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Prenylated protein methyltransferases do not distinguish between farnesylated and geranylgeranylated substrates

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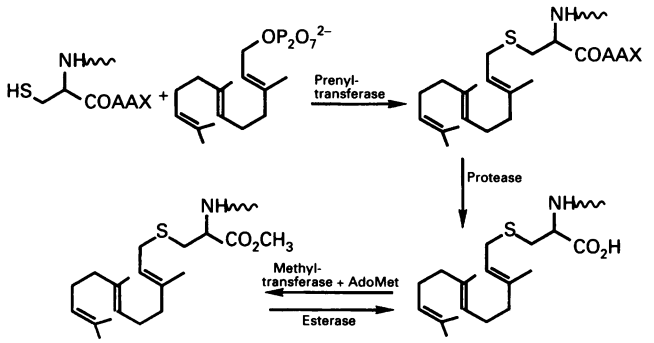
Proteins that are post-translationally modified by prenylation can be either farnesylated (C-15) or geranylgeranylated (C-20) by separate prenyltransferase enzymes. Prenylated proteins are also methylated at their *C*-terminal residue by S-adenosylmethionine-linked methylation. In this paper we show that the methylation of farnesylated and geranylgeranylated substrates can be accounted for by the presence of a single enzyme. It is demonstrated that the K_m and $V_{max.}$ values for the retinal rod outer segment methyltransferase, measured with small molecule farnesylated and geranylgeranylated substrates, are identical. These substrates mutually inhibit each other's methylation, with K_I values being equal to their K_m values. The K_m for *S*-adenosylmethionine was measured to be the same with either farnesylated or geranylgeranylated substrates. Competitive inhibitors of the methyltransferase containing either a geranylgeranyl or a farnesyl group equally block the methylation of synthetic geranylgeranylated and farnesylated substrates of the enzyme. Importantly, these inhibitors are also equipotent at inhibiting the methylation of the physiological substrates of the rod outer segment methyltransferase. These substrates are both farnesylated and geranylgeranylated. One of these substrates had previously been identified as the farnesylated γ subunit of transducin. Therefore it appears that the same enzymic activity can methylate both farnesylated and geranylgeranylated substrates.

INTRODUCTION

Signal-transducing G proteins are post-translationally modified by prenylation (Scheme 1) [1,2]. This hydrophobic modification is thought to direct the modified protein to a membrane [3–6]. In the prenylation process, proteins containing a CAAX box (where C is cysteine, A is an aliphatic amino acid and X is any amino acid) at their *C*-termini are first S-alkylated at cysteine with either farnesyl pyrophosphate (C-15) or geranylgeranyl pyrophosphate (C-20) to produce a prenylated thioether [7–15]. The products of the *ras* oncogenes are farnesylated [7–10] and the heterotrimeric G proteins can be either farnesylated or geranylgeranylated at their γ subunits [11–14]. After prenylation, a protease cleaves the C–A bond of the CAAX box [3,5,16]. Subsequent carboxymethylation of the modified cysteine by an *S*-adenosylmethionine (AdoMet)-dependent methyltransferase produces the final prenylated methyl ester [17–19] (Scheme 1). The methylation reaction is the only potentially reversible step in this pathway. Indeed, G protein methylation has been shown to be reversible in rod outer segments (ROS), suggesting a possible regulatory role for this modification [19].

G protein methyltransferase recognizes very simple, small molecule substrates [19,20]. For example, *S*-(farnesyl-3-thio)propionic acid (FTP) is readily methylated by the ROS enzyme with K_m and $V_{max.}$ values close to those found for *N*-acetyl-*S*-farnesyl-L-cysteine (AFC) [20]. The structures of these two derivatives are shown in Fig. 1. It is, therefore, unlikely that the peptide backbone is an important factor for substrate recognition by the methyltransferase. On the other hand, substantial specificity is directed at the farnesylthiopropionate moiety of the protein. The farnesyl group (C-15) is essential for binding to the enzyme. Substitution of geranyl for farnesyl greatly weakens substrate activity, and hydrogenation of the farnesyl side-chain abolishes it completely [20]. Moreover, farnesylated molecules, in which the propionate moiety has been



Scheme 1. Post-translational protein modification by prenylation

See the text for details.

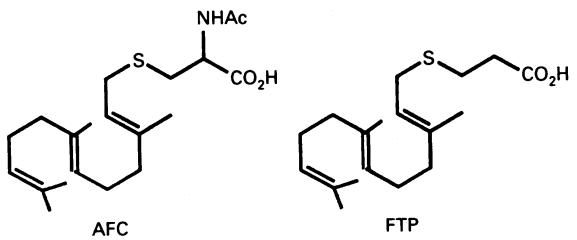


Fig. 1. Structures of AFC and FTP

Abbreviations used: AFC, *N*-acetyl-*S*-farnesyl-L-cysteine; FCM, *S*-farnesyl-L-cysteine methyl ester; FTP, *S*-(farnesyl-3-thio)-propionic acid; FTA, *S*-(farnesyl-2-thio)acetic acid; AGGC, *N*-acetyl-*S*-geranylgeranyl-L-cysteine; GGCM, *S*-geranylgeranyl-L-cysteine methyl ester; GGTA, *S*-(geranylgeranyl-2-thio)acetic acid; ROS, rod outer segment; AdoMet, *S*-adenosyl-L-methionine; Me_2SO, dimethyl sulphoxide; Tγ, γ subunit of transducin.

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altered, bind to the enzyme but are inactive as substrates, thus acting as competitive inhibitors. For example, *S*-(farnesyl-2-thio)-acetic acid (FTA) is a potent competitive inhibitor of the enzyme and inhibits AFC and transducin γ subunit methylation [20].

Because prenylated proteins can be either farnesylated or geranylgeranylated by distinct prenyltransferases [21,22], it is important to determine whether a specific methyltransferase is responsible for the methylation of farnesylated and geranylgeranylated proteins. In this article, farnesylated and geranylgeranylated molecules were studied as substrates for the ROS methyltransferase activity. These studies demonstrate that a single methyltransferase, or several very similar enzymes, in the ROS can process both the farnesylated and the geranylgeranylated substrates. This work also further defines the substrate specificity of G protein methyltransferases and makes it unlikely that inhibitors can be designed which will specifically inhibit only the methylation of either farnesylated or geranylgeranylated substrates.

EXPERIMENTAL

Materials

Frozen bovine retinas were obtained from W. Lawson Co. (Lincoln, NE, U.S.A.). S-Adenosyl[*methyl*-^{3}H]methionine ([^{3}H]AdoMet) (15 Ci/mmol and 85 Ci/mmol) and Amplify were purchased from Amersham. Tos-Phe-CH_2Cl-treated trypsin was from Fluka. Microsomal leucine aminopeptidase (type VI) and *N*-acetyl-L-cysteine was purchased from Sigma. *trans,trans,trans*-Geranylgeraniol was obtained from American Tokyo Kasei, Inc. *trans,trans*-Farnesyl bromide and mercaptoacetic acid were purchased from Aldrich.

Synthesis of analogues

trans,trans,trans-Geranylgeranyl bromide was synthesized from *trans,trans,trans*-geranylgeraniol [23]. *trans,trans*-Farnesyl-L-cysteine methyl ester (FCM) was prepared from *trans,trans*-farnesyl bromide and L-cysteine methyl ester hydrochloride using a previously described method for the preparation of *trans,trans,trans*-geranylgeranyl-L-cysteine methyl ester (GGCM) [14]. L-AFC was prepared from *trans,trans*-farnesyl bromide and *N*-acetyl-L-cysteine [24], and FTA was obtained from *trans,trans*-farnesyl bromide and mercaptoacetic acid [20]. *trans,trans,trans*-Geranylgeranyl-L-cysteine methyl ester was prepared as previously described [14]. *N*-Acetyl-*S*-geranylgeranyl-L-cysteine (AGGC) was prepared from *trans,trans,trans*-geranylgeranyl bromide and *N*-acetyl-L-cysteine using a previously described procedure [20]. *trans,trans,trans*-Geranylgeranyl bromide (593 mg, 1.68 mmol, 1.0 equiv.), *N*-acetyl-L-cysteine (492 mg, 3.02 mmol, 1.8 equiv.) and guanidine carbonate (362 mg, 2.01 mmol, 1.2 equiv.) were dissolved in acetone (75 ml). The resulting solution was stirred at room temperature for 18 h. The solvent was evaporated under reduced pressure and the residue was taken up in ethyl acetate (150 ml). The ethyl acetate solution was washed successively with 3.5% HCl (50 ml) and satd. NaCl (25 ml), dried (Na_2SO_4), and concentrated to a small volume. Chromatography of this material on a silica column eluted with ethyl acetate/methanol (4:1–1:2, v/v) gave AGGC as a white waxy solid in 64% yield: ^{1}H n.m.r. (500 MHz, $Me_2SO[^2H_6]$ δ (p.p.m.) 1.53 (9 H, s), 1.59 (3 H, s), 1.61 (3 H, s), 1.88–2.05 (12 H, m), 2.48 (3 H, s), 2.60 (1 H, dd, *J* 14 Hz, *J* 7.5 Hz), 2.81 (1 H, dd, *J* 14 Hz, *J* 4.0 Hz), 3.08 (1 H, dd, *J* 13.5 Hz, *J* 7.5 Hz), 3.14 (1 H, dd, *J* 13.5 Hz, *J* 7.5 Hz), 4.26 (1 H, m), 5.05 (2 H, m), 5.13 (1 H, bt, *J* 7.5 Hz), 7.91 (1 H, bs).

S-(Geranylgeranyl-2-thio)acetic acid (GGTA) was prepared by the method described above from *trans,trans,trans*-geranylgeranyl bromide and mercaptoacetic acid to provide GGTA as a colourless oil in 76% yield: ^{1}H n.m.r. (500 MHz, C^2HCl_3) δ (p.p.m.) 1.58 (3 H, s), 1.64 (3 H, s), 1.66 (9 H, s), 1.96 (4 H, m), 2.06 (8 H, m), 3.17 (2 H, s), 3.27 (2 H, d, *J* 7.5 Hz), 5.07 (3 H, m), 5.18 (1 H, bt, *J* 7.5 Hz).

The methyl esters of AFC and AGGC were obtained from their corresponding carboxylic acids by treatment with methanolic HCl (0.05–0.1 M) [25]. The isolated products gave n.m.r. spectra essentially identical to those of their parent compounds, except for singlet resonances, equivalent to 3 protons, at δ 3.74 p.p.m. for the methyl ester of AFC and at δ 3.76 p.p.m. for the methyl ester of AGGC.

Preparation of ROS membranes and methyltransferase assays

The ROS membranes used as the source of methyltransferase were obtained as previously described [19]. Attempts to solubilize the membrane-bound enzyme in a stable form have so far not been successful. Hence further purification of the enzyme has not been possible. Substrates were dissolved in Me_2SO and incubated with extensively washed ROS membranes (0.5 mg of protein/ml) and [^{3}H]AdoMet (10 μM, 15 Ci/mmol) in 100 mM-Hepes, pH 7.4, 100 mM-NaCl and 4 mM-$MgCl_2$ (buffer A) for 30 min at 37 °C. The final Me_2SO concentration in the assay was 4% (v/v). The amount of the corresponding ^{3}H-labelled methyl esters was determined by h.p.l.c. analysis of the chloroform extracts obtained from the incubation mixtures as described in [19]. Samples were injected on a normal-phase h.p.l.c. column (Dynamax 60, Rainin, Woburn, MA, U.S.A.) connected to an on-line radioactivity monitor (Berthold, Nashua, NH, U.S.A.). Elution was performed with 85% hexane/15% propan-2-ol at 1.5 ml/min. In all cases the methyl esters were readily separated from the parent acids.

Methylation of ROS proteins *in vitro*

Methylation reactions were carried out essentially as described [20]. Briefly, disrupted ROS or washed ROS membranes (1 mg of total protein/ml) were incubated with [^{3}H]AdoMet (2.34 μM; 85 Ci/mmol) in 100 μl of buffer A for 2 h at 37 °C. Purified transducin [26] was added to this mixture at 5 μM final concentration. Inhibitors were added in 2 μl of Me_2SO. The final Me_2SO concentration was 2% (v/v) in all mixtures. For electrophoresis, samples were precipitated in chloroform/methanol (1:2, v/v) [27]. The protein pellets were dissolved in sample buffer [28] containing 5% SDS, and boiled for 5 min. Samples were run in 15% polyacrylamide gels. To better visualize the γ subunit of transducin (Tγ), 0.1 M-sodium acetate was included in the anode buffer [29], and m-Cresol Purple was used as the tracking dye. After Coomassie Blue staining, gels were treated with Amplify and fluorographic exposures were carried out at −80 °C for 24 or 48 h with preflashed film. The fluorographic spots were quantified by densitometry.

RESULTS

Initial experiments were directed at studying AGGC as a substrate for the ROS methyltransferase. The K_m and $V_{max.}$ values obtained for AGGC (22 μM and 19 pmol/min per mg respectively) (Fig. 2) were almost identical to those found for AFC [20] (Table 1). When AFC and AGGC were used in competition assays (Fig. 2), they behaved as mutually competitive inhibitors. The K_I obtained for each compound was identical to its corresponding K_m as a substrate (Fig. 1 and Table 1).

FTA has previously been shown to be a potent inhibitor of the methylation of farnesylated substrates [20]. The geranylgeranyl analogue of FTA (GGTA) was synthesized and tested as an inhibitor of the methyltransferase. As shown in Fig. 3, GGTA is a potent inhibitor of the methylation of AFC. The calculated K_I

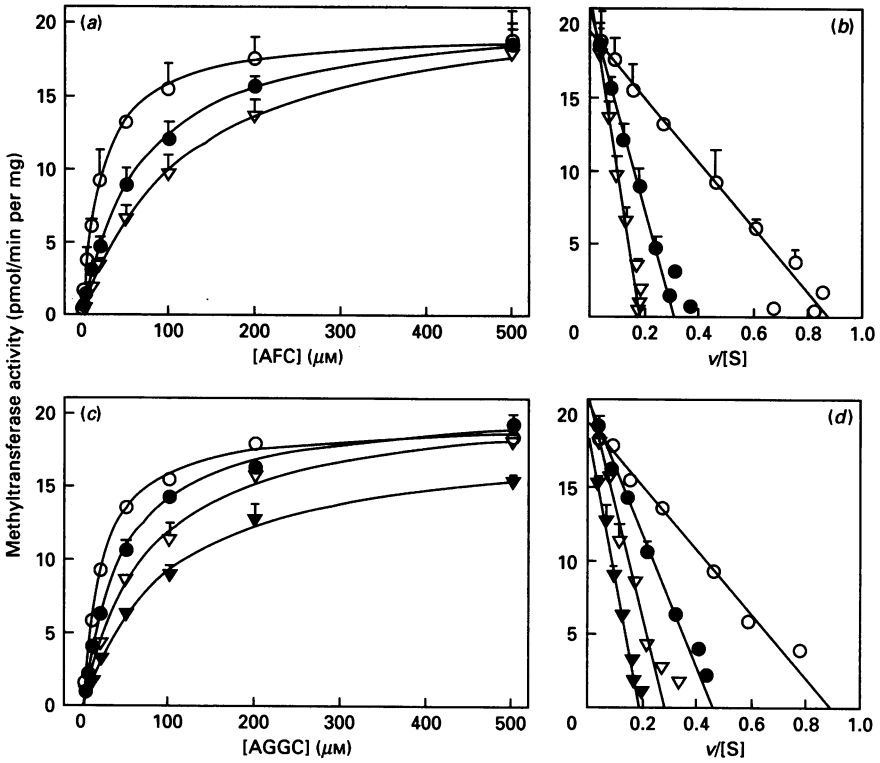
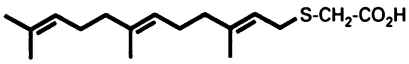
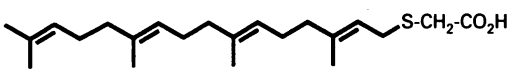


Fig. 2. Kinetic plots for the methylation of AFC and AGGC when used as competitive substrates

(*a*) Michaelis-Menten and (*b*) Eadie–Hofstee plots of the formation of AFC [^{3}H]methyl ester in the presence of no AGGC (○), 50 μM-AGGC (●) and 100 μM-AGGC (▽). (*c*) Michaelis–Menten and (*d*) Eadie–Hofstee plots of the formation of AGGC [^{3}H]methyl ester in the presence of no AFC (○) or 20 μM- (●), 50 μM- (▽) or 100 μM- (▼) AFC. Methyltransferase assays were performed as described in the Experimental section. AFC [^{3}H]methyl ester and AGGC [^{3}H]methyl ester were separated by h.p.l.c. in hexane/propan-2-ol/acetonitrile (17:2:1, by vol.) as eluant at a flow rate of 1.2 ml/min. Symbols represent average values of triplicate experiments and error bars are S.D. of the mean.

Table 1. Kinetic constants for prenylated molecules and AdoMet

Kinetic constants were obtained from plots shown in Figs. 2–4. Values marked * were obtained using AFC as substrate; values marked ** were obtained using AGGC as substrate. The K_I for FTA [20] is shown here for comparison.

	K_m (μM)	$V_{max.}$ (pmol/min per mg)	K_I (μM)
 AFC	22.3±2.9*	19.4±0.6*	22.6±4.2**
 AGGC	21.9±0.8**	19.2±0.2**	23.4±0.9*
 FTA	–	–	4.6±0.7*
 GGTA	–	–	3.9±0.7*
AdoMet	2.0±0.2*	25.3±0.6*	
AdoMet	1.9±0.2**	26.6±0.7**	

for GGTA is within the experimental error of the reported K_I value for FTA (Table 1). Moreover, in separate experiments both GGTA and FTA were able to inhibit the methylation of AFC and of AGGC to a similar extent. When the inhibitors were used at equimolar concentrations with the substrates (20 μM), GGTA inhibited AFC methylation by 68% and AGGC methylation by 75%, while FTA inhibited AFC methylation by 65% and AGGC methylation by 69%.

If the same methyltransferase processes both geranylgeranylated and farnesylated substrates, it would be expected that

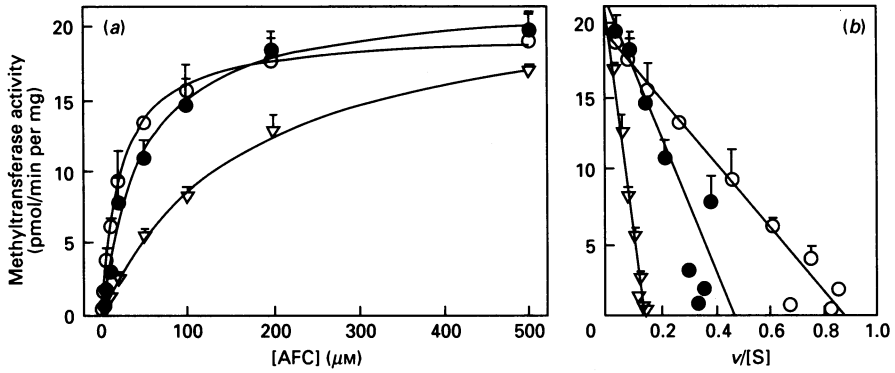


Fig. 3. Inhibition by GGTA of AFC methylation

(*a*) Michaelis–Menten and (*b*) Eadie–Hofstee plots of the formation of AFC [^{3}H]methyl ester in the presence of no GGTA (○), 5 μM-GGTA (●) and 20 μM-GGTA (▽). Symbols represent average values of three determinants and error bars are the S.D.

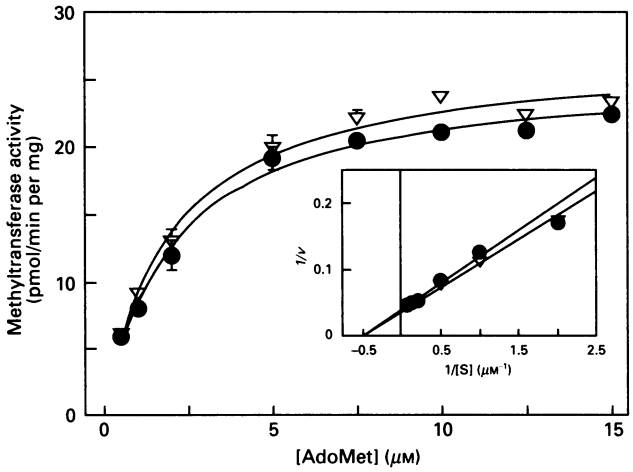


Fig. 4. Dependence of methyltransferase activity on AdoMet concentration

Michaelis–Menten and Lineweaver (insert) plots of the formation of AFC [^{3}H]methyl ester (●) and AGGC [^{3}H]methyl ester (▽) as a function of AdoMet concentration, with 200 μM-AFC (●) or -AGGC (▽) as substrate. Symbols represent average values of duplicate experiments and error bars are the S.D.

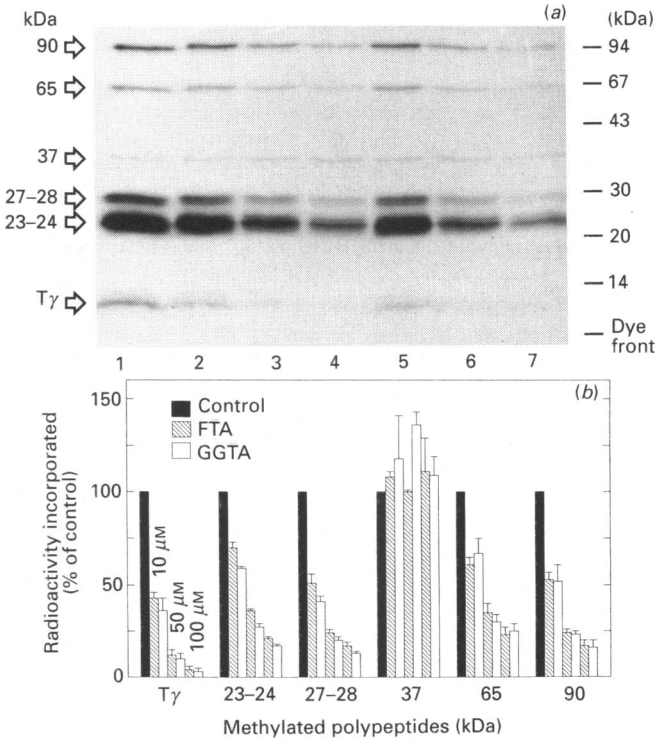


Fig. 5. Inhibition by FTA and GGTA of ROS protein methylation

Methylation reactions *in vitro* were carried out as described in the Experimental section, in the presence of several concentrations of FTA or GGTA. The experiment was repeated four times. A representative autoradiography is shown in (*a*). Lane 1, control; lane 2, 10 μM-FTA; lane 3, 50 μM-FTA; lane 4, 100 μM-FTA; lane 5, 10 μM-GGTA; lane 6, 50 μM-GGTA; lane 7, 100 μM-GGTA. The positions of the molecular mass standards in the Coomassie Blue staining of the same gel are indicated on the right. The apparent molecular masses of the methylated polypeptides and the position of Tγ are indicated on the left. Quantification of the radioactivity incorporated into Tγ and the other methylated polypeptides (identified by their molecular masses) is shown in (*b*). Values, expressed as percentage of control, are averages of four determinations and error bars represent the S.E.M.

the same K_m should be measured for AdoMet irrespective of whether a geranylgeranylated or a farnesylated substrate is used. When K_m values for AdoMet were determined by using AFC or AGGC as the second substrate (Fig. 4), the values obtained proved to be within experimental error of each other (Table 1). These results are also consistent with the idea that a single methyltransferase processes both the farnesylated and the geranylgeranylated substrates.

Tγ is a farnesylated protein known to be methylated in the ROS [11,19]. The abilities of GGTA and FTA to inhibit the methylation of this protein were compared (Fig. 5). Incubation of bovine ROS, supplemented with purified transducin, with [^{3}H]AdoMet results in the labelling of several polypeptides of molecular masses of approx. 6 (Tγ), 23–24, 27–28, 37, 65 and 90 kDa. Both GGTA and FTA inhibited the methylation of Tγ and the other proteins that are methylated in the ROS membranes to a similar extent, as quantified by densitometry (Fig. 5*b*). Both inhibitors were most effective at inhibiting Tγ methylation, and did not affect the methylation of the 37 kDa polypeptide.

Washed ROS membranes, with transducin absent, were labelled with [^{3}H]methyl groups and the nature of their adducted prenyl group(s) was determined. The proteins were proteolysed, as explained in the legend to Fig. 6, and the radioactive amino acids were separated by h.p.l.c. As seen in Fig. 6, virtually all of the labelled prenylated amino acids ran with the geranylgeranylated analogue. This would imply that, except for transducin, the preponderance of prenylated proteins in the washed

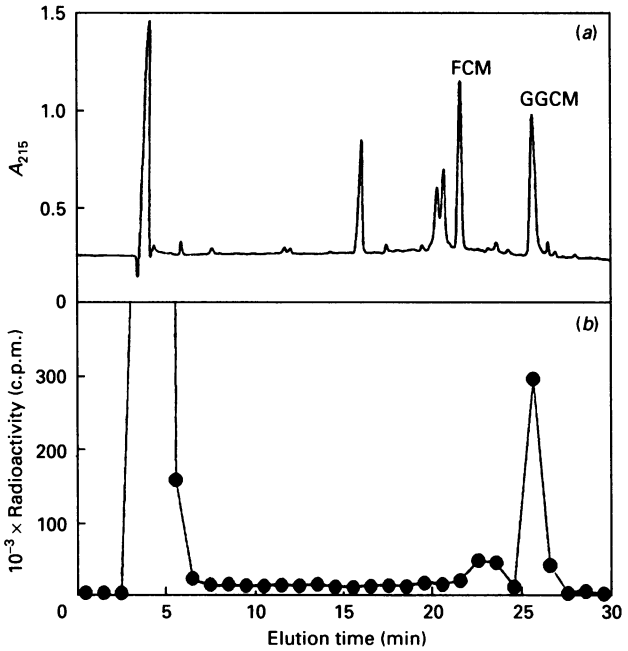


Fig. 6. H.p.l.c. chromatogram of Tos-Phe-CH_2Cl-treated trypsin-digested and microsomal leucine aminopeptidase-digested [^{3}H]methylated ROS proteins

Methylation of washed ROS membrane proteins *in vitro* was carried out as described in the Experimental section, except that purified transducin was not added to the incubation. Proteolysis of the methylated proteins was performed using a previously described procedure [15]. The digested sample was applied directly to a C_{18} reverse-phase column (Dynamax 300A). The column was eluted at 1 ml/min with a linear solvent gradient from 9.9% acetonitrile/0.1% trifluoroacetic acid/90% water (by vol., solvent A) to 100% acetonitrile (solvent B) in 30 min. (*a*) Proteolytic digest mixture plus synthetic FCM and GGCM standards. (*b*) Fractions (1 ml) were collected and diluted with 10 ml of scintillation fluid (Hydrofluor; National Diagnostics, Manville, NJ, U.S.A.), and radioactivity was counted.

ROS membranes are geranylgeranylated. The radioactivity at the front consists of unreacted [^{3}H]AdoMet and [^{3}H]methanol released by hydrolysis of [^{3}H]methyl esters during proteolytic digestion. Some of the ROS proteins are thus geranylgeranylated, rather than farnesylated. Therefore GGTA and FTA are effective at inhibiting the methylation of not only synthetic substrates but also of physiologically relevant farnesylated and geranylgeranylated substrates as well.

DISCUSSION

Extensive published structure–activity measurements on the G protein methyltransferase have revealed an interesting pattern. First, the nature of the peptide backbone seems not to be unduly important, because simple molecules such as AFC are excellent substrates [19,20]. Moreover, removal of the *N*-acetyl group of AFC produces FTP, which is still an excellent substrate for the methyltransferase [20]. All vestiges of the peptide backbone have been lost in FTP. However, small alterations in FTP can lead to molecules which are inactive as methyltransferase substrates. For example, oxidation of the sulphur to generate the sulphoxide, or deletion of a methylene group to produce FTA, transforms the substrate into a competitive inhibitor [20]. The farnesyl requirement is also strict. *N*-Acetyl-*S*-geranyl-L-cysteine is a very weak substrate for the enzyme, and fully hydrogenated AFC is inactive as a substrate [20]. The need for an intact farnesyl group has also been observed by using an *S*-farnesyl-L-cysteine-modified hexapeptide from *ras* as a substrate and rat liver microsomes as the source of methyltransferase [18]. Simple saturated analogues, such as the tridecyl and pentadecyl derivatives reported in [18], were relatively inactive as substrates. K_m values of 2.2 and 10.9 μM were estimated for the farnesylated and the geranylgeranylated hexapeptides respectively. However, the geranylgeranylated peptide appeared to be a non-specific inhibitor of the enzyme, allowing only a narrow range of concentrations of this peptide to be tested, all of which were substantially lower than the reported K_m [18]. Recently it has been stated that yeast membranes can also process the farnesylated and geranylgeranylated hexapeptide substrates, but that membranes from a methyltransferase-negative mutant (STE14) cannot [30]. This result would tend to support the idea that a single methyltransferase in yeast may be responsible for the methylation of synthetic geranylgeranylated and farnesylated substrates.

In the current study, AFC and AGGC were found to be processed by the ROS methyltransferase with almost identical kinetic constants. Moreover, AFC and AGGC showed identical cross-inhibition patterns. AFC inhibited AGGC methylation with a measured K_i virtually identical to its K_m as a substrate. The same was true for the AGGC inhibition of AFC methylation. The K_m for AdoMet measured with either AGGC or AFC as substrate was the same. Like FTA [20], GGTA also inhibited methylation of AFC. In fact, its measured K_i is the same as that reported for FTA [20]. In addition, GGTA and FTA inhibited the methylation of AFC and AGGC to the same extent. These experiments with synthetic substrates and inhibitors make it highly unlikely that functionally separate enzymes process farnesylated and geranylgeranylated substrates, and they strongly support the idea that a single enzyme is responsible for the methylation of both farnesylated and geranylgeranylated substrates. It should be noted that ROS membranes were used in the studies reported here because the membrane-bound enzyme has proved recalcitrant to solubilization and purification.

GGTA and FTA also inhibited the methylation of the endogenous ROS methyltransferase substrates. This shows that the observations made with the synthetic substrates are also relevant to the physiological substrates, some of which are farnesylated and some of which are geranylgeranylated. The major methylated products in the ROS are known to be prenylated [11,12,31], although only the post-translational modification on Tγ has previously been chemically characterized [11,12]. In this case the isoprenoid lipid has been shown to be *trans,trans*-farnesyl.

Some of the methylated proteins have not yet been identified. The identity of the previously unreported weakly labelled 37 kDa polypeptide is unclear, but the observation that neither FTA nor GGTA inhibited its methylation suggests that it may not be prenylated. The ~ 65 kDa protein has also yet to be identified, but is a good candidate for rhodopsin kinase. The methylated 90 kDa protein had previously been identified as the α subunit of the retinal phosphodiesterase [32]. It has recently been suggested that the α subunit of the ROS phosphodiesterase is farnesylated, while the β subunit is geranylgeranylated [31]. The small G proteins (20–30 kDa) are also prenylated [12,31]. It has been reported that two of these small G proteins can be ADP-ribosylated in the ROS by added botulinum toxin C3 [33]. This suggests that they are members of the rho family of small G proteins [34–37] which, on the basis of their *C*-terminal sequences [38,39], are likely to be geranylgeranylated. Interestingly, bovine ROS membranes have been reported to carboxymethylate the *C*-terminal cysteine residue of a 23 kDa G protein purified from brain [40], which has recently been identified as G25K and shown to be geranylgeranylated [15,21,22]. It is shown here that geranylgeranylated proteins are indeed present in ROS membranes and constitute a major form of prenylated proteins.

As described above, ROS contain both geranylgeranylated and farnesylated proteins. That GGTA and FTA inhibit the methylation of both classes of these prenylated substrates lends support to the idea that the ROS methyltransferase(s) is non-selective with respect to the farnesyl and geranylgeranyl side-chains, with both synthetic and physiologically relevant substrates.

It is likely that the methyltransferase enzyme(s) recognizes only the farnesyl side-chain, and that longer appendages do not strongly interact with the active-site region. As previously mentioned, side-chains shorter than the farnesyl moiety, such as geranyl, appear not to bind very well to the enzyme [20]. Thus, in terms of the structure–activity relationship of the side-chain, enzyme affinity appears to reach a plateau at farnesyl. Further structure–activity studies on the prenyl side-chain will be required to define the nature of the interactions between the enzyme and the side-chain moiety. With respect to methyltransferase inhibitor design, it is clear that even if there are multiple prenylated protein methyltransferases, they will not be distinguishable based on whether the side-chain is farnesyl or geranylgeranyl.

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