2 P. M. J. Burgers, and F. Eckstein, unpublished observation.
glycerol kinase (15) and the cyclic phosphodiesterase from beef heart (16) are also in agreement with the results obtained earlier with phosphorothioates.

The phosphorothioate analogues can also be used to determine which metal nucleotide chelate isomer is required in an enzymatic reaction (2). A change in the stereoselectivity of the enzyme for a particular isomer of ATPαS or ATPβS, when Co²⁺ or Cd²⁺ replaces Mg²⁺ as the metal ion, is taken as evidence that the metal is normally bound to the sulfur-containing phosphate during catalysis, and also to the corresponding phosphate in the natural substrate. A knowledge of the various screw sense isomers formed preferentially by the ATPαS and ATPβS diastereomers with either Mg²⁺ or Cd²⁺ leads to a precise description of the configuration of the metal nucleotide substrate in the enzymatic reaction. For enzymes which catalyze adenylyl transfer (17–19), it was found that substituting Co²⁺ or Cd²⁺ for Mg²⁺ in the reaction did not affect the enzyme stereoselectivity with ATPαS, but did do so for ATPβS, leading to the conclusion that the metal ion is βγ coordinated to the nucleotide. We attempted to establish whether the stereoselectivity of adenylyl cyclase for the S₆ isomer of ATPαS in the presence of Mg²⁺ would change to the R₆ isomer in the presence of Co²⁺ or Cd²⁺. Unfortunately, the enzyme was not active with ATPαS when either Co²⁺ or Cd²⁺ was the divalent metal ion. Elucidation of the structure of the metal nucleotide complex in the adenylate cyclase reaction will be of interest as a means of determining whether βγ bidentate metal coordination is a general case for nucleotide transfer reactions in which pyrophosphate is the leaving group.

REFERENCES