Steric Course of the Methyl Transfer from AdoMet to *S*-Farnesyl-3-thiopropionate by G-Protein Methyltransferase

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Methylation is one of the important posttranslational modifications in G-proteins containing a carboxy terminal CAAX or CAC motif. This methylation occurs at the carboxyl group of the terminal cysteine of these proteins, generally following isoprenylation (C_{15} farnesylation or C_{20} geranylgeranylation) of the sulfur atom of cysteine and then proteolysis at the CA linkage to expose the carboxyl group of the cysteine.[1] The methyl group is transferred from *S*-adenosylmethionine (AdoMet) by the G-protein methyltransferase, a membrane-bound enzyme. This enzymatic reaction is the only reversible step in these posttranslational modifications, suggesting a dynamic role for methylation and demethylation.[2,3] The posttranslational modifications are essential for the function of the G-proteins and serve to promote protein−membrane or protein−protein interactions.[1]

In the course of studying the methylation of G-proteins, interestingly, it was observed that the methyltransferase has the ability to use simple nonproteinaceous substrate analogues such as *N*-acetyl-*S*-farnesyl-L-cysteine (AFC)[3] and *S*-farnesyl-3-thiopropionate (FTP). However, a shortened substrate, *S*-farnesyl-2-thioacetate (FTA), was found to be a competitive inhibitor and an extended one, *N*-acetyl-*S*-farnesyl-L-homocysteine (AFHC), was inactive as a substrate.[4] The distance between the sulfur atom and the carboxyl group (two intervening carbon atoms) and the nature of the isoprenyl group, i.e., farnesyl or geranylgeranyl but not geranyl, are important factors in the methylation. Thus, FTP incorporating the minimum structural unit required for the G-protein methyltransferase is the simplest substrate used to investigate the nature of the enzyme.[4]

The majority of AdoMet-dependent methyltransferases catalyze a single, direct transfer of the methyl group from the sulfur of AdoMet to the acceptor nucleophile via an S_N2 transition state.[5] This might also be a plausible mechanism for G-protein methyltransferase. However, the absolute requirement for the sulfur atom in the substrates for this enzyme[1] suggests the alternative possibility that the transfer of the methyl group of AdoMet might occur in two steps, i.e., first transfer to the sulfur atom of the substrate and then a second, intramolecular transfer to the carboxylate. The two mechanisms can be easily distinguished on the basis of stereochemistry; the single direct transfer would result in inversion of methyl group configuration, whereas the alternative double transfer would give net retention of configuration. There are two known examples of methyltransferases operating with net retention, one involved in thiostrepton[6] and the other in thienamycin biosynthesis.[7] In addition, several transfers of methyl groups from unactivated positions also proceed by two-step mechanisms, in this case via methylated corrin intermediates. Thus, for example, the B_{12}-dependent methionine synthase from *Escherichia coli* has been shown to operate with net retention of methyl group configuration,[8] as does the enzyme from *Methanosarcina barkeri* transferring the methyl group of methanol to coenzyme M.[9]

To probe the steric course of the methylation by G-protein methyltransferase, (*methyl-R*)- and (*methyl-S*)-[*methyl*-$^2H_1$,^{3}H]AdoMet prepared earlier[10] were used as methyl donors and FTP as a model substrate. The samples of stereospecifically tritiated AdoMet were each incubated with FTP and G-protein methyltransferase from rod outer segment (ROS) membranes. It had been observed that the G-proteins of ROS are reversibly methylated and demethylated.[3] The tritiated reaction products (*R*)- and (*S*)-[$^2H_1$,^{3}H]methyl *S*-farnesyl-3-thiopropionate (FTP-OMe) were purified by HPLC, diluted with nonlabeled carrier FTP-OMe, and converted to [2-$^2H_1$,^{3}H]acetonitrile by reaction with KCN.[11] The KCN reaction mixture was heated at reflux in an evacuated closed system to evaporate immediately the product, acetonitrile, avoiding racemization by excess KCN. The isotopically chiral acetonitrile was converted to acetic acid by the sequence of steps shown in Figure 1.[10] The resulting [2-$^2H_1$,^{3}H]acetic acid was subjected to configurational analysis of the methyl group.[12−14] The acetic acid was converted to acetyl-CoA by acetate kinase and phosphotransacetylase with ATP and CoASH. The acetyl-CoA was then converted to malate by malate synthase with glyoxylate. In the latter reaction, a proton is abstracted from the chiral methyl group of acetyl-CoA, and a large primary kinetic isotope effect ($k_H/k_D = 3.8$)[15] results in an uneven tritium distribution between the two

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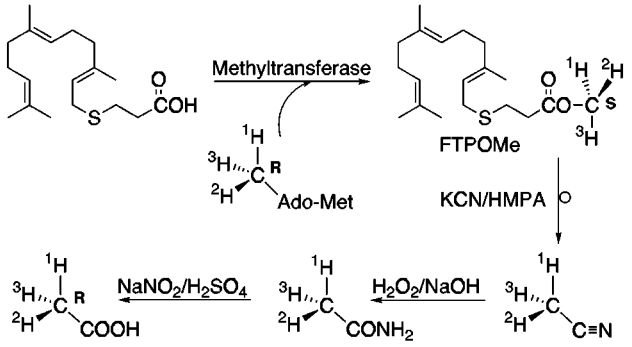

Figure 1. Methylation of *S*-farnesyl-3-thiopropionate by G-protein methyltransferase and degradation of methyl *S*-farnesyl-3-thiopropionate to acetate.

remaining methylene hydrogens. This tritium distribution is then determined by incubation of the L-malate with fumarase. The percentage of tritium retention in the fumarase reaction is called the *F* value, from which the enantiomeric excess can be calculated.[16] The *F* value of pure *(S)*-acetate is 21 and that of the pure *R* isomer is 79.

Configurational analysis of the acetic acid from the (*methyl-R*)-[*methyl*-$^2H_1$,^3H]AdoMet experiment gave an *F* value of 70, corresponding to 69% ee *R* configuration. A refence sample of optically pure (*S)*-acetate analyzed in parallel gave an *F* value of 23. The radioactivity of the acetate sample (1 nCi) obtained from (*methyl-S*)-[*methyl*-$^2H_1$,^3H]AdoMet was insufficient to complete the configurational analysis. Since the chemical conversion of the FTP-OMe to acetic acid involves one inversion of configuration in the cyanide displacement step (S_N2 reaction) to give acetonitrile, the configuration of the enzymatically generated FTP-OMe must be opposite to that of the acetic acid. The result, an *F* value of 70, therefore shows that the transfer of the methyl group from AdoMet to FTP catalyzed by G-protein methyltransferase proceeds with net inversion of configuration.

The stereochemistry established for this enzyme conforms to that of the majority of AdoMet-dependent methyltransferases which transfer the methyl group from AdoMet to a variety of different nucleophiles with inversion of configuration.[5] The AdoMet-dependent G-protein methyltransferase studied here thus most likely mediates a single direct transfer of the methyl group of AdoMet, activated as the sulfonium ion, through an S_N2 transition state to the acceptor nucleophile, the carboxylate anion of the FTP. The alternative possibility, transfer of the methyl group in two steps, first from AdoMet to the sulfur atom of FTP and then intramolecularly to the carboxylate, is ruled out by the above result.

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Experimental Section

Methylation of *S*-Farnesyl-3-thiopropionate (FTP) Using (*methyl*-R)- and (*methyl*-S)-[*methyl*-$^2H_1$,^3H]AdoMet. The substrate, 2 μL of FTP (2.5 mM in DMSO), was incubated with washed ROS membranes (0.5 mg of protein/mL) and *S*-adenosyl-[*methyl*-$^2H_1$,^3H]methionine (0.88 μCi *R*-AdoMet, sp act. 81 mCi/mmol; 0.46 μCi *S*-AdoMet, sp act. 100 mCi/mmol) in 100 mM Hepes (pH 7.4), 100 mM NaCl, and 5 mM $MgCl_2$ for 30 min at 37 °C (total incubation volume 50 μL). The amount of (*R*)- and (*S*)-[$^2H_1$,^3H]methyl *S*-farnesyl-3-thiopropionate (FTP-OMe) formed was determined by HPLC analysis of the chloroform extracts obtained from the incubation mixture as described previously.[4] Samples were injected in 15% 2-propanol/hexane on a normal phase HPLC column (Dynamax 60) connected to a Berthold radioactivity monitor and eluted with the same solvent at 1.5 mL/min to give FTP-OMe (t_R 3 min), 0.12 μCi from (*methyl-R*)-[*methyl*-$^2H_1$,^3H]AdoMet and 0.0085 μCi from (*methyl-S)*-[*methyl*-$^2H_1$,^3H]AdoMet.

Degradation of (*R*)- and (*S*)-[$^2H_1$,^3H]Methyl *S*-Farnesyl-3-thiopropionate (FTP-OMe). [*methyl*-$^2H_1$,^3H]FTP-OMe (0.12 μCi from (*methyl-R*)-[*methyl*-$^2H_1$,^3H]AdoMet and 0.0085 μCi from (*methyl-S)*-[*methyl*-$^2H_1$,^3H]AdoMet) diluted with nonlabeled carrier FTP-OMe (30 mg, 0.097 mmole), KCN (150 mg, 2.3 mmol), and dry hexamethylphosphoramide (HMPA, 1 mL) were placed in a 25 mL round-bottom flask. The flask was attached to a manifold for lyophilization which was connected to a receiving flask containing 1 mL of water. Both flasks were frozen in dry ice/2-propanol baths, and the system was evacuated to 500 Torr. The dry ice/2-propanol bath was removed from the reaction flask and replaced by in an oil bath (110 °C) with continued dry ice/2-propanol cooling of the receiving flask. The reaction mixture was stirred at 110 °C for 3 days and then the receiving flask was removed from the system. The content, [2-$^2H_1$,^3H]acetonitrile in water, was mixed with 30% H_2O_2 (3 mL) and 6 N NaOH (0.15 mL). The reaction flask equipped with a reflux condenser was heated at 55 °C overnight. The reaction solution containing [2-$^2H_1$,^3H]acetamide was evaporated to dryness in vacuo. The residue was dissolved in water (2 mL) and cooled with an ice bath, and then 5 N H_2SO_4 (2 mL) was added dropwise. One milliliter of $NaNO_2$ solution (400 mg) was added slowly and the solution was stirred at room temperature for 12 h to produce [2-$^2H_1$,^3H]acetic acid. The flask containing the solution was frozen in a dry ice/2-propanol bath and attached to a lyophilization manifold with a receiving flask. The system was evacuated to about 500 Torr and the bath was moved to the receiving flask, thus transferring the acetic acid. The collected [2-$^2H_1$,^3H]acetic acid was adjusted to pH 9 with 0.1 N NaOH and then lyophilized to dryness to give sodium [2-$^2H_1$,^3H]acetate (0.05 μCi) in 41% radiochemical yield from [*methyl*-$^2H_1$,^3H]FTP-OMe isolated from the incubation with (*methyl-R*)-[*methyl*-$^2H_1$,^3H]AdoMet. The sodium [2-$^2H_1$,^3H]acetate was subjected to the configurational analysis as described.[14] The radioactivity of the sodium [2-$^2H_1$,^3H]-acetate (0.001 μCi) from the FTP-OMe derived from the incubation with (*methyl-S*)-[*methyl*-$^2H_1$,^3H]AdoMet was not sufficient for configurational analysis.

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