Stereochemical Course of the Reaction Catalyzed by Guanylate Cyclase from Bovine Retinal Rod Outer Segments*

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The stereochemical course of the reaction catalyzed by guanylate cyclase from bovine retinal rod outer segments was investigated using phosphorothioate analogs of GTP as chiral probes. \((S_\alpha)-\text{Guanosine 5':O-}(1\text{-thiotriphosphate})\) \((S_\alpha)-\text{GTPoS}\) is a substrate, whereas \((R_\alpha)-\text{GTPoS}\) is a competitive inhibitor \((K_i = 0.1 \text{ mM})\), but not a substrate. \((S_\beta)-\text{GTPoS}\) is converted into \((R_\beta)-\text{guanosine 3':5'-monophosphorothioate}\), showing that the reaction proceeds with inversion of configuration at the \(\alpha\)-phosphorus atom. \(K_i\) and \(V_{\text{max}}\) for \((S_\alpha)-\text{GTPoS}\) (at low \([\text{Ca}^{2+}]\), 20 mM) are 3.7 mM and 1.1 nmol/min/mg of rhodopsin, respectively, compared with 1.1 mM and 23.1 nmol/min/mg of rhodopsin for GTP. \(V_{\text{max}}\) for the cyclization of \((S_\alpha)-\text{GTPoS}\), as for GTP, increases 10–20-fold when the calcium level is lowered. This activity change is centered at ~90 nm and has a Hill coefficient of 4.8. The configuration of the metal-substrate complex was determined by measuring the effectiveness of the \(S_\alpha\) and \(R_\alpha\) isomers of GTPoS and guanosine 5':O-(2-thiotriphosphate) \((\text{GTPoS})\) in the presence of \(\text{Mg}^{2+}\) or \(\text{Mn}^{2+}\). \((S_\alpha)-\text{GTPoS}\) is a substrate with either \(\text{Mg}^{2+}\) or \(\text{Mn}^{2+}\), whereas \((R_\alpha)-\text{GTPoS}\) is a substrate with only \(\text{Mn}^{2+}\). These findings suggest that the substrate is a metal-\(\beta\),\(\gamma\)-bidentate complex with \(\Delta\) screw sense. We also found that the cyclization reaction catalyzed by the membrane-bound guanylate cyclase from sea urchin sperm proceeds with inversion of configuration at the \(\alpha\)-phosphorus atom. The stereochemical course of the reactions catalyzed by all prokaryotic and eukaryotic adenylate cyclases and guanylate cyclases studied thus far is the same.

Visual excitation in vertebrate rods and cones is mediated by the hydrolysis of cGMP, which leads to the closure of cGMP-gated channels and the consequent hyperpolarization of the plasma membrane of the outer segment (for reviews, see Pugh and Cobbs, 1986; Stryer, 1986; Yau and Baylor, 1989). Recovery of the dark state depends on the resynthesis of cGMP. Guanylate cyclase, which is associated with the axoneme traversing the outer segment, catalyzes the synthesis of cGMP from the Mg\(^{2+}\) (or Mn\(^{2+}\)) complex with GTP (Goridis et al., 1973; Pannbacker, 1973; Fleischman and Denesich, 1979). Little is known about the catalytic mechanism of this enzyme, which thus far has resisted purification. However, recent studies have shown that the enzyme of this range (Pepe et al., 1986; Koch and Stryer, 1988). Electrophysiological studies have revealed that the calcium ion level drops markedly following a light pulse (McNaughton et al., 1986; Nakatani and Yau, 1988a, 1988b; Ratlo et al., 1988; Cervetto et al., 1989). These findings suggest that control of guanylate cyclase by calcium ion is important for recovery following illumination.

We have carried out studies of the reaction mechanism of guanylate cyclase from bovine retinal rod outer segments using \(\alpha\) and \(\beta\)-phosphorothioate analogs of GTP as chiral probes. Phosphorothioate analogs of nucleotides have provided a wealth of information concerning the mechanism of phosphoryl transfer reactions (Eckstein, 1985). In particular, this approach has been applied to mammalian and bacterial adenylate cyclases (Gerlt et al., 1988; Eckstein et al., 1981) and to soluble lung guanylate cyclase (Senter et al., 1983). Our experiments reveal the stereochemical course of the reaction catalyzed by the retinal enzyme and define the configuration of the metal-GTP complex that serves as substrate. They also show that regulation of the enzyme by calcium ion is preserved when a phosphorothioate analog is used as substrate. The membrane-bound guanylate cyclase from sea urchin sperm was also studied to learn whether evolutionarily distant guanylate cyclases have similar reaction mechanisms.

**MATERIALS AND METHODS**

GTP, cGMP, and 3-isobutyl-1-methylxanthine were from Sigma. Pure 2 was from Molecular Probes. \((S_\alpha)-\) and \((R_\alpha)-\text{GTPoS}\) and \((S_\alpha)-\) and \((R_\beta)-\text{GTPoS}\) were synthesized as described (Connolly et al., 1982; Senter et al., 1983). \((S_\alpha)-\) and \((R_\alpha)-\text{GMPoS}\) were synthesized according to Eckstein and Kutaku (1980). All other reagents were analytical or HPLC-grade.

**Preparation of Rod Outer Segments**—A stock of commercially available or freshly prepared bovine retinas were shaken in buffer A (115 mM \(\text{NaCl}\), 20 mM KCl, 1 mM \(\text{MgCl}_2\), 10 mM Hepes, pH 7.5) containing 35% (w/w) sucrose. This suspension was centrifuged for 10 min at 6000 rpm (4000 \(\times\) g), the pellet was discarded, and the supernatant was diluted 1:2 with buffer A. The suspension was centrifuged again for 10 min at 4000 rpm (1900 \(\times\) g), and the pellet was resuspended in 4 mM Mops, pH 7.1, 5 mM dithiothreitol at a
final rhodopsin concentration of ~0.1 mM.

**Determination of Guanylate Cyclase Activity**—Guanylate cyclase activities were determined either in whole ROS or in hypotonically extracted membranes of ROS. All incubation experiments were performed in complete darkness. Ten μl of a ROS suspension (20–70 μg of rhodopsin) and 20 μl of a Ca²⁺/EGTA solution were incubated with 20 μl of assay buffer containing 50 mM Mops, pH 7.1, 58 mM NaCl, 12 mM MgCl₂, 2 mM GTP or GTP-γ-S, 2 mM CaCl₂ or MnCl₂, 20% of either the S₉ or R₉ isomer of GTPαS. The free calcium concentration in our buffer system at the indicated ratio of Ca²⁺ to EGTA (listed within parentheses below) was determined with the calcium indicator fura-2 (Grynkiewicz et al., 1985). The following concentrations of free calcium were obtained: 144 (0.5), 114 (0.3), 96 (0.2), 81 (0.15), 70 (0.125), and 63 (0.1) μM. Free calcium was added to obtain 0.125 μM free Ca²⁺, and samples without EGTA and with added calcium contained ~20 nM free Ca²⁺. The obtained peaks for cGMP and (R₉)-cGMPS was used to calculate the amount of cyclic nucleotide formed during the time of incubation.

The hydrolysis of cGMP was measured separately by incubating ROS with [³H]cGMP and using a previously described TLC assay (Koch and Stryer, 1988). The detection of hydrolysis was usually between 10 and 20%.

**Determination of Kₚ and Vₐₜₚ at Low Free Ca²⁺**—ROS were incubated with assay buffer as described above. Calcium was buffered with 2 mM EGTA to get a free concentration of 20 nM. The concentration of substrate, either GTP or (S₉)-GTPαS, was varied in the 0.2–3 mM range. The ratio of M₉⁺ to GTP was held constant at 6:1. The formation of cGMP and cGMPS was measured by HPLC analysis. Kₚ and Vₐₜₚ were determined from Lineweaver-Burk plots.

**Inhibition Studies**—Two sets of experiments were performed to test the inhibitory effects of the phosphorothioate analogs on retinal rod guanylate cyclase. First, ROS suspensions were incubated with 2 or 0.5 mM GTP and different concentrations of the S₉ or R₉ isomer of GTPαS in the 0.01–2 mM range. Second, ROS suspensions were incubated with either 0.4 mM (R₉)-GTPαS or 1 mM (S₉)-GTPαS, and the concentration of the substrate GTP was varied in the 0.2–2 mM range.

**Substitution of M₉⁺ by Mn²⁺**—Typically, 200 μl of ROS (400–1400 μg) was washed twice with 2 ml of 10 mM Mops, pH 7.1. Ten μl of the ROS suspension was incubated with assay buffer containing 2 mM (S₉)- or (R₉)-GTPαS or GTP. The diastereomers of GTPαS were incubated with whole ROS. Either 12 mM M₉⁺ or 6 mM Mn²⁺ was added as cofactor. No EGTA was present in experiments with effects of M₉⁺ and Mn²⁺ were compared. The calcium concentration was in the micromolar range. The time of incubation was 1, 3, and 5 h with GTPαS, 1 h with GTPαS, and 5 or 10 min with GTP. All other steps and the HPLC analysis were performed as described above.

**Sea Urchin Sperm Guanylate Cyclase**—Experiments involving sea urchin sperm guanylate cyclase were performed in a reaction mixture containing 50 mM Tris, pH 8.0, 1 mM 1-isobutyl-3-methylxanthine, 7.2 mM NaCl, 4.5 mM MnCl₂, 0.1 mM each (S₉)- and (R₉)-GTPαS. The reaction was started by adding 10 μl (6–7 μg of protein) of the enzyme preparation to a total volume of 0.4 ml. After incubation at 30 °C for 30 min, the reaction mixture was boiled for 2 min and centrifuged. The nucleotide content of the supernatant was analyzed by the HPLC method described above.

**RESULTS**

**Sterochemical Course of Cyclization Reaction**—The S₉ and R₉ diastereomers of GTPαS were incubated with retinal rod outer segments, and the reaction mixture was analyzed by HPLC to determine whether they serve as substrates and, if so, the configuration of the product. The HPLC elution profile of a mixture of authentic cGMP and (R₉)- and (S₉)-cGMPS is shown in Fig. 1A. A good separation of these nucleotides was achieved, with elution times of 8.0, 8.3, and 8.8 min, respectively. cGMP formed by the action of retinal guanylate cyclase elutes at 8.0 min (Fig. 1B). The additional peak at 7.75 min is guanosine, which was formed from GTP, GDP, and GMP by the action of nonspecific phosphatases. The elution profile of the product formed from (S₉)-GTPαS is shown in Fig. 1C. The peak at 8.3 min corresponds to (R₉)-cGMPS, and the one at 7.75 min is guanosine. The product of this reaction was also analyzed by incubating it with retinal rod GMP phosphodiesterase. (S₉)-cGMPS is slowly hydrolyzed by this enzyme, whereas (R₉)-cGMPS is totally resistant to hydrolysis (Zimmerman et al., 1985). Incubation of the reaction mixture with trypsin-activated phosphodiesterase (Hurley and Stryer, 1982) for 2 h did not decrease the amount of product eluting at 8.3 min. Under these conditions, (S₉)-cGMPS would have been nearly completely hydrolyzed because the half-time for its hydrolysis would have been only 7 min. These experiments establish that (R₉)-cGMPS was formed from (S₉)-GTPαS. In contrast, neither diastereomer of cGMPS was formed on incubating (R₉)-GTPαS with ROS.

The action of the membrane-bound guanylate cyclase from sea urchin sperm on (S₉)- and (R₉)-GTPαS was also measured to determine whether this evolutionarily distant enzyme has a similar mechanism. As shown in Fig. 2, (S₉)-GTPαS was converted to (R₉)-cGMPS by the sea urchin enzyme. In contrast, (R₉)-GTPαS was not a substrate. Thus, the bovine retinal and sea urchin enzymes are specific for (S₉)-GTPαS. Furthermore, the cyclization reaction catalyzed by both guanylate cyclases proceeds with inversion of configuration at the α-phosphorus atom.
The effect was shown to be mediated by a regulatory protein that led to a 5-20-fold stimulation of cyclase activity. This stimulatory effect was separable from the catalytic moiety. As shown in Fig. 4, the rate of cyclization of (S)-GTPαS by the retinal enzyme is also highly dependent on the Ca2⁺ level. Between 100 and 200 nM Ca2⁺, the guanylate cyclase activity was low (between 0.04 and 0.1 nmol/min/mg of rhodopsin). Decreasing the free calcium level led to a 10-20-fold increase in cyclase activity, which reached 1 nmol/min/mg of rhodopsin at 20 nM Ca2⁺.

Competitive Inhibition by (S)- and (R)-GTPαS—The formation of cGMP from GTP was inhibited by both diastereomers of GTPαS. The apparent inhibition constants were 1 mM for (S)-GTPαS and 0.4 mM for (R)-GTPαS, as determined by measuring the effect of different concentrations of these analogs on the rate of formation of cGMP from GTP. The mode of inhibition was identified by varying the concentration of GTP in the presence of a fixed amount of phosphorothioate analog. The Lineweaver-Burk plot of these data shows that (S)- and (R)-GTPαS changed the apparent K₉₅ of GTP from (S)-GTPαS by membrane-bound guanylate cyclase from 9661. The Lineweaver-Burk plot of these data shows that (S)- and (R)-GTPαS changed the apparent K₉₅ of GTP from (S)-GTPαS by membrane-bound guanylate cyclase from 9661. In the control experiment, no analog was added.

Stereochemical Course of Retinal Guanylate Cyclase

Table I

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Guanylate cyclase activity</th>
<th>pmol/min/mg rhodopsin</th>
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<tbody>
<tr>
<td>With Mg²⁺</td>
<td>1325 ± 50</td>
<td>2150 ± 250</td>
</tr>
<tr>
<td>(S)-GTPαS</td>
<td>40 ± 16</td>
<td>57 ± 19</td>
</tr>
<tr>
<td>(R)-GTPαS</td>
<td>8 ± 2</td>
<td>160 ± 25</td>
</tr>
<tr>
<td>With Mn²⁺</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>(S)-GTPβS</td>
<td>50 ± 10</td>
<td></td>
</tr>
<tr>
<td>(R)-GTPβS</td>
<td>&lt;1</td>
<td></td>
</tr>
</tbody>
</table>

Effect of Mg²⁺ and Mn²⁺ on the cyclization of GTP and its α- and β-phosphorothioate analogs

The substrate concentration was 2 mM in each case. The total concentration of Mg²⁺ was 12 mM and that of Mn²⁺ was 6 mM. Calcium ion was present in micromolar concentration. Assay conditions were as described under “Materials and Methods.” Values are means ± S.D. and were taken from three or four different incubations.

Discussion

Phosphorothioate analogs of nucleoside triphosphates such as GTPαS and GTPβS exist as pairs of diastereomers because...
of the chirality of the phosphorus at the α- and β-positions. They are particularly well-suited for answering two mechanistic questions (Eckstein, 1985; Frey, 1989). First, does a reaction proceed by direct nucleophilic substitution or by formation of a covalent intermediate? Second, what is the configuration of the active metal-substrate complex? The stereochemical course of the axoneme-associated guanylate cyclase of retinal rod outer segments was determined by testing the diastereomers of GTPαS as substrates. The finding that (S)-GTPαS was converted into (R)-cGMP indicates inversion of configuration at the α-phosphorus atom (Fig. 1). The simplest explanation is that cyclization takes place by a single nucleophilic displacement (Fig. 5). In this proposed mechanism, the oxygen atom of the 3'-OH group of ribose attacks the α-phosphorus atom. The pentacoordinated phosphorus in this transition state has bipyramidal geometry, with the attacking 3'-OH group and the leaving pyrophosphate group in apical positions. This reaction scheme is the same as was proposed previously for adenylate cyclases (Gerlt et al., 1980; Eckstein et al., 1981) and for soluble lung guanylate cyclase (Senter et al., 1983).

The reaction rate was ~30 times slower with (S)-GTPαS than with GTP (Table I). Although a slower rate is often seen with phosphorothioate analogs, the question arises whether the reaction mechanism is the same as with the physiological substrate GTP. Ten enzymes have been analyzed by both the phosphorothioate method and the oxygen isotope method (for a review, see Eckstein, 1985). In all cases, the same stereochemical course is found with the two methods. Another test of the preservation of the reaction mechanism is to determine whether a regulatory aspect is conserved. Retinal rod guanylate cyclase exhibits a 20-fold increase in catalytic activity when the Ca<sup>2+</sup> level is lowered in the submicromolar range (Koch and Stryer, 1988). For GTP, the effect is highly cooperative, with a Hill coefficient of 3.9 ± 1.3 and a half-maximal change at 90 nM Ca<sup>2+</sup>. For (S)-GTPαS, the corresponding values are similar (4.8 ± 0.6 and 90 nM), suggesting that the reaction mechanisms for the physiological substrate GTP and the phosphorothioate analog are essentially the same. This is the first report that the control of a cyclase reaction is completely preserved when a phosphorothioate analog is used as substrate.

The configuration of the active metal-substrate complex was determined by taking advantage of the fact that some metal ions preferentially coordinate with sulfur or oxygen (Cohn, 1982; Eckstein, 1985; Pecoraro et al., 1984). Ideally, one would like to use Mg<sup>2+</sup> (which greatly prefers oxygen) and Cd<sup>2+</sup> (which greatly prefers sulfur) as a test pair. However, guanylate cyclase, like many other enzymes, is inactivated by Cd<sup>2+</sup>. Although less selective, Mn<sup>2+</sup> (which coordinates to both oxygen and sulfur) can be used instead of Cd<sup>2+</sup>. Turning to Table I, we see that (S)-GTPαS is a substrate, whereas (R)-GTPαS is not, irrespective of whether the divalent cation is Mg<sup>2+</sup> or Mn<sup>2+</sup> (Table I). Hence, the α-phosphorus atom is probably not coordinated to a metal ion. If it were, (R)-GTPαS would very likely become a substrate on substituting Mn<sup>2+</sup> for Mg<sup>2+</sup>. The observed selectivity for (S)-GTPαS is probably an expression of a specific interaction of the pro-R oxygen by sulfur appears to disrupt this critical interaction, which is essential for catalysis but not for binding. (R)-GTPαS binds to the enzyme with a Ki similar to the K<sub>m</sub> of GTP (Fig. 3).

In contrast, the substitution of Mn<sup>2+</sup> for Mg<sup>2+</sup> had a large effect on the rate of cyclization of (R)- and (S)-GTPαS (Table I). This finding indicates that the β-phosphorus atom is coordinated to the divalent metal ion. The coordination of the γ-phosphorus atom cannot be determined in the same way because the γ-phosphorothioate is not chiral. Coordination to the β-phosphorus atom, but not to the α-phosphorus atom, points to a metal β,γ bidentate complex because such structures are much more stable than monodentate-β-phosphate complexes. What is the configuration of the metal-β,γ-bidentate complex that serves as a substrate for guanylate cyclase? As shown in Fig. 6, the fact that (S)-GTPαS is a substrate with Mn<sup>2+</sup> whereas (R)-GTPαS is not suggests that the active form has the Δ screwsense (compound 2) and not the Λ screwsense (compound 1). This inference is reinforced by the finding that (R)-GTPαS becomes a substrate when Mn<sup>2+</sup> replaces Mg<sup>2+</sup>. The R<sub>δ</sub> isomer can assume the Δ screwsense in the presence of Mn<sup>2+</sup> (compound 6), but not with Mg<sup>2+</sup> (compound 1, which has the Λ screwsense). (S)-GTPαS is a substrate with Mn<sup>2+</sup> as well with Mg<sup>2+</sup> because Mn<sup>2+</sup> can coordinate to either oxygen or sulfur. The active Mn<sup>2+</sup> complex with (S)-GTPαS is compound 4, which has the Δ screwsense.

The membrane-bound guanylate cyclase from sea urchin sperm has the same specificity for (S)-GTPαS and catalyzes the same stereochemical course (Fig. 2) as does the bovine retinal rod enzyme (Fig. 1). Recent cDNA cloning studies have shown extensive amino acid sequence similarities between sea urchin and mammalian membrane-bound guanylate cyclases (Schulte et al., 1989). Likewise, the reaction catalyzed by bacterial and mammalian adenylate cyclases (Gerlt et al.,...
1980; Eckstein et al., 1981) and by soluble lung guanylate cyclase (Senter et al., 1983) proceeds with inversion of configuration. Thus, the stereochemical course of the reactions catalyzed by these prokaryotic and eukaryotic adenylate cyclases and guanylate cyclases is the same. It is also interesting to note that *Escherichia coli* DNA polymerase I and RNA polymerase, like these cyclases, use the Δ screw sense of the metal-nucleoside triphosphate complex as substrate (Eckstein, 1985). This similarity, although possibly fortuitous, may reflect nature’s tendency to conserve structural motifs in carrying out related reactions. DNA and RNA polymerases, like cyclases, catalyze the nucleophilic attack of a 3'-OH group on the α-phosphorus atom of a nucleoside triphosphate.

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**REFERENCES**


