The Stereochemical Course of the Reaction Catalyzed by Soluble Bovine Lung Guanylate Cyclase*

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The stereochemical course of the reaction catalyzed by the soluble form of bovine lung guanylate cyclase has been investigated using [α-32P]guanosine 5'-triphosphate (R, diastereomer) and guanosine 5'-O-(1-thiotriphosphate) (S, diastereomer) as substrates. The product from the 3-thiomorpholino-1',1'-dioxide syndnonime-stimulated enzymatic cyclization of [α-32P]guanosine 5'-triphosphate was esterified with diazomethane. 31P NMR analysis of the triesters indicated that all of the 18O label was present in the axial position. Guanosine 5'-O-(1-thiotriphosphate) (S, diastereomer) was cyclized under stimulated and basal enzyme activities and, in both cases, the R, diastereomer of guanosine 3',5'-cyclic phosphorothioate was formed. This was determined by direct comparison with material synthesized chemically from guanosine 5'-phosphorothioate. The results from these experiments show that the reaction catalyzed by guanylate cyclase proceeds with inversion of configuration at phosphorus and this indicates that the reaction proceeds by way of a single direct displacement reaction.

Guanylate cyclase catalyzes the formation of cGMP from GTP. The product, cGMP, has recently been shown to act as an intracellular messenger and is presumed to be involved in such processes as protein kinase activation, visual stimulation, and neurotransmission (1, 2). The soluble form guanylate cyclase activity is stimulated by a number of mild oxidants including N-nitroso compounds, nitrite, hydroxylamine, and peroxides (1, 2). Recent investigations indicate that the enzyme from mammalian tissue contains a heme prosthetic group which is thought to be involved in the stimulation process (3–5).

The mechanism by which guanylate cyclase converts GTP to cGMP is not well understood. It has been demonstrated that the reaction proceeds with cleavage of the P1-P2 bond (6). Recent reports concerning the synthesis of the diastereomers of GTPoSγ (7) and the substitution of sulfur by oxygen-18 (8–10) provide a means to delineate the stereochemical course of the cyclization reaction. Such an analysis can help to distinguish between various mechanistic pathways which include a direct displacement reaction or a covalent enzyme intermediate (11–13). In this report, we describe the cyclization of (S)-GTPoS and (R)-[α-32P]GTP and the stereochemical analyses of the resulting products in the reactions catalyzed by the soluble form of bovine lung guanylate cyclase.

MATERIALS AND METHODS

Soluble guanylate cyclase was purified from bovine lung (14). The specific activity of the enzyme was 0.1 unit/mg in the presence of Mg2+ and 2 units/mg in the presence of sodium nitroprusside and Mg2+. Sodium dodecyl sulfate-gel electrophoresis showed it to be 30% pure. Glyceral kinase (Candida mycoderma, 85 units/mg), pyruvate kinase (rabbit muscle, 200 units/mg), phosphoglycerate kinase (yeast, 450 units/mg), phosphoglyceraldehyde dehydrogenase (rabbit muscle, 80 units/mg), and creatine kinase (rabbit muscle, 37.8 units/mg) were obtained from Boehringer Mannheim (Germany). Inorganic pyrophosphatase (yeast, 835 units/mg) was obtained from Sigma. 3-Thiomorpholino-1',1'-dioxide syndnonime was kindly supplied by Cassella AG, Frankfurt (Germany). (R,)-GTPoS was a kind gift from Dr. Connolly, Göttingen, and was prepared according to the published procedure (7). 31P NMR spectra were measured at 81 MHz using a Bruker WP 200 SY spectrometer with quadrature detection and proton broad band decoupling. Positive chemical shifts are downfield from external 85% phosphoric acid. HPLC experiments were performed with a Waters Associates (Model 6000 A) liquid chromatograph using the following columns and solvent systems: anion exchange (Nucleosil 10 SB, Macherey-Nagel, Duren, Germany) eluted with 200 mM KH2PO4 containing 500 mM KOAc at pH 4.5; reverse phase (ODS Hypersil, 5 μ, Shandon Southern Products Ltd., Runcorn, England) eluted with 50 mM KH2PO4 at pH 6.0.

Guanosine 5'-O-(1-Thiodiphosphate), R, and S, Diastereomers—A suspension of 1.55 mmol of the pyridinium salt of GMPS (7) and 1.35 ml (3.09 mmol) of tri-n-octylamine in methanol was warmed until the nucleotide was completely dissolved. The solution was evaporated and the residue was dried by the addition and evaporation of five 2-ml portions of anhydrous pyridine. Dry nitrogen was introduced through a septum. To a solution of the residue in 20 ml of dioxane was added 0.64 ml (3.09 mmol) of diphenylphosphorochloridate and 1.35 ml (3.09 mmol) of tri-n-octylamine. The solution was stirred for 3 h, after which all the solvent was evaporated and 10 ml of ether/hexane (1:1) was added. The mixture was cooled to 0°C for 30 min and the solvent was separated from the residue with a syringe. Remaining solvent was evaporated under high vacuum.

In a separate flask, 5.8 ml (24.45 mmol) of tri-n-butylamine was added to a solution of 1.15 g (11.53 mmol) of crystalline 38% phosphorothioate; (R, and S,)-GMPS, the R, and S, diastereomers of guanosine 3',5'-cyclic phosphorothioate; (S,)-cAMP, the S, diastereomers of adenosine 3',5'-cyclic phosphorothioate; GMPCH3, guanosine 3',5'-cyclic phosphoric acid methyl ester; HPLC, high pressure liquid chromatography; TEAB, triethylammonium bicarbonate.

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The abbreviations used are: GTPoS, guanosine 5'-O-(1-thiotriphosphate); GMPS, guanosine 5'-phosphorothioate; R, and S, GTPoS, the R, and S, diastereomers of guanosine 5'-O-(1-thiotriphosphate); (S,)-GTPoS, the S, diastereomer of guanosine 5'-O-(1-thiytriphosphate).
phorothioate-A solution of 1.00 mmol of the tri-n-octylammonium then evaporated to dryness. Purification by flash chromatography in water (v/v) provided crude pyruvate kinase. The solution was allowed to stand at room temperature for 1 hr and transferred by means of a syringe to the activated nucleotide phosphorochloridate were used and by careful exclusion of air. After 10 h at 37 °C, the product by DEAE-Sephadex chromatography on a column (2 × 30 cm) with a linear gradient of 1.5 liters of 0.1 M and 0.5 M TEAB afforded 4.4 μmol of (Rp)-cGMPS in fractions 82–96. 31P-NMR, UV, and HPLC properties of the product were identical with previously prepared material (7).

Guanosine 5'-O-(1-Thiotriphosphosphate), S, Diastereomer—To an incubation mixture at pH 7.5 (total volume 100 ml) containing 8.12 mM (R, and S)-GDPaS (total amount of S, as determined by reverse phase HPLC was 414 μmol), 50 mM KCl, 15 mM glycerol, and 2.5 mM dithiothreitol was added 40 units of glycerol phosphatase, 10 mM MgCl₂, 1 mM EDTA, and 4.14 mM NAD were added 6300 units of phosphoglycerate kinase and 880 units of phosphoglycerate dehydrogenase. The solution was allowed to stand at room temperature for 4 hr. Purification of the product by DEAE-Sephadex chromatography on a column (1.5 × 50 cm) with a linear gradient of 0.5 ml each of 0.2 M and 0.6 M TEAB afforded 173 pmol (75%) of (S,)-GDPaS. HPLC analysis (reverse phase and ion exchange) showed only a single peak which was coincident with an authentic standard (7).

Guanosine 5'-O-(1-Thiotriphosphosphate), S, Diastereomer—To an incubation mixture (total volume 50 ml) containing 4.58 mM (S,)-GTPaS, 100 mM glycerol, pH 9.8 buffer, 10 mM MgCl₂, and 0.6 M NaCl was added 6030 units of phosphoglycerate kinase and 240 units of glyceraldehyde dehydrogenase. The solution was allowed to stand at room temperature for 2.5 hr. Purification of the product by DEAE-Sephadex chromatography on a column (2 × 80 cm) with a linear gradient of 0.25 liters each of 0.2 M and 0.7 M TEAB afforded 193 pmol of (R,)-cGMPS. 31P-NMR, UV, and HPLC properties of the product were identical with previously prepared material (7).

RESULTS

In order to improve the yields, the procedures used in the synthesis of (S,)-GTPaS were modified from those previously described (7). (R, and S,)-GDPaS were prepared chemically by reaction of GMPS with diphenylphosphorochloridate followed by displacement with inorganic phosphate. A good overall yield was obtained when two equivalents of diphenylphosphorochloridate were used and by careful exclusion of oxygen from the reaction mixture. Enzymatic phosphorylation of the diastereomeric mixture of GDPaS to (S,)-GTPaS was achieved using phosphoglycerate kinase and a limiting amount of 1,3-diphosphoglycerate as reported (7), but it was necessary to increase both the amount of enzyme and the reaction time. The (S,)-GTPaS obtained consisted of only one single diastereomer, as determined by HPLC.

For the substitution of sulfur by oxygen-18, (S,)-GTPaS was enzymatically converted to (S,)-GDPaS with glycerol kinase. This conversion is necessary because the substitution

An aqueous suspension of residue was extracted twice with ether and then evaporated to dryness. Purification by flash chromatography on a SiO₂ column (2 × 15 cm) with 220.75 cm³ 95% ethanol-water (v/v) provided crude (R, and S,)-cGMPS. Further purification of the product by DEAE-Sephadex chromatography on a column (2 × 30 cm) with a linear gradient of 1.5 liters each of 0.1 M and 0.5 M TEAB afforded 4.4 μmol of (Rp)-cGMPS in fractions 82–96. 31P-NMR, UV, and HPLC properties of the product were identical with previously prepared material (7).
reaction on nucleoside α-thiotriphosphates distributes the oxygen-18 between the α- and γ-phosphates (8–10). The conversion of (S)-GDPαS to [α-18O]GDP was achieved with N-bromosuccinimide in H218O and dioxane (Scheme 1) as described for the conversion of GTPαS to GTP (9). Enzymatic phosphorylation of [α-18O]GDP with pyruvate kinase and phosphoenolpyruvate gave [α-18O]GTP. The oxygen-18 was located exclusively at the α-phosphorus. This was shown by the 31P NMR spectrum where only the signal for the α-phosphorus and not the β-phosphorus exhibited the oxygen-18-induced upfield chemical shift. This material was 92% pure by HPLC and 31P NMR. The impurity, which we assume to be a base-modified product, could not be separated by further chromatography. The configuration of the [α-18O]GTP was not determined, but the substitution reaction was presumed to have proceeded with inversion of configuration as shown for the conversion of cAMP and (S)-ADPαS (9). As will be discussed later, this reaction does not always proceed with complete stereospecificity.

Guanylate cyclase-catalyzed cyclization of [α-18O]GTP was carried out with purified soluble guanylate cyclase (14) using 3-thiomorpholino-1,1'-dioxide sydnonimine to stimulate the enzyme activity. Creatine kinase and phosphocreatine were added to rephosphorylate any GDP produced by nonenzymatic hydrolysis of substrate during the incubation. The product, obtained in 54% yield, was esterified by diazomethane and analyzed by 31P NMR according to the method previously reported for the methyl and ethyl esters of cAMP and cGMP (16, 17).

The 31P NMR spectrum of the triester, cGMP[18O]CH3, shows two resonances (Fig. 1A). Based on the reported chemical shifts of cGMP[18O]CH3 (18), the high field resonance (−3.83 ppm) of the oxygen-18-labeled material is assigned to the axial triester and the low field resonance (−2.95 ppm) to the equatorial triester. The 18O-induced chemical shift was measured by mixing the cGMP[18O]CH3 with unlabeled material. The 31P NMR spectrum of the mixture (Fig. 1B) shows a greater difference in chemical shift for the equatorial triester (3.3 Hz) than for the axial triester (1.3 Hz). This difference in magnitude indicates that the P-18O bond order for the equatorial triester is greater than that for the axial triester (16, 17). Therefore, the oxygen-18 is in the axial position. This indicates inversion of configuration in the enzymatic cyclization of [α-18O]GTP.

The cyclization of (S)-GTPαS by stimulated guanylate cyclase was carried out under the same conditions used for the cyclization of [α-18O]GTP. The two reactions appeared to proceed at comparable rates. The nonstimulated enzymatic cyclization of (S)-GTPαS with MgCl2 and MnCl2 as metal cofactors was also investigated. In order to determine the
Mechanism of Action of Guanylate Cyclase

**Scheme 2.**

**DISCUSSION**

In this investigation, two independent methods have been used to determine the stereochemical course of the reaction catalyzed by bovine lung guanylate cyclase. The synthesis of the substrates used, (S)_\text{p}-\text{GTP}\alpha\text{S} and (R)_\text{p}-[\alpha^{-18}O]\text{GTP}, was achieved with minor modifications of previously published procedures (7, 9). N-Bromosuccinimide in aqueous dioxane was used for the conversion of (S)_\text{p}\text{-GDP}\alpha\text{S to [\alpha^{-18}O]}\text{GDP. This method was described as stereospecific and proceeding with inversion of configuration when carried out with (S)_\text{p}-\text{cGMP} and (S)_\text{p}-\text{ADP}\alpha\text{S (9). However, in more recent experiments with (S)_\text{p}-\text{cAMP}, we have consistently isolated approximately 80:20 mixtures of inversion to retention products. Under the same reaction conditions, (R)_\text{p}-\text{thymidine 3',5'-cyclic phosphorothioate gave the same product composition (10). The substitution of sulfur by oxygen-18 in (S)_\text{p}-\text{GDP}\alpha\text{S by N-bromosuccinimide in aqueous dioxane was assumed to proceed predominantly with inversion of configuration. However, the exact stereochemical composition of the [\alpha^{-18}O}\text{GDP formed was not determined at this stage. The two methods available for the configurational assignment of [\alpha^{-18}O]nucleoside diphosphates involve }^{31}\text{P NMR analysis of the nucleoside diphosphate cobalt tetrammine complexes (20) and the }^{31}\text{P-NMR analysis of the products obtained from the chemical cyclization of }^{[160,'70,180]\text{nucleoside monophosphate (10, 17). The sensitivity of the cobalt tetrammine analytical method is not high and 10 to 20% of the minor product obtained from retention of configuration could easily escape detection. The chemical cyclization procedure requires large quantities of [\alpha^{-18}O]nucleoside diphosphate and the limits of accuracy have not been determined. Because of these shortcomings, we did not undertake a separate stereochemical determination of the [\alpha^{-18}O]GDP obtained from (S)_\text{p}-\text{GDP}\alpha\text{S. Rather, we decided to defer the determination to a later stage and to use the enzymatic cyclization of (S)_\text{p}-\text{GTP}\alpha\text{S as an independent check for the stereochemical course of the reaction. The }^{31}\text{P NMR spectrum of the diastereomeric cGMP}^{18O}\text{CH}_3\text{ mixture obtained after esterification of [\text{PO}]cGMP shows that within the limits of experimental measurement, all of the oxygen-18 is present in the axial position (Fig. 1). If the substitution of sulfur by oxygen-18 or the enzymatic cyclization reaction had not been stereospecific, some of the oxygen-18 would have appeared in the equatorial configuration of the cGMP formed enzymatically, an independent synthesis of both diastereomers of cGMP was required (Scheme 2). Activation of GMPS with diphenylphosphorochloridate, followed by ring closure with potassium tert-butoxide provided (R)_\text{p}-\text{cGMP and (S)_p-cGMP. Although the yield was poor, the method is simple and direct, and enough product can be obtained for analytical purposes. The diastereomers were separated chromatographically on DEAE-Sephadex and were easily distinguished by anion exchange HPLC and }^{31}\text{P NMR. By analogy to cAMP, the diastereomer with the high field }^{31}\text{P NMR shift (54.6 ppm) was assigned the (R)_p configuration and the diastereomer with the low field shift (56.2 ppm) was assigned the (S)_p configuration. As with (R)_p-cAMP, the (R)_p diastereomer of cGMP eluted first on an anion exchange HPLC column (Fig. 2A). HPLC comparisons of the cGMP obtained from the enzymatic cyclizations of (S)_p-GTP\alpha\text{S under both stimulated (Fig. 2B) and basal conditions (not shown) with the diastereomers synthesized chemically, showed that only (R)_p-cGMP was formed enzymatically. Thus, inversion of configuration had occurred in the enzymatic cyclization reactions.**
position of the phosphate triester. The fact that the $^{31}$P NMR spectrum indicates that all of the oxygen-18 is in the axial position shows that the enzymatic reaction proceeded with inversion of configuration and also that the substitution of sulfur by oxygen-18 occurred stereospecifically with inversion of configuration. However, in an earlier experiment using different oxygen-18 substitution reaction conditions, 15% of the oxygen-18 was detected in the equatorial position. In this earlier experiment, the oxidation with N-bromosuccinimide was carried out for only 15 s instead of 2 min before being quenched with mercaptoethanol and disopropylethylamine was used to control the pH during the oxidation reaction. It is possible that these differences might promote mechanistic pathways leading to the formation of some of the retention product. Further detailed investigations are required to test this hypothesis.

The cyclization of (S)-GTP$\alpha$S by the activated enzyme proceeded to a similar extent under the conditions used to cyclize [α-32P]GTP. A more detailed kinetic study is necessary to establish whether the rates of conversion are also comparable or whether, as was found for mammalian adenylate kinase (21), the phosphorothioate is turned over at a lower rate. The $R_s$ diastereomer of GTP$\alpha$S was not a substrate for guanylate cyclase. This is in analogy to the mammalian and bacterial adenylyl cyclases, for which (R)-ATP$\alpha$S was not a substrate (21, 22). The $R_p$ diastereomer of cGMP$\alpha$S was formed from both the stimulated and basal enzymatic cyclizations of (S)-GTP$\alpha$S. The identification of the product was determined by HPLC comparison to chemically synthesized material (Fig. 2).

The result of this investigation is that guanylate cyclase from bovine lung cyclizes both (S)-GTP$\alpha$S and [α-32P]GTP with inversion of configuration. The simplest interpretation of these results is that the pyrophosphate leaving group in GTP is displaced by the 3'-hydroxyl group in a single, direct displacement reaction (Fig. 3) and does not involve a covalent enzyme intermediate (11–13). Thus, the stereochemical course of the reaction for bovine lung guanylate cyclase is the same as that for mammalian and bacterial adenylyl cyclases (20–22) and indicates that direct displacement is the mechanistic pathway for both classes of enzymes. Direct displacement appears to be the predominant pathway for enzymatic nucleotidyl and phosphoryl transfer reactions (13, 23, 24).


diagram of guanylate cyclase reaction

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