The kinetic mechanism of the rod outer segment (ROS) isoprenylated protein methyltransferase was investigated. This S-adenosyl-L-methionine (AdoMet)-linked enzyme transfers methyl groups to carboxyterminal isoprenylated cysteine residues of proteins, generating methyl esters. The enzyme also processes simple substrates such as N-acetyl-S-farnesyl-L-cysteine (L-AFC). Initial studies showed that a ping-pong Bi Bi mechanism could be eliminated. In a ping-pong Bi Bi mechanism plots of 1/v versus 1/[substrate A] at different fixed substrate B concentrations are expected to yield a family of parallel lines whose slopes equal \( K_m / V_{max} \). In fact, converging curves were found, which suggested a sequential mechanism. Dead-end inhibitors were used in order to further investigate the kinetic mechanism. S-Farnesylthioacetic acid is shown to be a dead-end competitive inhibitor with respect to the prenylated substrate L-AFC. On the other hand, S-farnesylthioacetic acid proved to be uncompetitive with respect to AdoMet, suggesting an ordered mechanism with AdoMet binding first. Further evidence for this mechanism came from product inhibition studies using the methyl ester of L-AFC (L-AFCMe) and S-adenosyl-L-homocysteine (AdoHcy). Since AdoMet binds first to the enzyme, one of the products (L-AFCMe or AdoHcy) should be a competitive inhibitor with respect to it. It could be shown that AdoHcy is a competitive inhibitor with respect to AdoMet, but L-AFCMe is a mixed-type inhibitor both with respect to AdoMet and to L-AFC. Therefore, AdoHcy combines with the same enzyme form as does AdoMet, and must be released from the enzyme last. Moreover, L-AFC and L-AFCMe must bind to different forms of the enzyme.

The isoprenylation of proteins is an important hydrophobic posttranslational modification, which is thought to target the modified protein to a membrane (1-4). Membrane association is assumed to be essential for the activation of isoprenylated proteins, which include the heterotrimeric G-proteins (5-8), the small G proteins (including ras) (1-3, 9-12), and lamin A precursor and lamin B (13, 14). The isoprenylation process consists of three distinct biochemical reactions. In the first reaction, a protein with a carboxyl-terminal CAAX (C = cysteine, A = aliphatic amino acid, and X is undefined) is condensed with either farnesyl pyrophosphate (C15) or geranylglycerol pyrophosphate (C20) at the cysteine residue (1, 15-23). The exact sequence at CAAX determines whether a farnesyl or geranylglycerol group is transferred to the cysteine residue (16, 24). After the isoprenoid is added to the cysteine, proteolytic cleavage of the protein occurs at the modified cysteine (1, 2). Finally, the carboxyl-terminal isoprenylated cysteine residue is methylated by an AdoMet-linked methyltransferase, producing the methyl ester (25-27). This latter reaction is the only reversible one in the pathway and thus the only reaction susceptible to regulation. Methylation is biochemically reversed by a methyl ester esterase (27). The role of this methylation process is currently unknown, but it could be related to increasing the hydrophobicity of the modified protein thus enhancing its association with membranes, or it could be important in protein-protein recognition, as in a receptor mediated event. Either way, it is quite possible that the methyltransferase plays a key control role in the isoprenylation pathway.

Although the methyltransferase is designed to methylate proteins with an isoprenylated carboxyl-terminal cysteine residue, the methyltransferase can be assayed with simple substrates. For example, hexapeptides terminating in a farnesylated cysteine residue are substrates (26), as are simple N-acetyl farnesylated cysteine analogs, such as N-acetyl-S-farnesyl-L-cysteine (L-AFC) (27). S-Farnesyl thioisopropionic acid (FTP) is also a substrate for the methyltransferase and appears to define the ultimate substrate recognition unit for the enzyme (28). S-Farnesyl thioacetic acid (FTA), which contains one less carbon atom than FTP, is not a substrate for the enzyme, but is a potent inhibitor of it (28). To begin to characterize the mechanism of action of the methyltransferase, its kinetic mechanism must first be determined. In a bisubstrate reaction of this type, several (often not mutually exclusive) alternatives present themselves, including ping-pong Bi Bi, rapid equilibrium random mechanisms, and ordered Bi Bi mechanisms. It is shown here that the mechanism is neither ping-pong Bi Bi nor a rapid equilibrium random mechanism. By using FTP as a dead-end inhibitor and L-AFC methyl ester as a product inhibitor, it is concluded that the kinetic mechanism is ordered, with AdoMet binding first and AdoHcy being released last.

**EXPERIMENTAL PROCEDURES**

**Materials**

Frozen bovine retinas were obtained from W. Lawson Co. (Lincoln, NE). S-Adenosyl[methyl-\(^{3}H\)]methionine (15 Ci/mmol) (AdoMet) was purchased from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: AdoMet, S-adenosyl-L-methionine; AdoHcy, S-adenosyl-L-homocysteine; L-AFC, N-acetyl-S-farnesyl-L-cysteine; L-AFCMe, N-acetyl-S-farnesyl-L-cysteine methyl ester; FTP, all-trans-farnesylthioacetic acid; FTP, all-trans-farnesylthioacetylproionic acid; DMSO, dimethyl sulfoxide; ROS, rod outer segment; HPLC, high-performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
from Amersham Corp. GTP, dithiothreitol, and phenylmethylsulfonyl fluoride were from Fluka Inc.; dimethyl sulfoxide was from Fisher; and S-adenosyl-L-homocysteine (AdoHcy) was from Boehringer Mannhein Inc.

Methods
Syntheses of Small Molecules—L-AFC, FTA, and L-AFCMe were synthesized as previously reported (28). Preparation of ROS Membranes—ROS membranes depleted of peripheral proteins were used as the source of methyltransferase. ROS membranes were obtained as described previously (29). The membranes were washed with low ionic strength buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, and 0.1 mM EDTA) and GTP, resuspended in 50 mM Hepes-Na, pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, and stored at -80 °C until used. The methyltransferase enzyme is membrane-bound and has thus far not been detergent-solubilized in a stable form, which precludes its purification.

Kinetic Measurements in the Absence of Inhibitor—L-AFC was dissolved in DMSO and mixed with S-adenosyl[methyl-3H]methionine (15 Ci/mmol) in the presence of buffer containing 200 mM Hepes, pH 7.4, 100 mM NaCl, and 5 mM MgCl₂. The substrates were pre-equilibrated at 37 °C for 5 min, then washed ROS membranes (0.5 mg of protein per ml) were added to the buffer. The final concentration of DMSO in the assay was 4% (v/v), and the incubation time for the assay was 15 min. The reaction was quenched with a mixed solution of chloroform and methanol (1:1), followed by the addition of water. After vortexing and centrifuging, the lower layer was removed and the solvent was gently evaporated with nitrogen. The residues were dissolved in isopropanol-hexane (15:85) and the samples were analyzed on a normal-phase HPLC column (Dynamax 60) connected to an on-line Berthold radioactivity monitor and eluted with isopropanol-hexane (15:85) at a flow rate of 1.5 ml/min.

Kinetic Measurements in the Presence of Inhibitors—The procedure is similar to that described above where no inhibitors were added. FTA, AdoHcy, or L-AFCMe were dissolved in DMSO, respectively, and pre-equilibrated with substrates at 37 °C for 5 min before ROS membranes were added. The incubation time was 15 min, and the final concentration of DMSO was 8% (v/v), except in the experiments shown in Fig. 2B where 4% of DMSO was used. The control experiments sans inhibitors were carried out with final concentrations of either 8 or 4% (v/v) DMSO.

RESULTS
In the initial series of experiments it was of interest to distinguish between a ping-pong Bi Bi mechanism and a rapid equilibrium random or ordered Bi Bi mechanism. This can sometimes be accomplished by measuring the velocity of product formation as a function of one of the substrates at different fixed concentrations of the other substrate. In a ping-pong Bi Bi mechanism, plots of 1/v versus 1/[substrate A] at different fixed substrate B concentrations should yield a family of parallel lines whose slopes equal $K_{cat}/V_{max}$ (30). On the other hand, both rapid equilibrium random and ordered mechanisms yield lines which intersect at the point where the 1/[substrate A] value is negative and the 1/v value is positive, negative, or zero (31). Therefore, kinetic studies of this kind are informative with respect to whether a ping-pong Bi Bi mechanism is operative or not. Rates of L-AFC methyl ester formation were determined by varying AdoMet concentrations at different fixed L-AFC concentrations and by varying L-AFC concentrations at different fixed AdoMet concentrations, as shown in Fig. 1. The curves in Fig. 1 rule out the possibility of a ping-pong Bi Bi mechanism since they converge, but they do not distinguish between random or ordered kinetic mechanisms.

To distinguish between a rapid equilibrium random mechanism and an ordered Bi Bi mechanism, a specific dead-end inhibitor is required. As mentioned above, FTA is a potent inhibitor of the enzyme (28). It is established in Fig. 2A that FTA is competitive with respect to L-AFC, with a measured $K_i = 1.21 \mu M$ under the conditions studied (Table I). By comparison, the apparent $K_{cat}$ for L-AFC is 20.2 μM. This means FTA binds the same enzyme form as L-AFC does. When FTA was tested as an inhibitor by varying AdoMet concentrations at different fixed FTA concentrations, the results in Fig. 2B were obtained. These results demonstrate that FTA is uncompetitive with respect to AdoMet, a result that is expected in an ordered mechanism if AdoMet binds first (32). If FTA were able to combine with free enzyme, i.e. L-AFC bound the enzyme before AdoMet did, Fig. 2B would have exhibited lines with different slopes and intercepts and converged at the point where both 1/v and 1/[AdoMet] values are negative. If a random mechanism were operative, then FTA should have been a mixed-type inhibitor with respect to AdoMet (33).

The experiments described above demonstrate an ordered reaction profile in which AdoMet binds to the enzyme first. In order to determine which product departs from the enzyme last, product inhibition studies were performed. First, the kinetics of product formation were determined at a fixed L-AFC concentration (50 μM), varying subsaturating AdoMet concentrations at different concentrations of AdoHcy (Fig. 3). As can be seen here, AdoHcy is a competitive inhibitor...
Kinetic Mechanism of Protein Methyltransferase

A

FIG. 2. A, Lineweaver-Burk plots for the dead-end inhibition of methylation of AFC in the presence of (■) no FTA, (○) 0.5 μM and (▲) 2 μM of FTA, and varied [AFC]. [AdoMet] = 10.67 μM, [DMSO] = 8%, and the incubation time = 15 min. The symbols represent the average values of triplicate experiments. B, Lineweaver-Burk plots for the dead-end inhibition of methylation of AFC in the presence of (■) no FTA, (○) 2 μM and (▲) 5 μM of FTA, and varied [AdoMet]. [AFC] = 20 μM, [DMSO] = 4%, and the incubation time = 15 min. The symbols represent the average values of triplicate experiments.

TABLE I

<table>
<thead>
<tr>
<th>Varied substrate</th>
<th>FTA</th>
<th>AdoHcy</th>
<th>L-AFCMe</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-AFC</td>
<td>Competitive (1.21 μM)</td>
<td>Mixed-type (3.26 μM)</td>
<td>Mixed-type (40.78 μM)</td>
</tr>
<tr>
<td>AdoMet</td>
<td>Uncompetitive (1.94 μM)</td>
<td>Competitive (73.4 μM)</td>
<td>Mixed-type (73.4 μM)</td>
</tr>
</tbody>
</table>

mixed-type inhibition with respect to the other substrate. As shown in Fig. 5, AdoHcy is a mixed-type inhibitor with respect to L-AFC with a $K_i = 3.26 \mu M$ (Table I). Finally, Fig. 6 shows that L-AFCMe is a mixed-type inhibitor with respect to L-AFC with a $K_i = 40.78 \mu M$ (Table I). These results are as expected and provide further evidence for an ordered sequential Bi Bi mechanism with AdoMet binding first and AdoHcy departing last.

DISCUSSION

In the experiments described here, the AdoMet-dependent isoprenylated protein methyltransferase from ROS was investigated with respect to its kinetic mechanism. This membrane-bound enzyme, which has yet to be successfully solubilized and purified, methylates proteins terminating in an isoprenylated cysteine residue. In the ROS this includes the β subunit of transducin, a group of small G proteins, the α subunit of the phosphodiesterase, and probably rhodopsin kinase (6, 27, 34, 35). Fortunately, the enzyme also methylates simple substrates, such as L-AFC and FTP, which makes
the release of AdoHcy. Transfer of the methyl group to L-AdoMet to an active-site residue of the enzyme, followed by a ping-pong mechanism studies feasible (27, 28). Moreover, simple dead-end inhibitors of the enzyme, such as FTA, are also available, which facilitates the kinetic analysis (28).

The experiments described here were initiated by determining whether or not a ping-pong Bi Bi mechanism was operative. As applied to a methyltransferase, a ping-pong mechanism would involve the initial transfer of a methyl group from AdoMet to an active-site residue of the enzyme, followed by the release of AdoHcy. Transfer of the methyl group to L-AFC would then occur, to generate the product L-AFCMe. In a bisubstrate Lineweaver-Burk plot, the nature of the slopes and intercepts of the family of lines provides an indication of the reaction mechanism type. In a ping-pong Bi Bi mechanism, the lines are parallel, and in a sequential reaction, either random or ordered, the lines will converge either on, above, or below the 1/[substrate] axis (31). The results of the experiments shown in Fig. 1 demonstrate that the methyltransferase mechanism must be of the sequential type and not a ping-pong Bi Bi mechanism. In a completely random mechanism, the enzyme can initially form a complex with either substrate, and if the binding of the first substrate increased the affinity of the enzyme for the second substrate, the lines should converge above the 1/[substrate] axis. The data in Fig. 1, however, do not permit a clear decision to be made with respect to the nature of the sequential mechanism type.

In order to distinguish between an ordered and a random mechanism, studies with a dead-end inhibitor of the methyltransferase were undertaken. First, the substrate analog FTA was shown to be a pure competitive inhibitor with respect to L-AFC, as shown in Fig. 2A. A compilation of the modes of inhibition observed along with the measured $K_i$ values are shown in Table I. The calculated $K_i$ for FTA was determined to be 1.2 $\mu$M. This value is slightly lower than had previously been reported (4.6 $\mu$M), but the conditions of the experiments were different, especially with respect to the DMSO concentrations (28). In a random mechanism, FTA would also be expected to be a mixed-type inhibitor with respect to AdoMet. That is, in a $1/v$ versus $1/[\text{AdoMet}]$ plot, at different FTA concentrations the lines should converge in the same quadrant with negative $1/[\text{AdoMet}]$ and positive $1/v$ values. In fact, as shown in Fig. 2B, the lines are parallel, which is indicative of an ordered Bi Bi mechanism (36). The inhibition pattern is clearly of the uncompetitive type, and is quite indicative of an ordered Bi Bi mechanism. The experiment shown in Fig. 2B is also informative with respect to the ordering of the substrates. If FTA combined with free enzyme, the Lineweaver-Burk plot of $1/v$ versus $1/[\text{AdoMet}]$ should have given a family of lines which had different slopes and intercepts. The intercept is affected when the dead-end inhibitor and varied substrate combine with a different form of the enzyme. The slope would be affected if FTA combined with the enzyme before the varied substrate (AdoMet) did. The parallel lines shown in Fig. 2B mean that FTA binds to a different enzyme form than does AdoMet, because the intercepts were affected but the slopes were not. Therefore, FTA adds to the enzyme after AdoMet, and the latter must thus bind first in an ordered Bi Bi mechanism.

To further investigate the nature of the ordering process, product inhibition studies were initiated. Since AdoMet binds first to the enzyme, one of the products, L-AFCMe or AdoHcy, should be a competitive inhibitor with respect to it. In Fig. 3 it could be shown that AdoHcy is a competitive inhibitor with respect to AdoMet, but L-AFCMe is a mixed-type inhibitor with respect to AdoMet (Fig. 4). Therefore, AdoHcy combines with the same enzyme form as does AdoMet and must be released from the enzyme last. In addition to product inhibition experiments being performed with respect to AdoMet, they were also performed with respect to L-AFC (Figs. 5 and 6). An ordered sequential Bi Bi mechanism requires that both products show either noncompetitive or mixed-type inhibition with respect to the other substrate. Fig. 5 shows that AdoHcy is a mixed-type inhibitor with respect to L-AFC, and Fig. 6 shows that L-AFCMe is a mixed-type inhibitor with respect to L-AFC. This latter result is expected because L-AFC and L-AFCMe must bind to different forms of the enzyme. These
results provide further evidence for an ordered sequential Bi Bi mechanism with AdoMet binding first and AdoHcy departing last.

In summary, the kinetic experiments described above show that an ordered Bi Bi mechanism describes the kinetic mechanism of the prenylated protein methyltransferase which is derived from the ROS. The cofactor AdoMet binds first, followed by the prenylated substrate. After methyl group transfer has occurred, the methylated product leaves, followed by AdoHcy (Scheme 1). This mechanism has important implications for any possible affinity chromatographic purification scheme for the enzyme. The membrane bound enzyme binding to the affinity gel would only occur in the presence of AdoMet. In addition, for any methyltransferase based on prenylated substrate analogs to function in cells, AdoMet will have to be present.

The question arises as to whether the ordered mechanism described here is a general one for methyltransferase enzymes. If so, it would suggest that the general ordering has mechanistic implications. However, this appears not to be the case. AdoMet:magnesium protoporphyrin methyltransferase from Euglena gracilis shows a random mechanism (37), as does the methyltransferase which operates on myelin basic protein (38). Ordered mechanisms are observed with the HhI methyltransferase (DNA first) (39) and with two DNA methyltransferases from Bacillus subtilis (20), where AdoMet binds first. Therefore, although the kinetic mechanism is, of course, of great importance for any particular enzyme, a general mechanistic theme for the AdoMet-dependent methyltransferases does not emerge, based on kinetic analysis alone.

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