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Identification of an Isoprenylated Cysteine Methyl Ester Hydrolase Activity in Bovine Rod Outer Segment Membranes†

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ABSTRACT: Proteins from eucaryotic cells which have a carboxyl-terminal CAAX motif are posttranslationally modified by isoprenylation. The pathway involves the linkage of an *all-trans*-farnesyl (C15) or an *all-trans*-geranylgeranyl (C20) moiety to the cysteine residue followed by proteolysis which generates the modified cysteine as the carboxyl-terminal residue. Carboxylmethylation of the modified cysteine residue completes the pathway. This latter methylation reaction is the only potentially reversible reaction in the pathway and thus of possible regulatory significance. A specific esterase is required to reverse the methylation. It is demonstrated here that simple isoprenylated cysteine derivatives, such as *N*-acetyl-*S*-farnesyl-L-cysteine methyl ester (L-AFCM) and *N*-acetyl-*S*-geranylgeranyl-L-cysteine methyl ester (L-AGGCM), are substrates for a rod outer segment (ROS) membrane esterase activity. The K_M and V_{max} values for L-AFCM and L-AGGCM are 186 μM and 2.2 nmol mg^{-1} min^{-1} and 435 μM and 4.8 nmol mg^{-1} min^{-1}, respectively. The enzyme(s) is stereoselective rather than stereospecific because D-AFCM is enzymatically hydrolyzed with K_M and V_{max} values of 157 μM and 0.46 nmol mg^{-1} min^{-1}, respectively. The enzyme(s) does not process *N*-acetyl-L-cysteine methyl ester, demonstrating that the isoprenyl moiety is required for substrate activity. Ebelactone B is a potent mechanism-based inactivator of the enzyme with a K_I = 42 μM and a k_{inh} = 3.7 × 10^{-3} s^{-1}. Importantly, L-AFCM, L-AGGCM, and ebelactone B all inhibit the demethylation of the endogenous ROS substrates, showing that the same enzymatic activity is involved in the processing of the synthetic and physiological substrates.

The γ subunits of heterotrimeric G proteins and the "small" G proteins, including ras, are posttranslationally modified by isoprenylation (Casey et al., 1989; Farnsworth et al., 1990; Hancock et al., 1989; Lai et al., 1990; Maltese, 1990; Mumby et al., 1990; Schafer et al., 1989). Isoprenylation involves three modifications at proteins containing a carboxyl terminus of CAAX, where C = cysteine, A = an aliphatic amino acid, and X is undefined (Casey et al., 1989; Hancock et al., 1989; Lowy

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& Willumsen, 1990; Schafer et al., 1989). In the first reaction, the cysteine is reacted with *all-trans*-farnesyl pyrophosphate or *all-trans*-geranylgeranyl pyrophosphate to generate the isoprenylated cysteine moiety (Manne et al., 1990; Reiss et al., 1990; Schaber et al., 1990). Farnesyl pyrophosphate and geranylgeranyl pyrophosphate transferases have been identified which distinguish among CAAX sequences (Reiss et al., 1991; Seabra et al., 1991). The isoprenylated protein is then proteolytically cleaved after the modified cysteine residue, and finally the free carboxyl group at the newly generated modified cysteine C terminus is methylated by means of an *S*-adenosyl methionine (AdoMet)[1] linked methyltransferase (Clarke et al., 1988; Fukada et al., 1990; Gutierrez et al., 1989; Kawata et al., 1990; Perez-Sala et al., 1991; Stephenson & Clarke, 1990; Yamane et al., 1990, 1991).

The overall role of the hydrophobic isoprenylation process is presumably to target the modified protein to the membrane (Gutierrez et al., 1989; Hancock et al., 1989, 1991; Holtz et al., 1989; Simonds et al., 1991). For example, the geranylgeranylated proteins, which account for the vast majority of isoprenylated proteins in mammalian cells (Epstein et al., 1990; Farnsworth et al., 1990; Rilling et al., 1990), are isolated in a membrane-bound form (Buss et al., 1991; Farnsworth et al., 1991; Johnson et al., 1990; Kawata et al., 1990; Mumby et al., 1990; Munemitsu et al., 1990; Shinjo et al., 1990; Yamane et al., 1990, 1991). However, how various isoprenylated G proteins are targeted to specific membranes remains completely unspecified. Although the hydrophobic isoprenylation process is normally thought to directly associate the modified protein to a membrane via increased hydrophobicity, the possibility of specific membrane receptors remains open.

The methylation reaction is the only reaction in the pathway which is potentially reversible and, hence, subject to regulation. Indeed, we have shown that G proteins are methylated and demethylated by rod outer segment (ROS) membranes, suggesting a dynamic role for methylation and demethylation (Perez-Sala et al., 1991). Possible roles for the methyl group are readily envisaged. If the role of isoprenylation is to simply anchor the modified G protein by hydrophobic interactions, then the role of the methyl group is clear: it neutralizes the charged modified cysteine residue, increasing its hydrophobicity. Another possibility is that a membrane-bound receptor is involved for isoprenylated G proteins, which only recognizes the methyl ester. Finally, it is also possible that the methyl ester is crucial for interactions of the isoprenylated G proteins with other proteins in the signal transduction cascade. In all cases, demethylation of the isoprenylated G protein would readily reverse the effects of methylation.

It has previously been shown that G proteins of rod outer segments (ROS) are reversibly methylated and demethylated in vitro (Perez-Sala et al., 1991). On the basis of this work, it was of interest to determine if a specific methyl ester hydrolase (methylesterase) activity can be detected that is capable of processing isoprenylated substrates. It is demonstrated here that ROS membranes contain an activity which can hydrolyze these substrates but not similar nonisoprenylated substrates. Moreover, it is shown that the enzymatic activity is irreversibly inactivated by ebelactone B, a serine esterase inhibitor (Köller et al., 1990; Umezawa et al., 1980).

[1] Abbreviations: L-AFC, *N*-acetyl-*S*-farnesyl-L-cysteine; L-AFCM, *N*-acetyl-*S*-farnesyl-L-cysteine methyl ester; L-AGGC, *N*-acetyl-*S*-geranylgeranyl-L-cysteine; L-AGGCM, *N*-acetyl-*S*-geranylgeranyl-L-cysteine methyl ester; FTPM, *S*-(farnesylthio)propionic acid methyl ester; L-FCM, *S*-farnesyl-L-cysteine methyl ester; D-FCM, *S*-farnesyl-D-cysteine methyl ester; DMSO, dimethyl sulfoxide; AdoMet, *S*-adenosyl-L-methionine; DFP, diisopropylfluorophosphate; L-ACM, *N*-acetyl-L-cysteine methyl ester; ROS, rod outer segment.

Materials and Methods

Materials. Frozen bovine retinas were obtained from Wanda Lawson, Co. (Lincoln, NE). *S*-Adenosyl[methyl-^{3}H]methionine (15 Ci/mmol and 78 Ci/mmol), [^{3}H]acetic anhydride (6 Ci/mmol) and Amplify were purchased from Amersham. Bio-Beads (SM-2) were from Bio-Rad. *N*-Acetyl-L-cysteine, L-cysteine dimethyl ester dihydrochloride, and bis(paranitrophenyl) phosphate were purchased from Sigma. *all-trans*-Geranylgeraniol was obtained from American Tokyo Kasei, Inc. *trans,trans*-Farnesyl bromide, diisopropyl fluorophosphate (DFP), Mosher's reagent, and guanidine carbonate were purchased from Aldrich. Protease inhibitors (kit) and ebelactone B were from Boehringer Mannheim.

Preparation of Washed ROS Membranes and Transducin. ROS membranes and transducin were obtained as previously described (Perez-Sala et al., 1991).

Esterase Assays. The radioactive substrates were dissolved in DMSO and incubated with extensively washed ROS membranes (0.5 mg of protein/mL) in 100 mM Hepes, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$ (buffer A), for 20 min at 37 °C. Final volume was 50 μL, and DMSO concentration in the assay was 4% (v/v). The amount of the corresponding radioactive carboxylic acid was determined either by HPLC analysis of the chloroform extracts obtained from the incubation mixtures as described in Pérez-Sala et al. (1991) or by partitioning with of *n*-butanol. With [^{3}H]AFCM, [^{3}H]-AGGCM, and FTP[^{3}H]M, the samples were injected on a normal phase HPLC column (Dynamax 60, Rainin, Woburn, MA) connected to an on-line radioactivity monitor (Berthold, Nashua, NH). For [^{3}H]AFCM and [^{3}H]AGGCM, the column was eluted with hexane/2-propanol/trifluoroacetic acid [85:15:0.1 (v/v/v)] at 1.5 mL/min. For FTP[^{3}H]M, elution was with hexane/2-propanol [100:0.2 (v/v)] at 1.5 mL/min. With [^{3}H]ACM, the incubation mixture was diluted with 150 μL of water and extracted with 3 × 100 μL of *n*-butanol. The amount of *N*-[^{3}H]acetylcysteine formed and [^{3}H]ACM consumed was determined by scintillation counting of the aqueous and organic phases, respectively. In all cases the acids were readily separated from the parent methyl esters.

Syntheses of Analogues. all-trans-Geranylgeranyl bromide was synthesized from *all-trans*-geranylgeraniol (Campbell et al., 1975). L-AFCM was prepared as previously described (Tan et al., 1991b). *N*-Acetyl-*S*-geranylgeranyl-L-cysteine methyl ester (L-AGGCM) was prepared from *all-trans*-geranylgeranyl bromide and *N*-acetyl-L-cysteine following the same procedure. Accordingly, *all-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 taken up in ethyl acetate (150 mL). The ethyl acetate solution was washed successively with 3.5% HCl (50 mL) and brine (25 mL), dried (Na_2SO_4), and concentrated to a small volume. Chromatography of this material on a silica column eluting with ethyl acetate/methanol (4:1–1:2) gave *N*-acetyl-*S*-geranylgeranyl-L-cysteine (L-AGGC) as a white waxy solid in 64% yield: ^{1}H NMR (500 MHz, DMSO-d_6) δ 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 = 14.0 Hz, J = 7.5 Hz), 2.81 (1 H, dd, J = 14.0 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 (3 H, m), 5.13 (1 H, bt, J = 7.5 Hz),

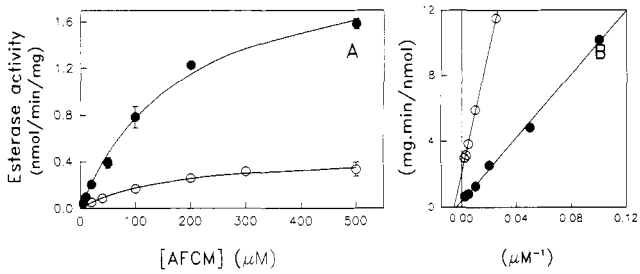


FIGURE 1: Methylesterase activity observed with L-[^{3}H]AFCM and D-[^{3}H]AFCM. Michaelis–Menten (A) and Lineweaver (B) plots of the formation of demethylated product as a function of L-[^{3}H]AFCM (●) and D-[^{3}H]AFCM (○) concentration. The small amount of control hydrolysis by heat-inactivated enzyme has been substracted from the values. Symbols represent mean values of three determinations and error bars the standard deviation of the mean. Error bars not shown are within symbols.

7.91 (1 H, bs). L-AGGCM was obtained from L-AGGC by treatment with methanolic HCl (0.05–0.1 M) (Means & Feeney, 1971). The product isolated gave NMR spectra essentially identical to those of the parent acid except for a singlet resonance, equivalent to three protons, at δ 3.76 ppm. [^{3}H]AFCM and [^{3}H]AGGCM were prepared by acetylating the methyl esters of *S*-(*all-trans*-farnesyl)cysteine and *S*-(*all-trans*-geranylgeranyl)cysteine (Yamane et al., 1990), respectively, with [^{3}H]acetic anhydride. Acetylation was effected by the addition of the isoprenylated cysteine derivatives (5 mol, equiv) to a methylene chloride solution of [^{3}H]acetic anhydride. The reaction mixture was kept at room temperature for 3 h and evaporated to dryness under a stream of nitrogen. The resulting residue was dissolved in a minimum volume of 2-propanol/hexane (15:85) and purified by normal-phase (Dynamax 60, Rainin, Woburn, MA) HPLC, eluting with the same solvent. L-ACM was obtained by treatment of *N*-acetyl-L-cystine with methanolic HCl (Means & Feeney, 1971). L-[^{3}H]ACM was prepared by acetylating L-cystine dimethyl ester dihydrochloride using the procedure described above. After the initial 3 h, an equivalent volume of 10% HCl was added, followed by the addition of zinc dust (20 mol equiv), and the reaction was stirred vigorously for 10 min. The organic phase was separated and the aqueous layer extracted twice with equivalent volumes of methylene chloride. The combined organic extracts were dried (Na_2SO_4) and evaporated to dryness under a stream of nitrogen. The residue was redissolved in a minimum volume of 2-propanol/hexane (1:1) and purified by normal-phase (Dynamax 60, Rainin, Woburn, MA) HPLC, eluting with the same solvent. FTP-[^{3}H]M was obtained enzymatically from FTP and [^{3}H]-AdoMet (73 Ci/mmol) as described in Tan et al. (1991b). All the radioactively labeled compounds coeluted with authentic standards on HPLC.

Enantiomeric Purity of L-*AFCM and* D-*AFCM.* DL-FCM, L-FCM, and D-FCM were reacted with chiral Mosher's reagent [(*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetic acid] (Dale et al., 1969) under conditions identical to those described by Evans and Ellman (1989). The diastereomers were readily separated by HPLC using an EM Lichrosorb Si-60 (5 μM) column using 98% *n*-hexane/2% isopropanol as eluant at a flow rate of 1.0 mL/min. Under these conditions, the retention time for the synthetic L- and D-enantiomers are 19.0 and 13.3 min, respectively. By this procedure, the L-enantiomer is 99.43% L and 0.57% D, and the D-enantiomer is 99.01% D and 0.99% L.

SDS–PAGE and Fluorography. For SDS–PAGE, aliquots of the reaction mixture were processed as described (Lai et al., 1990) and run on a 12% gel. To improve the resolution of low molecular weight polypeptides, 0.1 M sodium acetate was added to the anode buffer (Christy et al., 1989). Radioactive polypeptides were visualized by fluorography (Lai et al., 1990). Exposure was at −70 °C for 3 days.

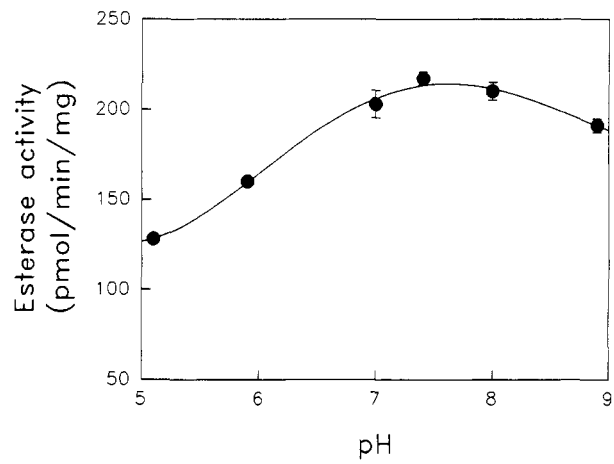


FIGURE 2: pH Dependence of methylesterase activity. Methylesterase activity was determined as described under Materials and Methods with 20 μM L-[^{3}H]AFCM as substrate in the following buffers (100 mM): Pipes (pH 5.1 and 5.9), Hepes (pH 7.0 and 7.4), and Tris (pH 8.0 and 8.9). All pH values were measured at 37 °C. Higher pH values were not used because methyl ester hydrolysis was expected to become significant. Symbols represent mean values of three determinations and error bars the standard deviation of the mean. Error bars not shown are within symbols.

RESULTS

Substrate Specificity of the Esterase. It had previously been shown that ROS membranes possess an enzymatic activity capable of hydrolyzing endogenous isoprenylated methyl esters of the ROS (Perez-Sala et al., 1991). The nature of the specificity of this enzymatic activity is addressed here. Initial experiments were directed at determining whether the synthetic isoprenylated substrates L-AFCM and L-AGGCM (structures shown below) were specifically processed by ROS membranes.

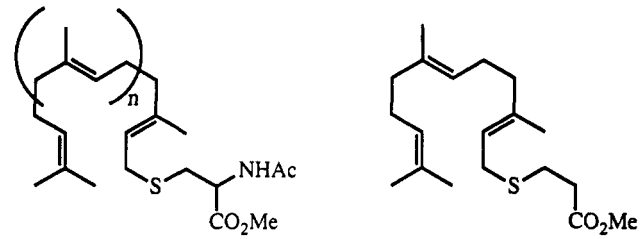


Figure 1 shows data for the hydrolysis of L-AFCM and D-AFCM. Enantiomeric purities of these compounds were >99% (see Materials and Methods). As can be seen here, the L-enantiomer is readily hydrolyzed to produce L-AFC. Saturation occurs as is expected of an enzymatic process, and the apparent K_M and V_{max} are 186 μM and 2.2 nmol/min^{-1} mg^{-1}, respectively. With L-AFCM as the substrate, the optimum pH for the esterase activity was determined to be approximately 7.5 (Figure 2). D-AFCM is processed much less readily than its L-enantiomer, with apparent K_M and V_{max} values of 157 μM and 0.457 nmol min^{-1} mg^{-1}, respectively. Thus the hydrolytic reaction is stereoselective. It was shown that FTPM (structure shown above) was enzymatically hydrolyzed (Figure 3). Complete kinetic determinations were not obtained for FTPM because of the limited supply of the radioactive form. Nevertheless, the compound could be shown

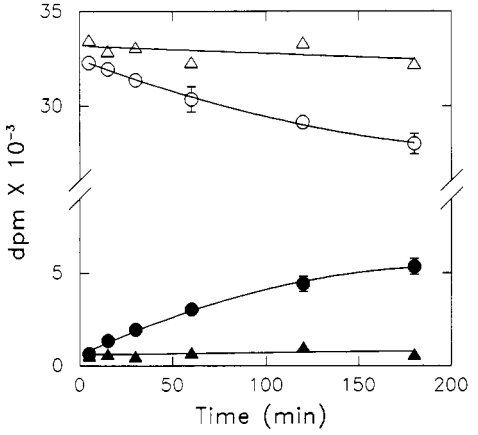


FIGURE 3: Demethylation of FTP[^{3}H]M. FTP[^{3}H]M (specific activity = 73 C_i/mmol, 50 nmol) obtained enzymatically (Perez-Sala et al., 1991) was incubated in 50 μL with intact (○, ●) and boiled (△, ▲) ROS membranes. The amount of FTP[^{3}H]M remaining (○, △) was determined by HPLC analysis, as described under Materials and Methods, and the radioactivity accumulated in the chloroform/methanol phase (●, ▲) was measured by scintillation counting. The efficiency of counting was 4% using an on-line radioactivity counter. Symbols represent mean values of three determinations and error bars the standard deviation of the mean. Error bars not shown are within symbols.

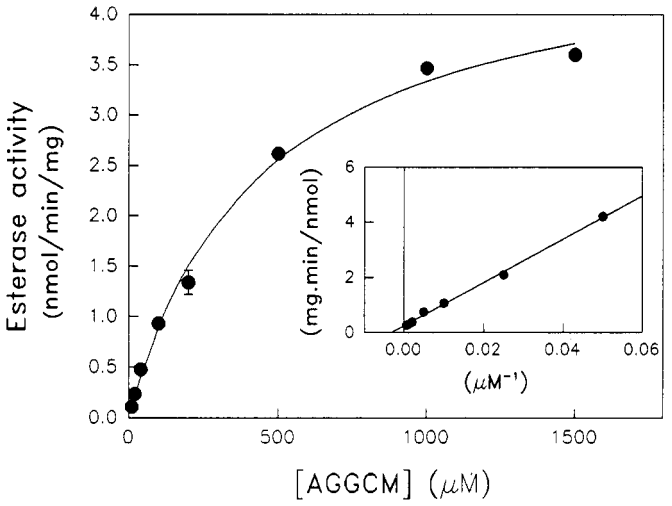


FIGURE 4: Methylesterase activity observed with L-[^{3}H]AGGCM. Michaelis–Menten and Lineweaver (inset) plots of the formation of demethylated product as a function of L-[^{3}H]AGGCM concentration. Symbols represent mean values of three determinations and error bars the standard deviation of the mean. Error bars not shown are within symbols.

to be readily enzymatically hydrolyzed even though it did not contain any vestiges of a peptide backbone. Under similar conditions, L-ACFM is hydrolyzed approximately 2–3-fold more rapidly than FTPM. In addition, 100 μM cold AFCM (K_M = 186 μM) inhibits the hydrolysis of 50 μM [^{3}H]FTP. The $t_{1/2}$ for hydrolysis is increased from 1.4 to 1.9 h of FTP. Also 200 μM unlabeled FTPM inhibits the hydrolysis of 20 μM labeled AFCM by 40%. These results suggest that the same enzyme(s) may be hydrolyzing both substrates. Further studies were aimed at geranylgeranylated substrates. When L-AGGCM was used as the substrate, the results shown in Figure 4 were obtained. Again, enzymatic hydrolysis was observed with a measured K_M = 435 μM and V_{max} = 4.8 nmol min^{-1} mg^{-1}. These results demonstrate that the ROS membrane associated esteratic activity can process simple isoprenylated substrates.

To explore the specificity of the ester hydrolase for the isoprenylated side chain, the potential hydrolysis of L-ACM

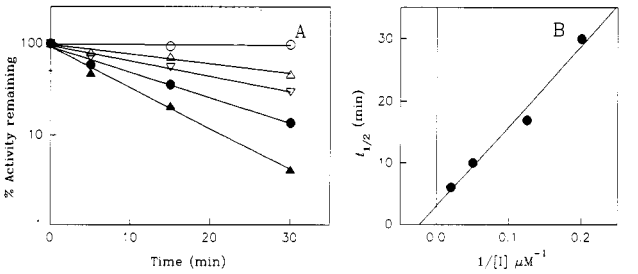


FIGURE 5: Irreversible inhibition of ROS methylesterase activity by ebelactone B and the determination of its K_I and k_{inh}. (A) Ebelactone B (○, 0 μM; △, 5 μM; ▽, 8 μM; ●, 20 μM; ▲, 50 μM) was incubated with ROS membranes (8 mg/mL protein concentration) in 100 mM Hepes buffer (Materials and Methods) with 4% DMSO at 23 °C for the indicated times. The incubation was washed twice with Bio-Beads (50 mg per 1 mg of protein), which had been preswollen in the same buffer (20 μL/mg Bio-Beads) for 2 h. The methylesterase activity in the membranes was determined as described under Materials and Methods. (B) Assuming that $E + I \overset{K_I}{\rightleftharpoons} EI \overset{k_{inh}}{\rightarrow} E - I'$ with $d(EI)dt = 0$, $I \gg E$, and where k_{cat} and k_{inh} refer to the same step (see Scheme I), the relationship between inactivation half-life ($t_{1/2}$) and inhibitor concentrations ([I]) can be represented by the equation $t_{1/2} = 0.69/k_{inh} + (0.69/k_{inh})K_I/[I]$ (Jung & Metcalf, 1975; Kitz & Wilson, 1964). The plot of $t_{1/2}$ against $1/[I]$ gives an apparent K_I of 42 μM and an apparent k_{inh} of 3.4×10^{-3} s^{-1}.

was studied. In this case, no enzymatic hydrolysis was observed. Furthermore, L-ACM was incompetent as an inhibitor of L-AFCM (20 μM) demethylation even at millimolar concentrations. This strongly suggests that the isoprenyl group is important in substrate recognition and that a nonspecific esterase is not being studied.

Inhibitors of Esterase Function. The issue of possible enzyme inhibitors of the methylesterase was addressed next. A selection of group-specific protease/esterase inhibitors were studied as possible inhibitors of L-AFCM demethylation. The effects of a group of inhibitors, including antipain, aprotinin, bestatin, chymostatin, diisopropylfluorophosphate, ebelactone B, EDTA, leupeptin, pepstatin, phosphoramidon, and bis-(paranitrophenyl) phosphate, were studied as potential inhibitors of the enzyme. Of all of the inhibitors tested, only ebelactone B proved to substantially inhibit the enzyme. Inhibition of the methylesterase by ebelactone B was slowed down when the enzyme was preincubated with approximately 500 μM AFCM. It has been suggested that ebelactone B is a serine esterase inhibitor (Köller et al., 1990; Umezawa et al., 1980). The nature of the ebelactone B-mediated inhibition of the methylesterase was explored further. First, the mode of inhibition appeared to be irreversible because when inhibited enzyme was washed with Bio-Beads to remove excess inhibitor, a substantial decrement in the activity of the enzyme was observed. Using this Bio–Beads washing procedure to remove excess unbound inhibitor, a time course for the inactivation of the enzyme was established (Figure 5). A $1/I$ vs $t_{1/2}$ (half-life) for the enzyme was plotted to give an apparent K_I = 42 μM and a $k_{inh} = 3.7 \times 10^{-3}$ s^{-1} at 25 °C.

Substrates and Inhibitors of Esterase Block Endogenous Substrate Hydrolysis. The experiments described above show that a methylesterase activity specific for isoprenylated methyl esters is found in the ROS membranes. It is also of interest to determine whether the methylesterase also processes the endogenous isoprenylated methyl esters found in the ROS. There are several groups of proteins which are isoprenylated and methylated in ROS membranes (Anant et al., 1991; Lai et al., 1990; Perez-Sala et al., 1991). These include a series of "small" G proteins (20–30 kDa), the γ subunit of transducin (Tγ, 6 kDa), the 65-kDa protein rhodopsin kinase (Lorenz et al., 1991), and the 90-kDa phosphodiesterase α subunit

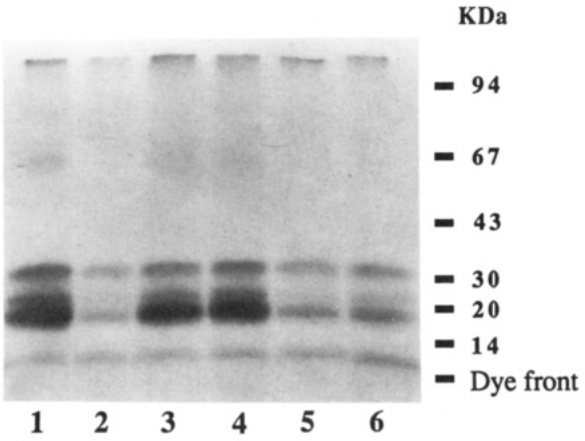


FIGURE 6: Inhibition of ROS protein demethylation. Transducin (5 μM) was incubated with ROS membranes (1 mg/mL protein concentration) and [^{3}H]Adomet (8 μM, 78 Ci/mmol) at 37 °C. After 1 h (lane 1), methylation was inhibited by the addition of 200 μM sinefungin. Inhibitors were added to aliquots of the methylation mixture (lane 2, no inhibitor; lane 3, 200 μM ebelactone B; lane 4, 500 μM ebelactone B; lane 5, 500 μM L-AGGCM; lane 6, 500 μM L-AFCM) and incubated at 37 °C for 2 h. The samples were then processed for SDS–PAGE and fluorography as described under Materials and Methods. The percent inhibition (relative to lane 2) observed with the 20–30-kDa proteins was determined by densitometry to be approximately 70% in lane 3, 90% in lane 4, 20% in lane 5, and 30% in lane 6. The other substrates had activities that were too weak for reliable percent inhibition determinations.

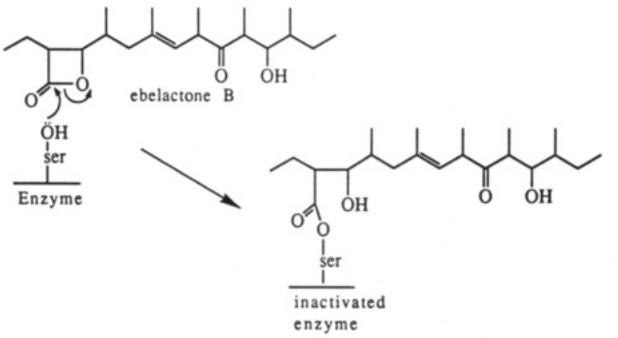
(Swanson & Applebury, 1983; Anant et al., 1991). In the current experiment (Figure 6), these membrane-associated proteins were first methylated using [^{3}H]AdoMet. Incubation of these methylated and labeled proteins led to their enzymatic demethylation. Clearly, Tγ is demethylated most slowly. The incorporation of ebelactone B at 200 and 500 μM strongly blocked their demethylation. Densitometric analysis showed that ebelactone B inhibited hydrolysis of the small G proteins by 70–90%. Moreover, the inclusion of L-AGGCM and L-AFCM both at 500 μM in the incubation medium also decreased the hydrolysis rate. Here the measured extents of inhibition were between 20 and 30%. These experiments show that molecules that are substrates for the methylesterase (L-ACFM and L-AGGCM) or inhibit the enzyme (ebelactone B) block the hydrolysis of natural substrates for the enzyme. These data strongly suggest that the methylesterase activity uncovered here processes the endogenous isoprenylated and methylated substrates in the ROS.

Discussion

Protein carboxymethylation appears to be an important component of diverse regulatory phenomena in procaryotes and eucaryotes. In bacteria, carboxymethylation in signal transduction occurs as a component of chemotaxis and phototaxis (Springer et al., 1979; Koshland, 1981; Boyd & Simon, 1982; Taylor & Panasenko, 1984). In yeast, the carboxymethylation of the a-factor mating factor phenomena is essential to its function (Anderegg et al., 1988). Finally, in mammals carboxymethylation occurs at L-isoaspartyl and D-aspartyl residues (McFadden & Clarke, 1982; Murray & Clarke, 1984; Aswad, 1984). Carboxymethylation has also been shown to be important in the functioning of lamin B assembly into the nuclear envelope (Chelsky et al., 1987, 1989) and in the membrane binding of p21$^{k\text{-}ras(B)}$ to membranes (Hancock et al., 1991).

Methylation is the only reaction in the isoprenylation pathway that is potentially reversible. In fact, it had been previously been shown that G protein methylation in the ROS is a reversible process (Perez-Sala et al., 1991). In order for

Scheme I



methylation to be reversible, a specific methylesterase is required. In this paper, it is shown that ROS membranes contain a methylesterase activity which can hydrolyze farnesylated and geranylgeranylated cysteine methyl ester derivatives (L-AFCM and L-AGGCM). The observed enzymatic activity, of course, could result from a single or multiple enzymes. The hydrolytic process is stereoselective since L-AFCM is a much better substrate for the enzyme than is its D-enantiomer. In addition to the enzymatic hydrolysis being stereoselective, there also appeared to be a requirement for the isoprenoid side chain since L-ACM proved to be neither a substrate nor an inhibitor for the enzyme. These data taken together suggest that the methylesterase activity is specific for isoprenylated cysteine derivatives.

Neither L-AFCM nor L-AGGCM had low K_M values, although their V_{max} values were substantial. The relatively high K_M values suggest that the methylesterase might also recognize the peptide portion of the substrate. Further structure–activity studies will be required to determine the role of the peptide backbone in substrate recognition. However, it should be noted that FTPM also appeared to be a substrate for the enzyme. In this case, all vestiges of the peptide backbone of endogenous substrates have been removed. Along these lines, it is also interesting to note that the *S*-adenosyl-L-methionine-linked isoprenylated protein methyltransferase does not appear to require substantial interaction with the peptide backbone in substrate recognition (Tan et al., 1991b). Very simple substrates which contain no remnants of the peptide backbone, such as *S*-farnesylthiopropionic acid (FTP), are excellent substrates for the methyltransferase with K_M values in the 10–20 μM range (Tan et al., 1991b).

Although a large bank of protease/esterase inhibitors were studied as potential inhibitors of the esterase, none proved to be effective. Interestingly, ebelactone B was shown to be a potent inactivator of the enzyme. Ebelactone B is a well-known serine esterase inhibitor whose mechanism of action has apparently not been studied. It is shown here that it is a time-dependent irreversible inhibitor of the methylesterase under study. The kinetics of the inhibition process suggest an active-site-directed mechanism reminiscent of mechanism-based ("suicide") enzyme inactivators (Rando, 1974). If the esterase in question is a serine enzyme, then a possible mechanism for the inactivation process is shown in Scheme I. In this mechanism, the activated serine attacks the β-lactone of ebelactone B, generating a dead-end acyl–enzyme intermediate which cannot be further hydrolyzed. In fact, the mechanism proposed is similar to the accepted one for penicillin action where a transpeptidase forms a stable acyl–enzyme intermediate with the β-lactam moiety, inactivating the enzyme (Waxman & Stominger, 1983).

The experiments described above show that there is a membrane-associated ROS methylesterase enzyme(s) capable

of hydrolyzing isoprenylated L-cysteine derivatives which is inhibitable by the esterase inhibitor ebelactone B. It was of interest to determine if the enzyme also hydrolyzes the endogenous methyl esters of isoprenylated proteins in the ROS. Several groups of ROS proteins are methylated (Swanson & Applebury, 1983). Of this group, several have also been shown to be isoprenylated as evidenced by the incorporation of exogenously added mevalonic acid (Anant et al., 1991; Fukada et al., 1990; Lai et al., 1990). Of the proteins that are isoprenylated, only the chemical nature of the isoprenyl group adducted to the γ subunit of transducin (Tγ, 6 kDa), in this case *all-trans*-farnesyl, has been established (Fukada et al., 1990; Lai et al., 1990). Nevertheless, the nature of several of the methylated proteins is relatively certain. The 90-kDa protein is the α subunit of the phosphodiesterase (Swanson & Applebury, 1983; Anant et al., 1991), and the 65-kDa protein is rhodopsin kinase (Lorenz et al., 1991). Finally, proteins between 20 and 30 kDa are probably of the class of "small" G proteins. These proteins are readily methylated by adding [^{3}H]CH_3-labeled AdoMet to the membranes (Perez-Sala et al., 1991). Incubation of these membranes in the absence of [^{3}H]AdoMet results in the loss of the labeled methyl groups (which can be reincorporated by reincubating with [^{3}H]CH_3-labeled AdoMet). This demethylation reaction is due to the enzymatic hydrolysis of the methyl esters. It was of interest to determine if this hydrolytic process could be blocked by ebelactone B, L-AFCM, and L-AGGCM. The hydrolyses were blocked by all of these compounds, providing a link between the interactions of the small molecules with the ester hydrolase and the processing of the endogenous ROS substrates. The inhibition of the hydrolysis of the endogenous substrates by ebelactone B was more pronounced than it was by either L-AFCM or L-AGGCM. This is expected because of the higher potency of ebelactone B coupled with the fact that it is an irreversible inhibitor. The fact that the K_M values for L-AFCM and L-AGGCM were relatively large did not allow us to achieve close to saturating concentrations with either substrate, diminishing the extents of inhibition.

The physiological role(s) of the isoprenylated protein methyl ester hydrolase activity remains to be determined. The fact that there is an methylesterase activity suggests a dynamic role for the methylation of isoprenylated proteins. If the terminal isoprenylated cysteine residue were unmethylated, the free carboxyl group would be charged and substantially hydrophilic. If the role of isoprenylation is simply to increase the hydrophobicity of the modified proteins and anchor them to membranes, then the function of the methylation reaction would be to enhance hydrophobicity. Alternatively, specific isoprenylated protein–receptor interactions might be involved whereby only the methyl ester is recognized. Along these lines, it is also possible that intramolecular interactions in the isoprenylated protein are governed by the state of carboxyl methylation. Future experiments will be required to distinguish between these various alternatives.

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The Secondary Structure of the Colicin E3 Immunity Protein As Studied by ^1H-^1H and ^1H-^{15}N Two-Dimensional NMR Spectroscopy†

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ABSTRACT: By performing ^1H-^1H and ^1H-^{15}N two-dimensional (2D) nuclear magnetic resonance (NMR) experiments, the complete sequence-specific resonance assignment was determined for the colicin E3 immunity protein (84 residues; ImmE3), which binds to colicin E3 and inhibits its RNase activity. First, the fingerprint region of the spectrum was analyzed by homonuclear ^1H-^1H HOHAHA and NOESY methods. For the identification of overlapping resonances, heteronuclear ^1H-^{15}N (HMQC-HOHAHA, HMQC-NOESY) experiments were performed, so that the complete ^1H and ^{15}N resonance assignments were provided. Then the secondary structure of ImmE3 was determined by examination of characteristic patterns of sequential backbone proton NOEs in combination with measurement of exchange rates of amide protons and $^3J_{HN\alpha}$ coupling constants. From these results, it was concluded that ImmE3 contains a four-stranded antiparallel β-sheet (residues 2-10, 19-22, 47-49, and 71-79) and a short α-helix (residues 31-36).

Colicin E3 is an antibacterial protein (551 residues) which kills sensitive *Escherichia coli* cells through binding to a common outer membrane receptor, BtuB (Pusgley & Oudega, 1987). E3 is a special kind of RNase which inactivates 16S RNA of 70S ribosomes, and this activity is exclusively defined by the C-terminal T2A domain of E3 (Boon, 1971; Bowman et al., 1971; Suzuki & Imahori, 1978; Ohno-Iwashita & Imahori, 1980). E3 is encoded by an *E. coli* plasmid, ColE3. The plasmid protects the host cell from both exogenous and endogenous colicin actions, a phenomenon referred to as immunity, by synthesizing the immunity protein (Imm), which specifically binds to the T2A domain and inhibits its RNase activity (Jakes & Zinder, 1974; Mochitate et al., 1981; Masaki & Ohta, 1985).

ColE6-directed colicin E6 is an E3 homologue, and the sequence differences of the two are almost confined in their

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