Substrate Specificity of the Isoprenylated Protein Endoprotease†

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ABSTRACT: Proteins containing a CAAX motif at their carboxyl termini are subject to isoprenylation at the cysteine residue. Proteolytic trimming of isoprenylated proteins is essential in the activation of these proteins. A microsomal endopeptidase activity has been identified which cleaves all-trans farnesylated cysteine containing tetrapeptides between the modified residue and the adjacent amino acid to liberate the modified cysteine residue and an intact tripeptide. Structure/activity studies are reported here on this endopeptidase activity which are consistent with the premise that this protease is identical to the one normally involved in the cellular isoprenylation pathway. The protease only processes peptides which possess an isoprenyl moiety. Within the isoprenyl series, the enzyme hydrolyzes *all-trans*-farnesyl-, *all-trans*-geranylgeranyl-, and geranyl-containing peptides. The protease also recognizes the AAX sequence, because the protease behaves either stereospecifically or stereoselectively with respect to the individual amino acids of the tripeptide. The enzyme only measurably hydrolyzes isoprenylated peptides possessing L-amino acids at C and A. On the other hand, there is a small but measurable hydrolysis of isoprenylated peptides containing a D-amino acid at X.

A substantial group of proteins, including both the heterotrimeric and the "small" G proteins, is 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). In this set of modifications, shown in Scheme I, a protein with a carboxyl terminal CAAX (Casey et al.; Hancock et al., 1989; Schafer et al., 1989; Lowy & Willumsen, 1990) or, less frequently, CXC sequence (where C = cysteine, A = aliphatic amino acid, and X = undefined amino acid) (Horiuchi et al., 1991; Farnsworth et al., 1991; Khosravi-Far et al., 1991) is first isoprenylated at the cysteine residue(s) with either *all-trans*-farnesyl (C15) or *all-trans*-geranylgeranyl (C20) pyrophosphate (Manne et al., 1990; Reiss et al., 1990; Schaber et al., 1990). In the case of modifications at a CAAX motif, proteolysis follows, to generate the isoprenylated cysteine residue as the new carboxyl terminus (Hancock et al., 1989). This set of modifications is completed by the biologically reversible carboxymethylation of the isoprenylated cysteine residue (Seabra et al., 1991; Hancock et al., 1991; 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 molecular enzymology of this pathway is just beginning to be clarified. Only the soluble isoprenyl transferases have already been purified and characterized (Reiss et al., 1991; Seabra et al., 1991). The membrane-bound methyltransferase (Perez-Sala et al., 1991; Stephenson & Clarke, 1990) and methylesterase (Tan & Rando, 1992) have been identified, and substrate structure/activity studies have been performed (Tan & Rando, 1992; Tan et al., 1991; Gilbert et al., 1992). However, neither enzyme has been purified.

Although proteolytic processing could occur by several different routes, recent evidence shows that proteolysis occurs in mammals primarily by endoproteolytic cleavage between the modified cysteine residue and the adjacent aliphatic amino acid to liberate the intact AAX tripeptide (Ashby et al., 1992; Ma & Rando, 1992). A liver and pancreatic microsomal proteolytic activity was identified that produces a single cut between the modified cysteine residue and the AAX tripeptide, using a synthetic tetrapeptide substrate L-AFC-Val-Ile-Ser[1] (Ma & Rando, 1992). The protease also specifically cleaves the tripeptide L-AFC-Val-Ile and the dipeptide L-AFC-Val, but not L-AFC amide (Ma & Rando, 1992). Therefore, minimally, a dipeptide is required for substrate activity. Moreover, the enzyme does not cleave substrates containing D-AFC as the first amino acid, demonstrating that the cleavage reaction is stereospecific at the scissile bond. Although this enzymatic activity appears to be an excellent candidate for the isoprenylated protein protease, further studies are needed to establish this. It is important to demonstrate (1) that the enzyme requires an isoprenoid moiety in the substrate and (2) that it recognizes the AAX portion of the substrate. Since the enzyme hydrolyzes simple farnesylated peptides, the required structure/activity studies are readily performed. In this paper, the structural requirements of the protease are characterized with respect to both the isoprenyl moiety and the stereochemistry of the amino acids. It is shown here that the isoprenyl group is essential for activity and that either stereospecificity or stereoselectivity is observed at AAX. By structure/activity criteria, this enzymatic activity behaves as expected of an isoprenylated protein protease.

MATERIALS AND METHODS

Materials

Amino acids and dipeptides were purchased from Bachem Inc. β-Mercaptoethanol, *all-trans*-farnesyl bromide, *all-trans*-farnesol, geraniol, 4,4-(dimethylamino)pyridine, 1-hydroxybenzotriazole hydrate, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, *N*-methylmorpholine, and 1-hydroxybenzotriazole hydrate were purchased from the Aldrich Chemical Co. *all-trans*-Geranylgeraniol was from

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[1] Abbreviations: DMSO, dimethyl sulfoxide; TFA, trifluoroacetic acid; AFC, *N*-acetyl-*S*-*all-trans*-farnesyl-L-cysteine; AGGC, *N*-acetyl-*S*-*all-trans*-geranylgeranyl-L-cysteine; AGC, *N*-acetyl-*S*-geranyl-L-cysteine; AC, *N*-acetyl-L-cysteine.

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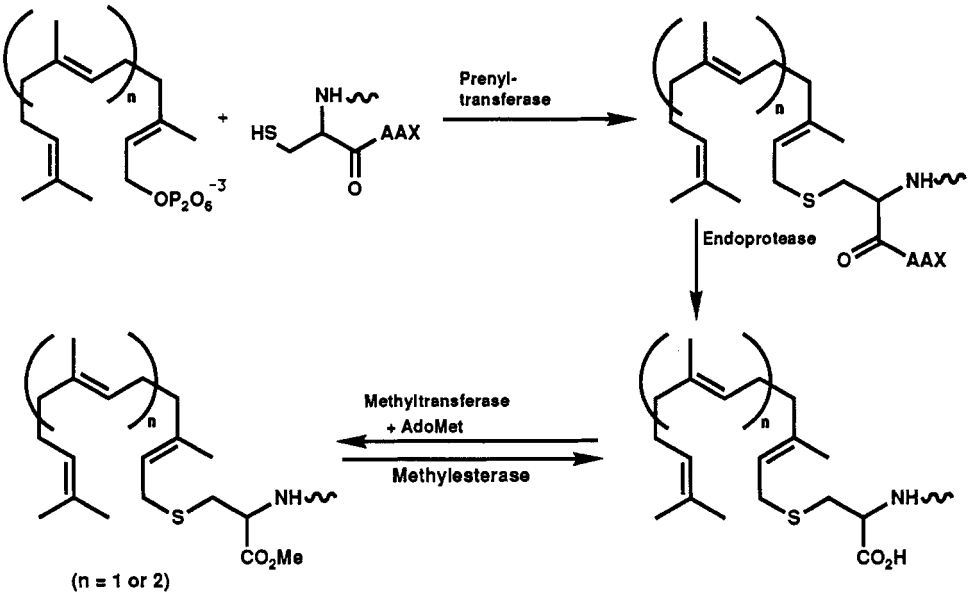
Scheme I: Biochemical Reactions in the Isoprenylation Pathway



TCI, Inc. HPLC solvents were from J. T. Baker, Inc. All chemicals and solvents purchased were of the highest purity available. [^{3}H]Acetic anhydride was from New England Nuclear, Inc. Fresh bovine calf liver was obtained from a local slaughterhouse.

Methods

Syntheses of Analogs. Cys-Val-Ile and *tert*-butylthio-Cys-Val-Ile were synthesized by Dr. Chuck Dahl at the Harvard Medical School peptide synthesis facility. The isoprenylated peptide analogs were prepared by methods similar or identical to those published earlier (Ma & Rando, 1992). Complete details of the synthetic protocols are provided as supplementary material. The NMR data and chromatographic behavior of the analogs are described below.

N-[^{3}*H*]*Acetyl-S-all-trans-farnesyl*-L-*Cys*-D-*Val*-L-*Ile*. The peptide was purified by HPLC (Rainin silica, 4.6 × 250 mm, hexane/2-propanol/TFA (92:8:0.01) as eluant at a flow rate of 1.5 mL/min with UV detection at 210 nm). The retention time of the L-D-L isomer was 8.60 min: ^{1}H NMR (DMSO-d_6, 500 MHz), δ 8.11 (1 H, *d*, J = 9.5 Hz), 8.09 (1 H, d, J = 7.5 Hz), 8.01 (1 H, d, J = 7.5 Hz), 5.15 (1 H, t, J = 8 Hz), 5.04 (2 H, t, J = 8 Hz), 4.59 (1 H, dd, J = 8, 15 Hz), 4.34 (1 H, dd, J = 6, 9 Hz), 4.18 (1 H, dd, J = 6.5, 8.5 Hz), 3.17 (2 H, dd, J = 4, 7.5 Hz), 2.65 (1 H, dd, J = 6.5, 13.5 Hz), 2.51 (1 H, dd, J = 7.5, 13.5 Hz), 2.08–1.90 (9 H, m), 1.80 (3 H, s), 1.79 (1 H, m), 1.61 (3 H, s), 1.60 (3 H, s), 1.54 (6 H, s), 1.38 (1 H, m), 1.19 (1 H, m), 0.79–0.87 (12 H, m).

N-[^{3}*H*]*Acetyl-S-all-trans-farnesyl*-L-*Cys*-L-*Val*-D-*Ile*. The retention time of the L-L-D isomer was 8.19 min: ^{1}H NMR (DMSO-d_6, 500 MHz) δ 8.19 (1 H, d, J = 8.5 Hz), 7.89 (1 H, br s), 7.77 (1 H, d, J = 8.5 Hz), 5.14 (1 H, t, J = 8 Hz), 5.04 (2 H, t, J = 6 Hz), 4.46 (1 H, dd, J = 7, 9 Hz), 4.26 (1 H, dd, J = 6, 13 Hz), 4.15 (1 H, br s), 3.15 (1 H, d, J = 8 Hz), 3.12 (1 H, d, J = 8 Hz), 2.73 (1 H, dd, J = 5.5, 12.5 Hz), 2.51 (1 H, dd, J = 8.5, 12.5 Hz), 2.06–1.90 (9 H, m), 1.83 (3 H, s), 1.78 (1 H, m), 1.61 (3 H, s), 1.60 (3 H, s), 1.53 (6 H, s), 1.39 (1 H, m), 1.18 (1 H, m), 0.79–0.84 (12 H, m).

N-[^{3}*H*]*Acetyl-S-all-trans-farnesyl*-L-*Cys*-L-*Val*-L-*Ile*-D-*Met*. The peptide was purified by HPLC chromatography (Rainin silica, 250 × 4.6 mm, 1.5 mL/min, hexane/2-propanol/TFA (90:10:0.01); UV detection was at 210 nm). The retention time was 9.38 min: ^{1}H NMR (DMSO-d_6, 500 MHz) δ 8.15 (1 H, d, J = 8 Hz), 8.13 (1 H, d, J = 8.5 Hz), 7.86 (1 H, d, J = 9 Hz), 7.75 (1 H, d, J = 8.5 Hz), 5.15 (1 H, t, J = 7 Hz), 5.04 (2 H, d, J = 3.5 Hz), 4.46 (1 H, dd, J = 8, 14 Hz), 4.28–4.16 (3 H, m), 3.13 (2 H, d, J = 7.5 Hz), 2.71 (1 H, dd, J = 6, 14 Hz), 2.51 (1 H, dd, J = 8.5, 14 Hz), 2.46–2.39 (3 H, m), 1.99 (3 H, s), 2.03–1.90 (10 H, m), 1.82 (3 H, s), 1.68 (1 H, m), 1.61 (3 H, s), 1.59 (3 H, s), 1.53 (6 H, s), 1.39 (1 H, m), 1.03 (1 H, m), 0.78 (12 H, m).

N-[^{3}*H*]*Acetyl-S-all-trans-farnesyl*-L-*Cys*-L-*Val*-L-*Ile*-L-*Met*. The peptide was purified by HPLC (Rainin silica, 250 × 4.6 mm, 1.5 mL/min, hexane/2-propanol/TFA (90:10:0.01); UV detection was at 210 nm). The retention time was 6.68 min: ^{1}H NMR (DMSO-d_6, 500 MHz) δ 8.14 (1 H, d, J = 8 Hz), 8.12 (1 H, d, J = 8 Hz), 7.85 (1 H, d, J = 8.5 Hz), 7.81 (1 H, d, J = 9.5 Hz), 5.15 (1 H, t, J = 8 Hz), 5.05 (2 H, m), 4.46 (1 H, ddd, J = 2, 6, 14.5 Hz), 4.39 (1 H, dd, J = 5.5, 9.5 Hz), 4.33 (1 H, ddd, J = 5, 10, 14 Hz), 4.23 (1 H, dd, J = 7, 8.5 Hz), 3.17 (1 H, dd, J = 8, 14 Hz), 3.12 (1 H, dd, J = 7.5, 14 Hz), 2.74 (1 H, dd, J = 6, 13.5 Hz), 2.52 (1 H, dd, J = 7.5, 13.5 Hz), 2.46–2.35 (3 H, m), 2.00 (3 H, s), 2.04–1.90 (10 H, m), 1.83 (3 H, s), 1.61 (3 H, s), 1.60 (3 H, s), 1.53 (6 H, s), 1.24 (2 H, m), 1.08 (1 H, m), 0.80 (12 H, m).

N-[^{3}*H*]*Acetyl-S-trans-geranyl*-L-*Cys*-L-*Val*-L-*Ile*. The peptide was purified by HPLC (Rainin silica, 250 × 4.6 mm, 1.5 mL/min, hexane/2-propanol/TFA (92:8:0.01); UV detection was at 210 nm). The retention time of the L-L-L isomer was 9.65 min: ^{1}H NMR (DMSO-d_6, 500 MHz) δ 8.11 (1 H, d, J = 8 Hz), 7.89 (1 H, d, J = 8 Hz), 7.76 (1 H, d, J = 8.5 Hz), 5.15 (1 H, t, J = 8 Hz), 5.03 (1 H, t, J = 6.5 Hz), 4.46 (1 H, dd, J = 8.5, 9 Hz), 4.25 (1 H, dd, J = 7, 9 Hz), 4.12 (1 H, dd, J = 6, 8 Hz), 3.13 (2 H, t, J = 9 Hz), 2.72 (1 H, dd, J = 6, 14 Hz), 2.53 (1 H, dd, J = 9, 14 Hz), 1.94–2.08 (5 H, m), 1.96 (1 H, m), 1.83 (3 H, s), 1.61 (3 H, s), 1.59 (3 H, s), 1.54 (3 H, s), 1.40 (1 H, m), 1.18 (1 H, m), 0.78–0.88 (12 H, m).

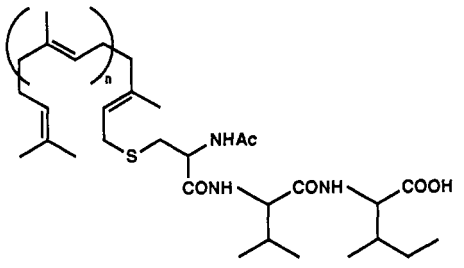
N-[^{3}*H*]*Acetyl-S-all-trans-geranylgeranyl*-L-*Cys*-L-*Val*-L-*Ile*. The peptide was purified by HPLC (Rainin silica, 250 × 4.6 mm, 1.5 mL/min, hexane/2-propanol/TFA (92:8:0.01); UV detection was at 210 nm). The retention time of the L-L-L isomer was 7.08 min: ^{1}H NMR (DMSO-d_6, 500 MHz) δ 8.11 (1 H, d, J = 8 Hz), 7.89 (1 H, d, J = 8 Hz), 7.76 (1 H, d, J = 8.5 Hz), 5.15 (1 H, t, J = 8 Hz), 5.03 (1 H, t, J = 6.5

Hz), 4.46 (1 H, dd, J = 8.5, 9 Hz), 4.25 (1 H, t, J = 7 Hz), 4.11 (1 H, t, J = 6 Hz), 3.12 (2 H, d, J = 6.5 Hz), 2.73 (1 H, dd, J = 5.5, 13.5 Hz), 2.53 (1 H, dd, J = 8.5, 13.5 Hz), 1.92–2.08 (13 H, m), 1.83 (3 H, s), 1.76 (1 H, m), 1.61 (3 H, s), 1.60 (3 H, s), 1.54 (9 H, s), 1.41 (1 H, m), 1.17 (1 H, m), 0.78–0.86 (12 H, m).

Protease Assays. The substrates were dissolved in DMSO and incubated with calf liver microsomal membranes (0.2 mg of protein/mL) (Walter & Biobel, 1983) according to the detailed previously published procedure (Ma & Rando, 1992). The amount of radioactive product was also determined as before (Ma & Rando, 1992). The reaction was quenched with 500 μL of $CHCl_3$/MeOH (1:1, v/v), and the radioactive N-terminal amino acid, i.e., *N*-[^{3}H]AFC, was extracted after thorough agitation of this mixture. Phase separation was achieved by adding 500 μL of 1 M citric acid. After the chloroform layer was evaporated under nitrogen, the residue was resuspended in *n*-hexane/2-propanol/TFA (90:10:0.01) and nonradioactive AFC was added as a standard for UV detection (210 nm). The sample was injected on a normal-phase HPLC column (Dynamax 60A, Rainin), and elution was performed with the same solvent at a flow rate of 1.5 mL/min. Radioactivity was counted with an on-line Berthold (Nashua, NH) LB 506-C HPLC radioactivity monitor. The limit of efficiency of this assay was 3× the background counts per minute (~150 cpm), which would readily allow us to assay molecules as substrates whose activities were 0.4% of the V_{max} of AFC-Val-Ile-Ser (1.13 pmol min^{-1} mg^{-1}). The retention times for AGC, AFC, and AGGC are 7.58, 7.26, and 7.00 min, respectively, and for AGC-Val-Ile, AFC-Val-Ile, and AGGC-Val-Ile, they are 5.48, 6.54, and 5.08 min, respectively.

RESULTS

Initial experiments were focused on elucidating the role of the isoprenoid side chain for substrate activity. The three tripeptides shown below were synthesized and tested as possible



1 AFC-V-I (n=1); **2** AGGC-V-I (n=2); **3** AGC-V-I (n=0)

substrates for the enzyme. AFC-V-I (**1**) had previously been shown to be hydrolyzed by the enzyme (Ma & Rando, 1992). The *all-trans*-geranylgeranyl derivative (AGGC-V-I, **2**), and the *all-trans*-geranyl derivative (AGC-V-I, **3**) were prepared and studied as possible substrates for the protease (Figure 1 A,B). As can be seen in Figure 1, both analogs were good substrates for the enzyme. The geranylgeranyl derivative **2** was hydrolyzed with a K_M = 4.01 μM and a V_{max} = 26.2 pmol min^{-1} mg^{-1} (Table I). The K_M and V_{max} values for the geranyl derivative **3** were measured to be 14.6 μM and 19.35 pmol min^{-1} mg^{-1}, respectively (Table I). As expected, stereospecificity was observed with respect to the isoprenylated amino acid because neither D-AGGC-V-I nor D-AGC-V-I is measurably processed enzymatically (Table I). D-AFC-V-I had been previously shown not to be a substrate for the protease (Ma & Rando, 1992).

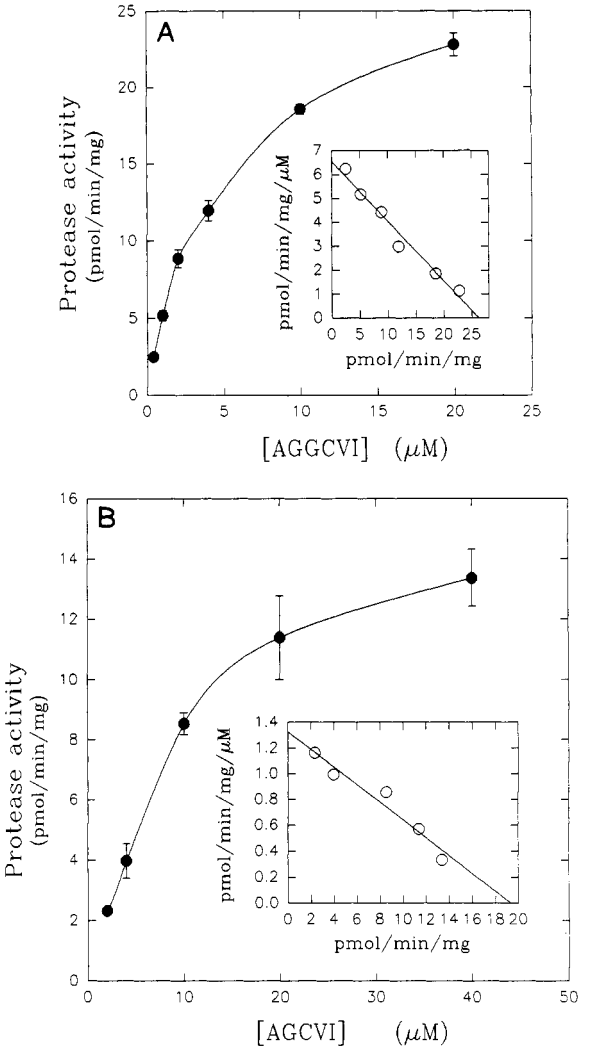
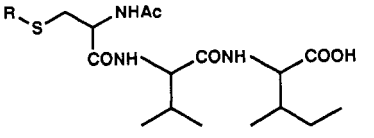


FIGURE 1: Protease activity with *N*-[^{3}H]Ac-*S*-geranyl-L-Cys-L-Val-L-Ile and *N*-[^{3}H]Ac-*S*-geranylgeranyl-L-Cys-L-Val-L-Ile as substrates. Michaelis–Menten and Eadie–Hofstee plots (inset) of the formation of *N*-[^{3}H]AGC or [^{3}H]AGGC as a function of *N*-[^{3}H]-Ac-*S*-geranylgeranyl-L-Cys-L-Val-L-Ile (A) or *N*-[^{3}H]Ac-*S*-geranyl-L-Cys-L-Val-L-Ile (B) concentrations. Symbols represent mean values of two determinations and error bars represent the standard deviation from the mean. Error bars not shown are within symbols. The assays were performed as described in Materials and Methods and in Ma and Rando (1992).

The experiments described above demonstrate that the protease exhibits a broad substrate specificity profile with respect to isoprenoid side chains. An important issue to resolve is whether or not the isoprenoid side chain is essential for substrate activity. To explore this issue further, the noniso-prenylated derivatives **4** and **5** were prepared and studied.



4 R=H ; **5** R=t-Bu-thio

Derivative **4** is the cysteine analog C-V-I, and derivative **5** is the *tert*-butylthio derivative of C-V-I. Neither analog was measurably processed by cleavage between the cysteine and valine residues (Table I). These experiments demonstrate a strong requirement for an isoprenoid moiety attached to the sulfur atom in order for substrate activity to be manifested.

Table I: Substrate Activities of Analogs[a]

substrate	isomer	K_M (μM)	V_{max} (pmol min^{-1} mg^{-1})
AFC-Val-Ile (**1**)	L-L-L	9.2 ± 0.2	57.7 ± 5
AGGC-Val-Ile (**2**)	L-L-L	4.01 ± 0.4	26.20 ± 0.97
	D-L-L	inactive[b]	
AGC-Val-Ile (**3**)	L-L-L	14.60 ± 2.14	19.35 ± 1.12
	D-L-L	inactive[b]	
Cys-Val-Ile (**4**)	L-L-L	inactive[b]	
tert-butylthio-Cys-Val-Ile (**5**)	L-L-L	inactive[b]	
AFC-Val-Ile (**6**)	D-L-L	inactive[b]	
AFC-Val-Ile (**7**)	L-D-L	inactive[b]	
AFC-Val-Ile (**8**)	L-L-D	inactive[b]	
AFC-Val-Ile-Met (**9**)	L-L-L-L	2.96 ± 0.35	126.3 ± 4.7
AFC-Val-Ile-Met (**10**)	L-L-L-D	weakly active[c]	

[a] The enzymatic assays were performed as indicated under Materials and Methods. Values given are average values of two determinations ± SD. We had previously reported (Ma & Rando, 1992) **1** to have K_M and V_{max} values of 12.6 μM and 65.6 pmol min^{-1} mg^{-1}. These values are slightly lower than those reported here. However, the former measurement was performed on a different microsome preparation from a different animal. The measurements reported in this paper were all determined using the same enzyme preparation. [b] Analogs referred to as inactive did not show detectable activity as substrate when used at a concentration of 10 μM and with prolonged incubation time (16 h). The sensitivity of the assay used allows the detection of protease activities above 1.13 pmol min^{-1} mg^{-1} (0.4% of the activity seen with AFC-V-I-S). [c] Where marginal activity is noted, detectable activity is observed under prolonged incubation periods, but the activity is <5% that observed with AFC-V-I-S.

The experiments described above demonstrate the necessity of an isoprenoid moiety attached to the cysteine residue for substrate activity. A second set of structure/activity experiments was aimed at exploring the stereospecificity requirements at the nonisoprenylated amino acid residues in the AFC-V-I series. The L-D-L **7** and the L-L-D **8** analogs were prepared and shown to be incompetent as substrates for the protease (Table I). The same stereospecificity for the L stereoisomer at AFC, V, and I was also observed in the AFC-V-I-S series. Moreover, all analogs studied in the AFC-V-I series in which there was more than one D-amino acid, such as the D-D-D, the D-D-L, the D-L-D, and the L-D-D diastereomers, were also entirely inactive as substrates of the protease. Finally, in order to explore the stereospecificity at the X position of CAAX, L-AFC-V-I-M was prepared with both L- and D-methionine residues, and the derivatives were tested as substrates for the enzyme (Figure 2 and Table I). As shown in Table I, only the all-L tetrapeptide **9** proved to be a good substrate for the enzyme. However, the L-L-L-D derivative **10** proved to be processed to some extent, although the minimal amount of product formed precluded kinetic analysis. These data taken together show that the protease is stereospecific with respect to the CAA moieties and stereoselective with respect to X.

DISCUSSION

Hancock et al. have shown that dog pancreas microsomes can specifically proteolyze P21K-*ras*(B) to generate mature *ras* protein (Hancock et al., 1991). In the same studies, these authors demonstrated that proteolysis of pro-*ras* was essential for the efficient binding of *ras* protein to membranes (Hancock et al., 1991). Recently, rat liver microsomes have been shown to process *ras* in a way consistent with the presence of an endoproteolytic activity being present that cleaves after the farnesylated cysteine residue (Ashby et al., 1992). Although crude extracts from yeast contain multiple proteolytic activities capable of hydrolyzing farnesylated peptides, the activities uncovered thus far apparently function in a nonspecific exoproteolytic manner (Hrycyna & Clark, 1992). Further studies will be required to determine whether yeast contain

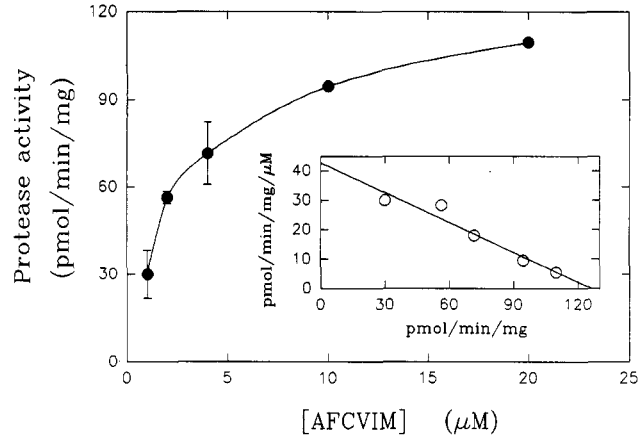


FIGURE 2: Protease activity with *N*-[^{3}H]Ac-S-farnesyl-L-Cys-L-Val-L-Ile-L-Met as substrate. Michaelis–Menten and Eadie–Hofstee plots (inset) of the formation of *N*-[^{3}H]AFC as a function of *N*-[^{3}H]Ac-S-farnesyl-L-Cys-L-Val-L-Ile-L-Met concentrations. Symbols represent mean values of two determinations and error bars represent the standard deviation from the mean. Error bars not shown are within symbols. The assays were performed as described in Materials and Methods and in Ma and Rando (1992).

a endoproteolytic activity specific for isoprenylated proteins. In a previous study we had demonstrated that bovine liver and dog pancreatic microsomes contain an endoproteolytic activity capable of processing L-AFC-L-Val-L-Ile-L-Ser and L-AFC-L-Val-L-Ile to L-AFC plus the intact tripeptide Val-Ile-Ser, and to L-AFC plus the dipeptide Val-Ile, respectively (Ma & Rando, 1992). The sequence of the tetrapeptide is taken from the carboxyl terminus of retinal transducin (Hurley et al., 1984). K_M and V_{max} values for the tetrapeptide were measured to be 4.8 μM and 236 pmol min^{-1} mg^{-1} protein, respectively (Ma & Rando, 1992). Proteolytic cleavage of the substrates is stereospecific, because the substitution of a farnesylated D-cysteine residue for the L-amino acid leads to the abolition of substrate activity. A free terminal carboxyl group is also required for substrate activity, because methyl esterification renders the substrates inert. The hydrolysis of the farnesylated tetrapeptide is not inhibited by a 5-fold excess of the corresponding nonfarnesylated tetrapeptide, suggesting that isoprenylation is important for substrate activity (Ma & Rando, 1992). These experiments are consistent with the notion that the proteolytic activity in question is the same as the one involved in the proteolysis of isoprenylated proteins, such as the *ras* products. Indeed, an inhibitor of the protease which is based on the tetrapeptide substrates blocks the proteolytic processing of pro-*ras* proteins (Hancock and Rando, unpublished experiments)).

An important issue to address is the nature of the specificity of the protease for substrates. In the current study, a series of tripeptides was studied with respect to their abilities to be processed by the protease. The studies were initiated by focusing on the role of the isoprenoid in determining whether or not a particular tripeptide would be a substrate or not. The basic tripeptide under consideration is L-Cys-L-Val-L-Ile (**4**). To begin with, this tripeptide is not proteolytically processed by the microsomal protease preparation. Moreover, the *tert*-butylthio analog (**5**) is also inert as a substrate. These experiments show that the protease is not of a nonspecific type, capable of processing random tripeptides. When the tripeptide was linked via the sulfur atom to an isoprenoid moiety, such as geranyl (**3**) or geranylgeranyl (**2**), substrate activity was reestablished. These experiments show that the isoprenoid side chain is an important determinant of substrate activity. These results are also consistent with the previous

finding that the hydrolysis of a farnesylated tetrapeptide is not inhibited by a 5-fold excess of the corresponding non-farnesylated tetrapeptide (Ma & Rando, 1992).

The finding that the farnesyl **1**, geranylgeranyl **2**, and geranyl **3** analogs are specifically hydrolyzed does not necessarily imply the presence of a single protease with these activities. Cross-inhibition studies are not uniquely helpful here, because studies of this type are uninformative with respect to actual substrate turnover. For example, geranylgeranyl pyrophosphate potently inhibits farnesyltransferase even though this enzyme does not utilize geranylgeranyl pyrophosphate as a substrate (Reiss et al., 1991; Seabra et al., 1991).

The roles of the nonisoprenylated amino acids in determining substrate specificities were also studied. Given the role that the protease(s) must play, the enzyme(s) would not be expected to be highly specific with respect to amino acid composition, since the protease(s) presumably must process many different isoprenylated proteins, with diverse CAAX sequences at their carboxyl termini. It is unlikely that different proteases exist to process the large (>50) and expanding number of distinct isoprenylated proteins. Along these lines, it is interesting to note that the protease processed both AFC-V-I-S and AFC-V-I-M. The former sequence is derived from retinal transducin, and the latter sequence is derived from the CAAX terminus of *ras*. Finally, it should also be noted that new isoprenylated proteins are being discovered in which the CAAX motif must be expanded to CXXX. It has been found that the Cys-Arg-Pro-Gln carboxyl terminus of the delta virus large antigen is isoprenylated (Glann et al., 1992). It is likely that proteins of the latter type are also proteolyzed, which further expands the number of possible substrates that require proteolytic cleavage.

Rather than attempting to catalog the various combinations of amino acid residues allowable at the AAX locations, we decided that it would be more profitable to study the stereospecificity of the interactions of the AAX amino acids with the protease. Initially, the stereospecificity of proteolytic processing in the AFC-V-I series was studied. The D-L-L **6**, the L-D-L **7**, and L-L-D **8** analogs are not substrates for the protease. Moreover, analogs studied with multiple *D*-amino acids, such as the D-D-D, the D-D-L, the D-L-D, and the L-D-D diastereomers, were also entirely inactive as substrates for the protease. These experiments show that there must be significant interactions between the protease and the AA portion of the AAX motif. While strict stereospecificity was observed at the AA sites, stereoselectivity was observed at X, because AFC-V-I-M was weakly processed when D-methionine was present. This may be interesting because binding at the X site must be less stringent than at the AA site, since sequence analysis shows that X can be any amino acid. However, there must be significant interactions between the protease and the AAX amino acids in order to explain our results. Further structure/activity studies, however, will decide whether quantitative differences exist among different peptides with respect to substrate activity. It will be especially interesting to determine whether only aliphatic amino acids are allowed at the AA sites of the CAAX motif. If so, it would suggest that the specificity of the protease is similar to the isoprenyltransferases. Whether the protease recognizes peptide sequences prior to the CAAX site is unknown. However, we have found that the deletion of the *N*-acetyl group from isoprenylated peptides that are otherwise substrates for the enzyme virtually abolishes substrate activity.

The finding that simple peptides can serve as substrates for the isoprenylated protein endopeptidase suggests that the design of specific inhibitors of this enzyme should be relatively straightforward. The structure/activity studies described here should provide useful starting points in the design of putative inhibitors of the enzyme. Mechanistic information on the class of proteases that the enzyme belongs to will also be essential for inhibitor design. It has been reported that standard protease class specific reagents do not inhibit the enzyme (Ashby et al., 1992). We have also made similar observations (Ma and Rando, unpublished experiments). The endoprotease proved to be unaffected by (1) serine protease inhibitors including phenylmethanesulfonyl fluoride, aprotinin, and leupeptin, (2) cysteine protease inhibitors including E-64 and leupeptin (the enzyme was, however, inhibited by *p*-hydroxymercuribenzoate), (3) metalloprotease inhibitors including phosphoramidon, EDTA, and 1,10-phenanthroline, and (4) the aspartyl protease inhibitor pepstatin. Given that the protease is designed to recognize modified amino acids, these results are not surprising. It will probably be essential to incorporate group-specific inhibitor moieties (Abeles & Alston, 1990; Dreyer et al., 1989; Bateman & Hersch, 1987) into the kinds of substrate molecules described here in order to begin to determine the mechanistic class that this protease belongs to.

SUPPLEMENTARY MATERIAL AVAILABLE

Details of the syntheses and ^{1}H NMR data for the modified peptides (11 pages). Ordering information is given on any current masthead page.

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