Complete Amino Acid Sequence of the Medium-Chain *S*-Acyl Fatty Acid Synthetase Thio Ester Hydrolase from Rat Mammary Gland†

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ABSTRACT: The complete amino acid sequence of the medium-chain *S*-acyl fatty acid synthetase thio ester hydrolase (thioesterase II) from rat mammary gland is presented. Most of the sequence was derived by analysis of peptide fragments produced by cleavage at methionyl, glutamyl, lysyl, arginyl, and tryptophanyl residues. A small section of the sequence was deduced from a previously analyzed cDNA clone. The protein consists of 260 residues and has a blocked amino-terminal methionine and calculated M_r of 29 212. The carboxy-terminal sequence, verified by Edman degradation of the carboxy-terminal cyanogen bromide fragment and carboxypeptidase Y digestion of the intact thioesterase II, terminates with a serine residue and lacks three additional residues predicted by the cDNA sequence. The native enzyme contains three cysteine residues but no disulfide bridges. The active site serine residue is located at position 101. The rat mammary gland thioesterase II exhibits approximately 40% homology with a thioesterase from mallard uropygial gland, the sequence of which was recently determined by cDNA analysis [Poulose, A. J., Rogers, L., Cheesbrough, T. M., & Kolattukudy, P. E. (1985) *J. Biol. Chem. 260*, 15953–15958]. Thus the two enzymes may share similar structural features and a common evolutionary origin. The location of the active site in these thioesterases differs from that of other serine active site esterases; indeed, the enzymes do not exhibit any significant homology with other serine esterases, suggesting that they may constitute a separate new family of serine active site enzymes.

The ability of the lactating mammary glands of nonruminants to synthesize the medium-chain fatty acids characteristic of milk fat is attributable to the presence in the epithelium of a tissue-specific chain-terminating enzyme, medium-chain *S*-acyl fatty acid synthetase thio ester hydrolase, trivial name thioesterase II (Knudsen et al., 1976; Libertini et al., 1976; Libertini & Smith, 1978). Thioesterase II enzymes are monomers of approximately 30 000 daltons which, although not covalently linked domains of the fatty acid synthetase, are able to hydrolyze acyl chains of intermediate chain length from the 4′-phosphopantetheine prosthetic group of the synthetase (Libertini & Smith, 1979; Smith & Libertini, 1979). The integrity of single residues of serine (Libertini & Smith, 1978), cysteine (Witkowski & Smith, 1985), and histidine (Smith et al., 1986) has been shown to be essential for catalytic activity. Recently, the amino acid sequence of a 57-residue segment of the enzyme containing a diisopropyl phosphofluoridate labeled serine residue has been elucidated and indicates that thioesterase II is a serine active site esterase (Randhawa et al., 1987). The sensitive cysteine residue has been implicated in the interfacing of the enzyme with the fatty acid synthetase, but a precise role for the sensitive histidine residue has yet to be established (Smith et al., 1986). This paper presents the complete primary structure of the enzyme. Elucidation of the thioesterase II amino acid sequence will facilitate establishment of the catalytic mechanism and the mode of interaction with the fatty acid synthetase and may reveal information regarding the evolutionary history of the enzyme.

Experimental Procedures

Materials. N^{α}-Tosyl-L-phenylalanine chloromethyl ketone–trypsin (TPCK–trypsin) and *Staphylococcus aureus* V8 protease were purchased from Millipore and Miles Laboratories, respectively. Cyanogen bromide, TFA,[1] carboxypeptidase Y, and constant boiling hydrochloric acid were purchased from Pierce Chemical Co. Iodoacetic acid (Nutritional Biochemicals) was recrystallized twice from chloroform. Radiolabeled compounds were obtained from New England Nuclear. HPLC-grade methanol and propan-2-ol were purchased from Fisher, and acetonitrile was from Burdick and Jackson. Tetrabutylammonium hydroxide (40% solution) was purchased from Sigma.

Peptide Nomenclature. Peptides were named according to the digestion method used for their generation: CB (cyanogen bromide), W (tryptophan cleavage by iodosobenzoic acid), T (trypsin), SV8 (*S. aureus* V8 protease), and ^{14}C-M ([^{14}C]-methionyl-labeled tryptic peptide). The peptides generated by each digestion were numbered sequentially from the amino terminus.

Preparation and Carboxymethylation of Thioesterase II. The enzyme was purified from lactating rat mammary glands and migrated as a single major zone on electrophoresis in polyacrylamide gels (Libertini & Smith, 1978). Prior to carboxymethylation (Perham, 1978), thioesterase II was fully reduced by incubating 75 nmol of the enzyme in 0.5 mL of 6 M guanidine hydrochloride/3 mM dithiothreitol/10 mM EDTA/0.1 M Tris-HCl (pH 8.5) under nitrogen for 0.5 h at 37 °C. Sodium iodo[2-^{14}C]acetate (12.5 μCi, 3.12 μmol) was added, and the reaction mixture was incubated in an atmosphere of nitrogen, in the dark, at 37 °C. In some experiments nonradioactive iodoacetate was used. After 30 min, 40 μL of 0.1 M dithiothreitol was added and the carboxymethylated enzyme was dialyzed overnight, under an atmosphere of nitrogen, in the dark at 4 °C against 0.2 N acetic acid and lyophilized.

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[1] Abbreviations: TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Cyanogen Bromide Cleavage. A solution containing 67 nmol of carboxymethylated thioesterase II was prepared in 0.5 mL of 70% (v/v) formic acid and purged with nitrogen for 5 min. Cyanogen bromide, in 50-fold molar excess over protein methionine (Gross & Witkop, 1962), was added, and the reaction mixture was maintained under nitrogen atmosphere at 20 °C for 24–48 h. Excess cyanogen bromide was then removed in a fume hood with a stream of nitrogen, and the mixture of peptides was adjusted to 5% (v/v) propan-2-ol, in preparation for HPLC. The presence of a carboxy-terminal homoserine residue in the peptides was verified by carboxypeptidase Y digestion and/or amino acid analysis.

Iodosobenzoic Acid Cleavage at Tryptophan. About 3 nmol of ^{14}C-carboxymethylated peptide dissolved in 80% (v/v) acetic acid/4 M guanidine hydrochloride was incubated in the dark at 20 °C with twice its weight of iodosobenzoic acid and $^1/_{20}$th of its weight of *p*-cresol (Mahoney et al., 1981). Fragments were separated directly by HPLC method A.

Exchange Radiolabeling of Methionyl Residues. The S-carboxymethylated thioesterase II (16 nmol) was dissolved in 0.25 mL of 6 M guanidine hydrochloride, and the ^{14}C exchange was achieved by the method of Shoji et al. (1983). The radiolabeled enzyme was digested with trypsin, and the peptides were purified by methods A–C.

Tryptic Cleavage. The S-carboxymethylated, methionyl-labeled thioesterase II or the cyanogen bromide fragment purified therefrom was dialyzed against 0.1 M NH_4HCO_3/10 mM $CaCl_2$ (pH 8.5). A portion of the sample containing 15 nmol of the protein in 0.5 mL was digested with 0.75 nmol of trypsin at 37 °C for 12 h. An additional 0.75 nmol of trypsin was then added and the incubation continued for 12 h. Cyanogen bromide fragments were trypsinized for 3 h by using a substrate:protease ratio of 25:1. The tryptic digests were acidified and fractionated by using HPLC methods A–C.

Staphylococcal V8 Protease Digestions. The ^{14}C-carboxymethylated thioesterase II was dialyzed against 0.1 M K_2HPO_4/2 mM EDTA/0.4% (w/v) sodium dodecyl sulfate (pH 7.8).[2] A portion of the sample containing 3 nmol of the labeled peptide (0.5 mL) was incubated with 0.1 nmol of *S. aureus* V8 protease under nitrogen at 37 °C for 12 h. Additional V8 protease (0.1 nmol) was added and the incubation continued for 24 h. The digest was acidified with TFA (1% v/v final concentration) and centrifuged, and the peptides in the supernatant were fractionated by HPLC method A. Peptides present in the acid-insoluble precipitate were solubilized in 0.4% (w/v) sodium dodecyl sulfate/0.1% (w/v) tetrabutylammonium hydroxide/5% (v/v) propan-2-ol and purified by method D.

Carboxypeptidase Y. Carboxymethylated thioesterase II or cyanogen bromide fragments derived therefrom were incubated with dialyzed carboxypeptidase Y (substrate:enzyme, 30:1 w/w) in 250 μL of 0.2 M sodium citrate buffer (pH 5.5) for up to 4 h at 37 °C (Hayashi, 1977). Samples were adjusted to pH 2.2 with HCl and subjected to amino acid analysis directly.

High-Performance Liquid Chromatography. Separation of the cyanogen bromide fragments was carried out by reversed-phase HPLC on a Waters gradient Model 6000A chromatograph equipped with an automatic sample injector (WISP 710B), variable- (Model 450) and fixed- (Model 440) wavelength detectors, system controller (Model 720), and data module recorder. For some HPLC separations an Altex-Beckman system was used in conjugation with a Gilson Holochrome detector (with 8-μL flow cell). Four separation procedures were used: method A, Synchropak-RP-P (0.41 × 25 cm, Synchrom, Inc.), propan-2-ol gradient, 5–50% in 0.1% TFA (pH 2.0), 0.7 mL/min (Mahoney & Hermodson, 1980); method B, Synchropak-RP-P, CH_3CN gradient, 5–50% in 0.1% TFA (pH 2.0), 0.7 mL/min (Pohl et al., 1984); method C, Lichrosorb C18 (0.46 × 25 cm, Altex), CH_3CN gradient, 0–40% in 10 mM ammonium acetate (pH 6.0), 1.0 mL/min (Schroeder et al., 1980); method D, Supelcosil C4 (0.46 × 2.7 cm, Supelco, Inc.), propan-2-ol gradient, 10–90% in 0.25% tetrabutylammonium hydroxide (pH 8.0), 0.5 mL/min (Randhawa et al., 1987).

Peptides were detected by their absorbance at 220 and 280 nm and characterized by amino acid analysis. The HPLC effluent was collected (1-mL fractions, unless otherwise stated) in plastic racks by using an FC-80K microfractionator (Gilson) in a fume hood. The plastic racks were cleaned with No-ChroMix (Godax Laboratories) and thoroughly washed with distilled water prior to use.

Criteria for Peptide Homogeneity. Prior to submitting peptides to automated Edman degradation, purity was established according to the following criteria: (i) single, symmetrical zone by HPLC; (ii) amino acid compositional analysis consistent with single peptide; (iii) release of a single amino-terminal residue by manual Edman degradation (Chang, 1983); (iv) single zone on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (large peptides only).

Automated Microsequence Analysis. The sequence analysis of intact protein or relatively large amounts of peptide (>5 nmol) was performed with a Beckman Model 890 M liquid-phase sequencer using 0.1 M Quadrol buffer and Polybrene (2 mg). PTH derivatives were identified by reversed-phase HPLC (Bhown et al., 1978), gas chromatography (Pisano & Bronzert, 1972), and thin-layer chromatography (Kulbe, 1974). The identification of each PTH derivative was made by at least two of the three methods.

Peptides available in only small amounts (0.1–2.0 nmol) were sequenced by using an Applied Biosystems Model 470A gas-phase Protein sequencer similar to that described by Hewick et al. (1981). PTH-amino acids were identified on-line by using an Applied Biosystems Model 120A PTH analyzer equipped with a reversed-phase column (2.1 mm × 22 cm PTH C-18) as described by Hunkapiller (1987).

Amino Acid Analysis. Routine amino acid analyses of 22-h hydrolysates were performed on a Dionex analyzer with a postcolumn fluorometric detection system (Smith et al., 1985). Occasionally, a Beckman Model 6300 high-performance amino acid analyzer equipped with a postcolumn ninhydrin detection system was used to quantitate proline residues. Tryptophan was not determined.

In order to determine the extent of cyanogen bromide cleavage or to determine total homoserine content in purified peptides, homoserine lactone was converted to homoserine after acid hydrolysis. The hydrolysate was dried by rotary evaporation and treated with pyridine–acetate (pH 6.5) for 1 h in vacuo at 110 °C (Ambler, 1965). The sample was dried and dissolved in sodium citrate buffer immediately before amino acid analysis. The well-resolved homoserine peak elutes ahead of glutamic acid with a retention time of 15.2 min. Cysteine was estimated as (carboxymethyl)cysteine and occasionally as cysteic acid (Smith et al., 1985).

RESULTS

Sequence Strategy. The sequence data that comprise the proof of the primary structure are summarized in Figure 1. Most of the sequence (190 out of 260 residues) was obtained

[2] G. R. Drapeau, personal recommendation.

Table I: Amino Acid Compositions[a] of Peptides Isolated after Cleavage of ^{14}C-Carboxymethylated Thioesterase II with Cyanogen Bromide

CNBr peptide (CB)	CB-I	CB-II	CB-III	CB-IV	CB-V	CB-VI	thioesterase II
residue no.	1	2–120	121–175	176–232	233–241	242–260	1–260
RT (HPLC-A) (min)[b]	5	77	44	67	22	55	
Asp/Asn (D/N)		11.4 (11)	5.3 (5)	7.0 (7)	1.1 (1)	4.0 (4)	28.0 (28)
Thr (T)		3.7 (4)	2.9 (3)	3.2 (3)			8.6 (10)
Ser (S)		5.2 (5)	4.0 (4)	4.4 (4)		1.5 (1)	14.0 (14)
Glu/Gln (E/Q)		11.4 (11)	8.1 (8)	3.7 (3)		2.3 (2)	25.0 (24)
Pro (P)		ND[c] (5)	ND (5)	ND (2)	ND (1)	ND (1)	14.0 (14)
Gly (G)		8.2 (8)	3.3 (3)	5.4 (5)	1.0 (1)		16.5 (17)
Ala (A)		14.1 (14)	2.0 (2)	3.6 (3)		1.0 (1)	19.5 (20)
Cys (C)[d]		1.6 (2)				0.9 (1)	3.2 (3)
Val (V)		4.9 (6)	3.6 (4)	2.3 (3)			12.2 (13)
Met (M)[e]	+ (1)	+ (1)	0.7 (1)	0.7 (1)	0.8 (1)		5.1 (5)
Ile (I)		9.2 (10)	1.8 (2)	4.3 (5)		1.6 (2)	18.6 (19)
Leu (L)		12.2 (12)	7.6 (7)	7.4 (7)	2.0 (2)	2.0 (2)	29.2 (30)
Tyr (Y)		3.9 (4)			0.9 (1)	0.8 (1)	6.0 (6)
Phe (F)		8.0 (8)	1.9 (2)	4.3 (5)	1.0 (1)	0.8 (1)	17.0 (17)
His (H)		2.7 (3)	5.2 (5)	1.4 (1)	0.9 (1)		10.0 (10)
Lys (K)		9.0 (9)	1.3 (1)	6.6 (7)		3.0 (3)	20.0 (20)
Arg (R)		3.6 (4)	3.4 (3)				7.4 (7)
Trp (W)[f]		+ (2)		+ (1)			3.0[g] (3)
no. of residues	1	119	55	57	9	19	260
yield (%)	ND	37	53	45	58	25	

[a] Determined by amino acid analysis after 22-h acid hydrolysis and calculated from sequence (in parentheses). [b] RT, retention time. [c] ND, not determined. [d] Determined as *S*-(carboxymethyl)cysteine. [e] Methionine was measured, in acid hydrolysates, as homoserine, except for the intact enzyme. [f] Presence of tryptophan is based on A_{280nm}. [g] Data taken from Libertini and Smith (1978).

by direct sequence analysis of five fragments resulting from cleavage at methionyl residues by cyanogen bromide. When cyanogen bromide fragments could not be sequenced directly to completion, additional specific cleavage methods were employed to generate smaller overlapping peptides, viz., iodosobenzoic acid cleavage at tryptophan, *S. aureus* V8 digestion at glutamic acid, and trypsin cleavage at arginine and lysine residues. Alignment of the various cyanogen bromide fragments was achieved by analysis of overlapping peptides generated by SV8 digestion and by trypsin digestion of the ^{14}C-methylated protein. The time course for release by carboxypeptidase Y of residues from the intact protein and cyanogen bromide fragments was also used to confirm the carboxy-terminal sequences of the whole enzyme and its fragments.

Analysis of Cyanogen Bromide Fragments. Sequenator analysis of the intact carboxymethylated protein (10 nmol) yielded no PTH derivatives in five cycles of the degradation, suggesting that the amino terminus of the thioesterase II is blocked. Cyanogen bromide cleavage of the reduced and S-[^{14}C]carboxymethylated thioesterase II, which contains five methionyl residues, gave six fragments as expected (Figure 2). Amino acid analysis of the purified fragments revealed that all except CB-VI contained homoserine (Table I), thus assigning CB-VI to be the likely carboxy-terminal fragment of the intact protein.

Of the remaining five cyanogen bromide fragments, one species (CB-I), after further purification (HPLC method C) and acid hydrolysis, yielded only homoserine. Since no amino acid was detectable before acid hydrolysis, homoserine was tentatively assumed to have arisen from the blocked amino-terminal residue.

Analysis of CB-II yielded the sequence of residues 2–49 and 51 and revealed the location of two tryptophans at positions 34 and 45. As shown in Table II, two peptides (W-1 + W-2 and W-3) were purified from CB-II after oxidative cleavage of tryptophan. The molecular weight (8000) and purity of the W-3 peptide were confirmed by amino acid analysis and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (details not shown). Analysis of W-3 yielded the sequence of residues 46–90 including the internal overlap 46–51 within the CB-II fragment, thus completing the sequence for residues 2–90 for CB-II. The remaining sequence of residues 91–120, including the overlap, was completed by sequencing peptides purified from the SV8 digestion of CB-II and the intact thioesterase II.

Table II: Amino Acid Compositions[a] of Peptides Isolated after Cleavage of Cyanogen Bromide Fragment ([^{14}C]Carboxymethyl)-CB-II at Tryptophan Residue(s) with Iodosobenzoic Acid

tryptophan-oxidized peptide (W)	W-1 + W-2	W-3	CB-II
residue no.	2–45	46–120	2–120
RT (HPLC-A) (min)[b]	60	85	80
Asp/Asn (D/N)	5.2 (5)	5.6 (6)	11.4 (11)
Thr (T)	1.3 (1)	2.6 (3)	3.7 (4)
Ser (S)	2.1 (2)	3.2 (3)	5.2 (5)
Glu/Gln (E/Q)	3.0 (3)	7.6 (8)	11.4 (11)
Pro (P)	ND[c] (3)	ND (2)	ND (5)
Gly (G)	3.0 (3)	5.0 (5)	8.2 (8)
Ala (A)	4.6 (5)	7.5 (9)	14.1 (14)
Cys (C)[d]	2.0 (2)		1.6 (2)
Val (V)	3.1 (3)	2.4 (3)	4.9 (6)
Met (M)[e]	0 (0)	+ (1)	+ (1)
Ile (I)	1.7 (2)	4.0 (8)	9.2 (10)
Leu (L)	2.7 (3)	5.2 (9)	12.2 (12)
Tyr (Y)	0.5 (1)	1.4 (3)	3.9 (4)
Phe (F)	3.0 (3)	4.4 (5)	8.0 (8)
His (H)	1.0 (1)	1.7 (2)	2.7 (3)
Lys (K)	3.6 (4)	4.2 (5)	9.0 (9)
Arg (R)	1.2 (1)	2.7 (3)	3.6 (4)
Trp (W)	+ (2)		+ (2)
no. of residues	44	75	119
yield (%)	64	51	

[a] Determined by amino acid analysis and calculated from sequence (in parentheses). [b] RT, retention time. [c] ND, not determined. [d] Determined as *S*-(carboxymethyl)cysteine. [e] Methionine was identified qualitatively as homoserine.

We experienced particular difficulty in solubilizing and purifying subfragments derived from CB-II, possibly due to their unusual hydrophobic character. The problems were resolved by the use of short-alkyl reversed-phase columns and alkaline solvents (HPLC method D). A complete account of the experimental evidence supporting the proposed sequence for residues 65–121, which includes the active site region and the overlap at Met-120 between CB-II and CB-III, has been presented previously (Randhawa et al., 1987).

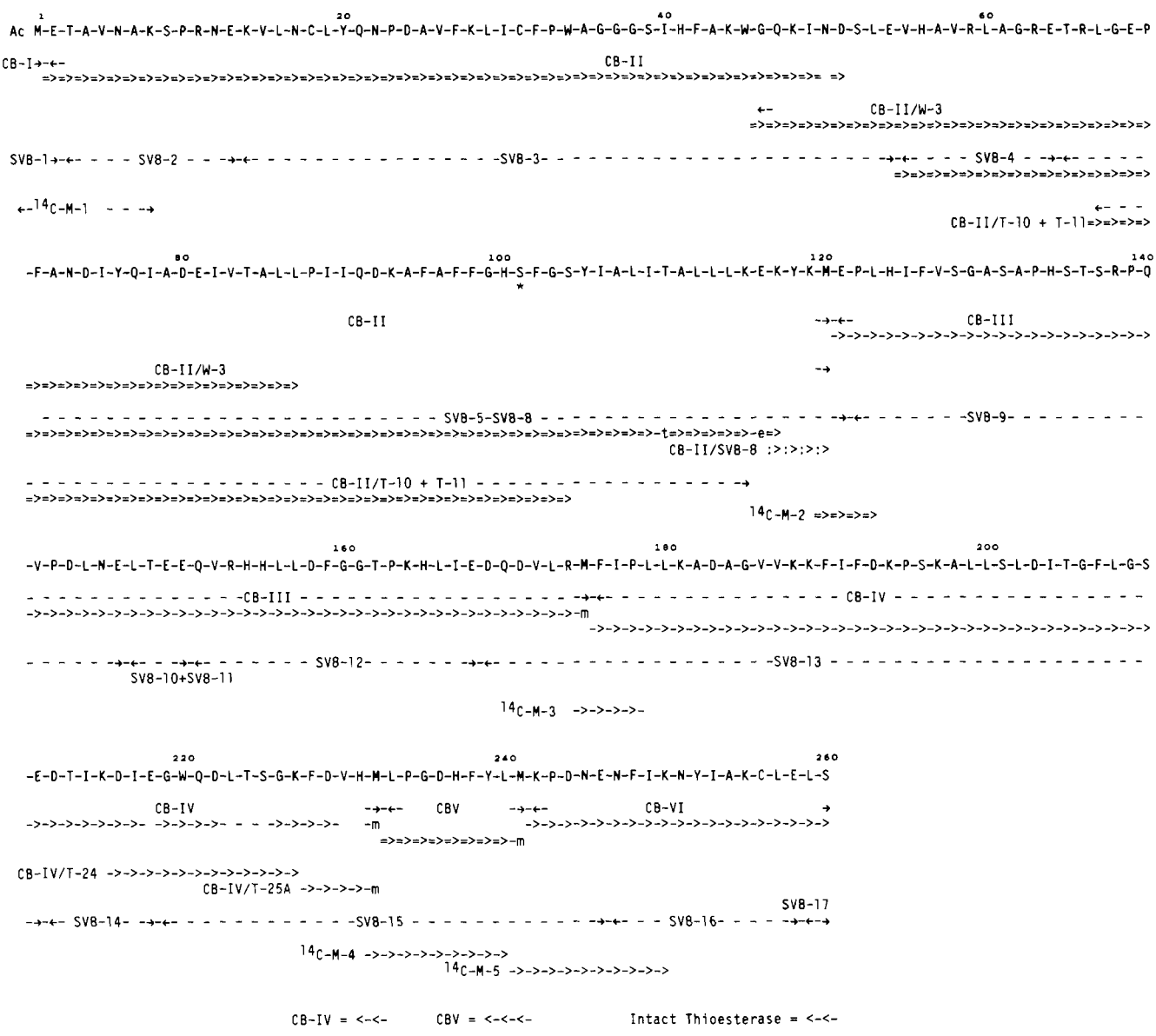

FIGURE 1: Summary of the proof of the sequence of thioesterase II. Abbreviations: CB, cyanogen bromide fragment; SV8, *S. aureus* V8 protease fragment; W, iodosobenzoic acid cleaved fragment; T, tryptic peptide; ^{14}C-M, [^{14}C]methyl iodide labeled tryptic peptide. Symbols: =>, automated Edman degradation (gas phase); ->, automated Edman degradation (liquid phase); :>, manual Edman degradation; ---, fragment purified and analyzed for amino acid composition; lower case letter, assigned sequence position based on amino acid composition; *, active site serine; <-, carboxypeptidase Y release (time course). The peptides generated by each digestion were numbered sequentially from the amino terminus. In a case where an expected cleavage did not occur and a longer peptide was obtained, the peptide was assigned two numbers, e.g., T-10 + T-11.

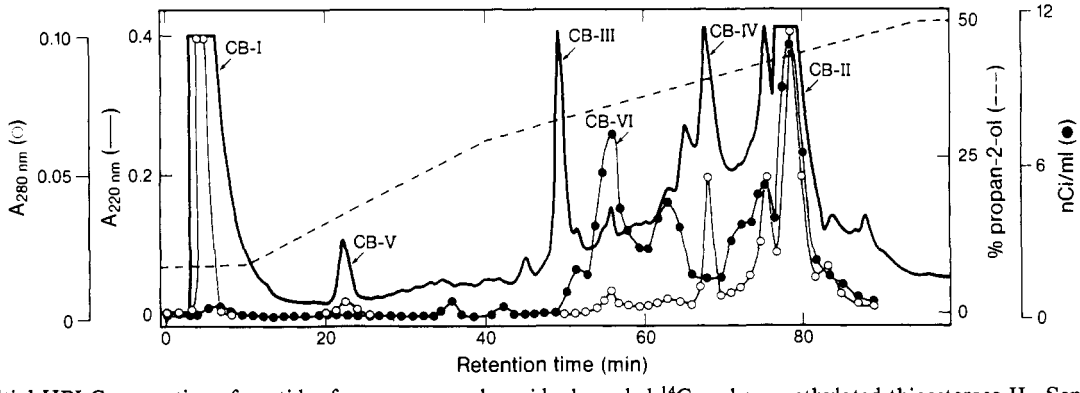

FIGURE 2: Initial HPLC separation of peptides from cyanogen bromide degraded ^{14}C-carboxymethylated thioesterase II. Separation of 10 nmol of the degraded enzyme was performed by HPLC method A. The pooled fraction of zones containing CB-II, CB-III, CB-IV, and CB-V were subjected to sequence analysis without further purification. A_{280} and radioactivity profiles were used as an indicator for the presence of tryptophan and labeled (carboxymethyl)cysteine residues, respectively.

Table III: Amino Acid Compositions[a] of Tryptic Peptides from Cyanogen Bromide Fragment CB-III

tryptic peptide (T)	T-14B + T-15	T-16	T-17	T-18A	CB-III
residue no.	121–153	154–164	165–174	175	121–175
RT (HPLC-B) (min)[b]	42	24	22	5	48
Asp/Asn (D/N)	2.1 (2)	1.0 (1)	1.9 (2)		5.3 (5)
Thr (T)	1.7 (2)	1.0 (1)			2.9 (3)
Ser (S)	3.4 (4)				4.0 (4)
Glu/Gln (E/Q)	5.5 (6)		1.9 (2)		8.1 (8)
Pro (P)	ND[c] (4)	ND (1)			ND (5)
Gly (G)	1.0 (1)	1.8 (2)			3.3 (3)
Ala (A)	2.0 (2)				2.0 (2)
Cys (C)[d]					
Val (V)	2.8 (3)		1.1 (1)		3.6 (4)
Met (M)[e]				+ (+)	0.7 (+)
Ile (I)	1.0 (1)		1.0 (1)		1.8 (2)
Leu (L)	3.0 (3)	2.0 (2)	2.1 (2)		7.6 (7)
Tyr (Y)					
Phe (F)	1.0 (1)	1.0 (1)			1.9 (2)
His (H)	2.2 (2)	2.2 (2)	1.0 (1)		5.2 (5)
Lys (K)		1.0 (1)			1.3 (1)
Arg (R)	1.9 (2)		1.1 (1)		3.4 (3)
Trp (W)					
no. of residues	33	11	10	1	(55)
yield (%)	70	84	74	ND	

[a] Determined from amino acid analysis and calculated from sequence (in parentheses). [b] RT, retention time. [c] ND, not determined. [d] Determined as *S*-(carboxymethyl)cysteine. [e] Methionine was identified qualitatively as homoserine.

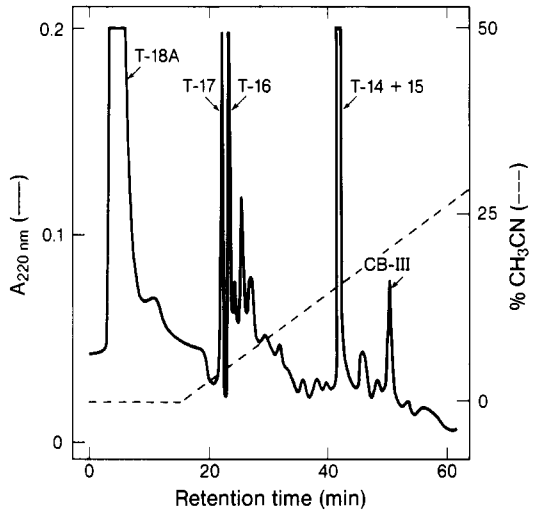

FIGURE 3: HPLC separation of tryptic peptides from CB-III fragment. Purified CB-III (3.5 nmol) was digested with trypsin, and the resulting peptides were equilibrated with 5% CH_3CN/0.1% TFA (pH 2.0) and fractionated by HPLC method B.

Of the remaining four cyanogen bromide fragments, CB-III and CB-V were sequenced up to, but not including, their carboxy-terminal homoserine residues, and CB-VI was sequenced in its entirely. The proposed sequence for CBIII was supported by analysis of the component tryptic peptides (Figure 3 and Table III). The sequence for CB-IV was completed by subdigestion with trypsin. Purification and analysis of these peptides are illustrated in Figure 4 and Table IV. Selected tryptic peptides (T-24 and T-25a) were subjected to automated Edman degradation by the gas-phase sequenator. CB-VI was sequenced to completion as verified by tryptic peptide mapping (Figure 5 and Table V) and by the time course of release of residues by carboxypeptidase Y (details not shown). A cyanogen bromide fragment with a retention time of 63 min was found to have an amino acid composition identical with that of CB-VI, retention time 55 min (Figure 2). We have assumed that these two peptides are identical in sequence but have different chromatographic properties, perhaps as a result of modification of one or more residues on one of the peptides.

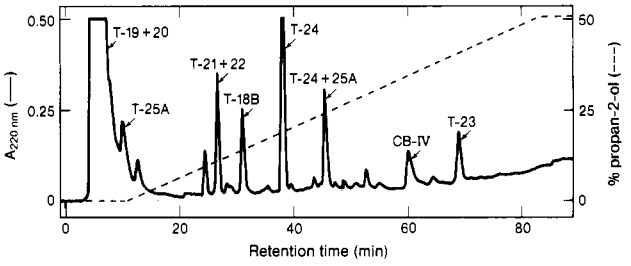

FIGURE 4: HPLC separation of tryptic peptides from the ^{14}C-carboxymethylated cyanogen bromide fragment CB-IV. The ^{14}C-carboxymethylated CB-IV fragment was first rechromatographed by HPLC method C (retention time 75 min) and trypsinized for 3 h. About 2 nmol of the digest was fractionated by HPLC method C, and the purified peptides were subjected to amino acid analysis (Table IV).

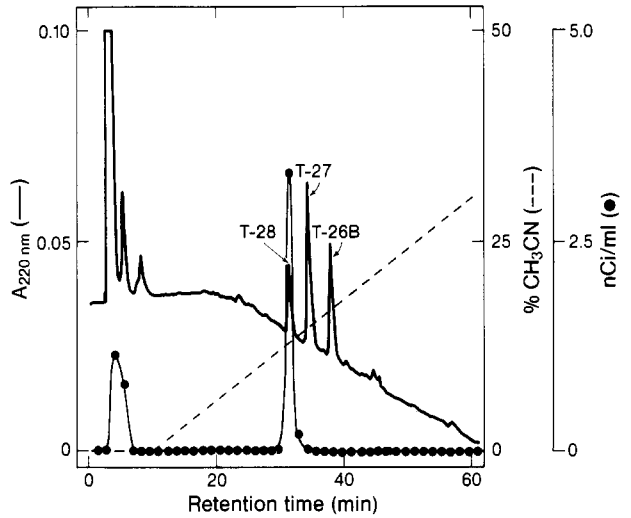

FIGURE 5: HPLC separation of tryptic peptides from CB-VI fragment. About 2 nmol of the CB-VI fragment was trypsinized, and the digest was fractionated by HPLC method A. Fraction volume was 1.5 mL.

Contiguity of the Cyanogen Bromide Fragments. Analysis of selected *S. aureus* V8 peptides (Figure 6 and Table VI) and [^{14}C]methionine-labeled tryptic peptides provided the required overlap sequence data. The amino acid compositional analysis

Table IV: Amino Acid Compositions[a] of Tryptic Peptides from Cyanogen Bromide Fragment CB-IV

tryptic peptide (T)	T-18B	T-19 + T-20	T-21 + T-22	T-23	T-24	T-25A	CB-IV
residue no.	176–181	182–189	190–197	198–215	216–227	228–232	176–232
RT (HPLC-A) (min)[b]	31	4	26	69	38	9	67
Asp/Asn (D/N)		0.9 (1)	0.9 (1)	2.1 (2)	2.0 (2)	1.0 (1)	7.0 (7)
Thr (T)				1.6 (2)	0.9 (1)		3.2 (3)
Ser (S)			1.1 (1)	2.0 (2)	1.1 (1)		4.4 (4)
Glu/Gln (E/Q)				1.1 (1)	2.1 (2)		3.7 (3)
Pro (P)	ND[c] (1)		ND (1)				ND (2)
Gly (G)		0.8 (1)		2.1 (2)	2.0 (2)		5.4 (5)
Ala (A)		2.2 (2)		1.1 (1)			3.6 (3)
Cys (C)[d]							
Val (V)		2.0 (2)				0.9 (1)	2.3 (3)
Met (M)[e]						+ (+)	+ (+)
Ile (I)	1.0 (1)		0.8 (1)	1.2 (2)	0.9 (1)		4.3 (5)
Leu (L)	2.0 (2)			3.0 (4)	1.0 (1)		7.4 (7)
Tyr (Y)							
Phe (F)	1.0 (1)		2.1 (2)	0.7 (1)		1.0 (1)	4.3 (5)
His (H)						0.7 (1)	1.4 (1)
Lys (K)	1.1 (1)	1.7 (2)	2.2 (2)	1.0 (1)	0.9 (1)		6.6 (7)
Arg (R)							
Trp (W)					+ (1)		
no. of residues	6	8	8	18	12	5	57
yield (%)	87	73	67	51	75	37	

[a] Determined by amino acid analysis and calculated from sequence (in parentheses). [b] RT, retention time. [c] ND, not determined. [d] Determined as *S*-(carboxymethyl)cysteine. [e] Methionine was identified qualitatively as homoserine.

Table V: Amino Acid Compositions[a] of Tryptic Peptides from Cyanogen Bromide Fragment ([^{14}C]Carboxymethyl)-CB-VI

tryptic peptide (T)	T-26B	T-27	T-28	CB-VI
residue no.	242–250	251–255	256–260	242–260
RT (HPLC-C) (min)[b]	38	35	31	
Asp/Asn (D/N)	2.7 (3)	1.1 (1)		4.0 (4)
Thr (T)				
Ser (S)			1.5 (1)	1.5 (1)
Glu/Gln (E/Q)	1.0 (1)		1.1 (1)	2.0 (2)
Pro (P)	ND[c] (1)			ND (1)
Gly (G)				
Ala (A)		1.0 (1)		1.0 (1)
Cys (C)[d]			0.6 (1)	0.9 (1)
Val (V)				
Met (M)				
Ile (I)	0.7 (1)	0.9 (1)		1.2 (2)
Leu (L)			2.0 (2)	2.0 (2)
Tyr (Y)		1.0 (1)		0.8 (1)
Phe (F)	0.7 (1)			0.8 (1)
His (H)				
Lys (K)	1.4 (2)	0.7 (1)		3.0 (3)
Arg (R)				
Trp (W)				
no. of residues	9	5	5	19
yield (%)	57	48	65	

[a] Determined from amino acid analysis and calculated from sequence (in parentheses). [b] RT, retention time. [c] ND, not determined. [d] Determined radiochemically and fluorometrically as *S*-(carboxymethyl)cysteine.

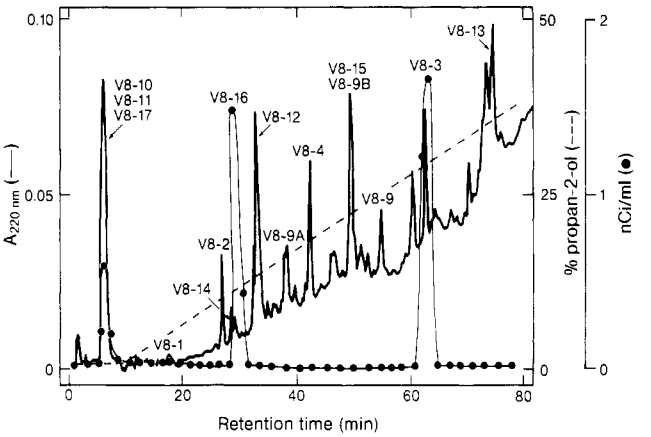

FIGURE 6: HPLC separations of *S. aureus* V8 protease digest of ^{14}C-carboxymethylated thioesterase II. Acid-soluble peptides (1 nmol) were fractionated by HPLC method A. The fractions from the center of the zones were pooled for further analysis. Amino acid analysis and recovery data for each zone are given in Table IV.

of ^{14}C-M-1 was identical with that predicted for a peptide derived from the first six residues of CB-II except that an additional residue, the radiolabeled methionine, was present (Table VII). In addition, amino acid compositional analysis of SV8-1 (Met, Glu) was consistent with the location of this peptide as the overlap between CB-I and CB-II. The cyanogen bromide fragment CB-I was found to cochromatograph, in two different HPLC solvent systems, with authentic *N*-acetylhomoserine (details not shown). Therefore, we tentatively identified *N*-acetylmethionine as the blocked amino-terminal residue of the intact protein.

Amino acid analysis of SV8-13, a methionine-containing peptide, was entirely consistent with its location as the overlap peptide between CB-III and CB-IV. One peptide, SV8-15, was purified that contained two methionine residues. The amino acid analysis of this peptide allowed its unambiguous positioning as the overlap peptide linking CB-IV and CB-V as well as CB-V and CB-VI. Thus the ordering of CB-I/CB-II and CB-III/CB-IV/CB-V/CB-VI was verified. Since the amino terminus of CB-I is blocked and the carboxy terminus of CB-VI is not a methionine, these two sections of the protein must be ordered with CB-III following CB-II. That no intervening residues are present was confirmed by the sequence of the [^{14}C]methionine-containing tryptic peptide, ^{14}C-M-2, which contains the amino-terminal sequence of CB-III preceded immediately by a methionine residue (Table VII). Three additional [^{14}C]methionyl-containing tryptic peptides were isolated, making a total of five, as predicted (Table VII). The peptides ^{14}C-M-3, ^{14}C-M-4, and ^{14}C-M-5, were located in the proposed sequence but gave no additional overlap information since methionine was the amino-terminal residue in all cases.

Several lines of evidence indicate that the carboxy-terminal residues identified by Edman degradation of CB-VI do indeed represent the true carboxy-terminal sequence of the enzyme. First, an *S. aureus* V8 peptide was identified, SV8-17, that contained only Leu and Ser; the absence of Glu and Asp

Table VI: Amino Acid Compositions[a] of Peptides Isolated after Hydrolysis of ^{14}C-Carboxymethylated Thioesterase II with SV8 Protease

peptide (SV8)	SV8-1	SV8-2	SV8-3	SV8-4	SV8-5–8	SV8-9	SV8-10 + SV8-11	SV8-12	SV8-13	SV8-14	SV8-15	SV8-16	SV8-17	TE-II
residue no.	1–2	3–13	14–54	55–64	65–121[b]	122–146[c]	147–150	151–168	169–211	212–218	219–246	247–258	259–260	1–260
RT (HPLC-A) (min)[d]	18	26	63	43	–	55	5[e]	33	75	28	50[e]	29	5[e]	
Asp/Asn (D/N)		2.0 (2)	4.0 (5)		4.0 (4)	2.4 (2)		1.0 (1)	5.2 (5)	2.0 (2)	4.5 (5)	2.0 (2)		28.0 (28)
Thr (T)		0.9 (1)			2.4 (3)	1.0 (1)	1.0 (1)	0.7 (1)	0.9 (1)	1.1 (1)	0.8 (1)			8.6 (10)
Ser (S)		1.1 (1)	2.0 (2)		2.3 (2)	3.0 (4)			3.0 (3)		1.1 (1)		1.0 (1)	14.0 (14)
Glu/Gln (E/Q)	1.0 (1)	1.0 (1)	2.5 (3)	1.0 (1)	5.5 (6)	1.6 (2)	2.0 (2)	2.0 (2)	2.1 (2)	1.0 (1)	2.0 (2)	1.0 (1)		25.0 (24)
Pro (P)		ND[f] (1)	ND (2)		ND (2)	ND (4)	ND	ND (1)	ND (2)	ND	ND (2)			14.0 (14)
Gly (G)			(4)	1.2 (1)	3.4 (3)	1.4 (1)		2.4 (2)	3.5 (3)		2.4 (3)			16.5 (17)
Ala (A)		1.8 (2)	(3)	2.0 (2)	5.5 (7)	2.1 (2)			3.0 (3)			1.2 (1)		19.5 (20)
Cys (C)[g]			1.6 (2)									0.7 (1)		3.2 (3)
Val (V)		1.0 (1)	2.2 (2)	1.7 (2)	1.2 (1)	1.3 (2)		0.9 (1)	2.6 (3)		1.4 (1)			12.2 (13)
Met (M)	0.9 (1)				0.7 (1)				0.9 (1)		2.0 (2)			5.1 (5)
Ile (I)			2.4 (3)		4.6 (7)	0.7 (1)		1.0 (1)	3.0 (3)	2.0 (2)		2.0 (2)		18.6 (19)
Leu (L)			3.1 (4)	0.9 (1)	5.5 (7)	1.8 (2)	1.0 (1)	2.1 (3)	5.2 (7)		2.8 (3)	1.0 (1)	1.0 (1)	29.2 (30)
Tyr (Y)			1.1 (1)		2.2 (3)						1.1 (1)	0.8 (1)		6.0 (6)
Phe (F)			2.8 (3)		4.7 (5)	1.0 (1)		1.0 (1)	4.0 (4)		1.6 (2)	1.1 (1)		17.0 (17)
His (H)			1.0 (1)	1.0 (1)	1.0 (1)	2.0 (2)		2.8 (3)			2.0 (2)			10.0 (10)
Lys (K)		1.2 (1)	4.0 (4)		4.0 (4)			1.0 (1)	5.5 (5)	1.0 (1)	2.0 (2)	2.3 (2)		20.0 (20)
Arg (R)		1.0 (1)		2.0 (2)	1.3 (1)	1.0 (1)		1.0 (1)	1.0 (1)					7.4 (7)
Trp (W)			+ (2)								+ (1)			3.0 (3)
no of residues	2	11	41	10	57	25	4	18	43	7	28	12	2	260
yield (%)	90	55	44	ND	60	46	86	60	42	42	32	42	82	

[a] Determined by amino acid analysis and calculated from sequence (in parentheses). [b] Randhawa et al. (1987). [c] Nonspecific cleavage after Ser-135 resulted in SV8-9A and SV8-9B fragments with retention times of 39 and 50 min, respectively (see Figure 6). [d] RT, retention time. [e] Amino acid composition was established after rechromatography of the zone. [f] ND, not determined. [g] Determined as *S*-(carboxymethyl)cysteine.

indicates this peptide is probably derived directly from the carboxy terminus. Second, amino acid analysis of T-28, a tryptic peptide derived from CB-VI, revealed the absence of both Arg and Lys residues, indicating that this peptide too was derived directly from the carboxy terminus of CB-VI (Table V). The overall amino acid composition of this peptide was entirely consistent with the sequence of the last five residues of CB-VI. Final confirmation of the carboxy-terminal residues of thioesterase II was provided by carboxypeptidase Y digestion of the whole enzyme (Table VIII). Ser and Leu were released sequentially in stoichiometric amounts.

Discussion

The task of sequencing thioesterase II was compounded by the presence of a blocked amino terminus, the hydrophobic character of the central core region (CB-II), and a surprisingly high number of bonds that showed unusual behavior toward chemical and enzymatic cleavage procedures. For example, the bond Lys-93–Ala-94 was resistant to trypsin, bonds Glu-81–Ile-82 and Glu-149–Glu-150 were resistant to *S. aureus* V8 protease, and the bond Met-120–Glu-121 was somewhat resistant to cyanogen bromide attack. This latter case was shown to account for the presence of an additional peptide in the cyanogen bromide digest which eluted from the reversed-phase column at 76 min (Figure 2) slightly ahead of CB-II (details not presented). In addition, the bonds His-231–Met-232 and Leu-240–Met-241 were cleaved unexpectedly by trypsin, and the bond Glu-120–Pro-121 was cleaved by *S. aureus* V8 protease; Glu–Pro bonds are not usually cleaved by this enzyme (Hausinger & Howard, 1982) (for example, Glu-69–Pro-70). In the case of both unexpected tryptic cleavages the residue on the carboxy side was a methionine that had been radiolabeled by the [^{14}C]methyl iodide exchange procedure. It is unclear whether this treatment may have influenced susceptibility to tryptic attack. We also observed partial hydrolysis of the Arg-66–Leu-67 and Ser-135–Thr-136 bonds (40% and 25%, respectively) by *S. aureus* V8 protease. Although this behavior is inconsistent with the established specificity of the V8 protease (Houmard & Drapeau, 1972), other exceptions have been reported (Hausinger & Howard, 1982; Reimer et al., 1983; Karplus et al., 1984).

Two observations made during our study indicate that modified amino acids may be present in the enzyme. First, although residue 250 was assigned as Lys on the basis of amino acid analysis and the observed susceptibility of the residue 250–251 bond to trypsin, during Edman degradation of CB-VI this residue could not be positively identified by HPLC or gas chromatographic methods. Second, during Edman degradation of CB-II, a substantial drop in the PTH yield was observed at residue 49. This region contains the sequence Asn-X-Ser, so that Asn-50 is a potential site for glycosylation (Hart et al., 1979; Pohl et al., 1984). Both observations require additional investigation.

The thioesterase II sequence as determined by protein sequencing terminates with a Ser residue and lacks an additional three residues, Ser-Leu-Thr, predicted by the cDNA sequence (Safford et al., 1987; J. Naggert and S. Smith, unpublished results). Whether this difference is due to proteolysis by a physiological posttranslational modification or by an artifactural in vitro proteolysis, during isolation of the enzyme, remains an open question.

The uropygial glands of certain waterfowl also synthesize medium-chain fatty acids by utilizing both fatty acid synthetase and a separate medium-chain *S*-acyl fatty acid synthetase thio ester hydrolase (De Renaboles et al., 1980). Recently, the cDNA sequence encoding the medium-chain

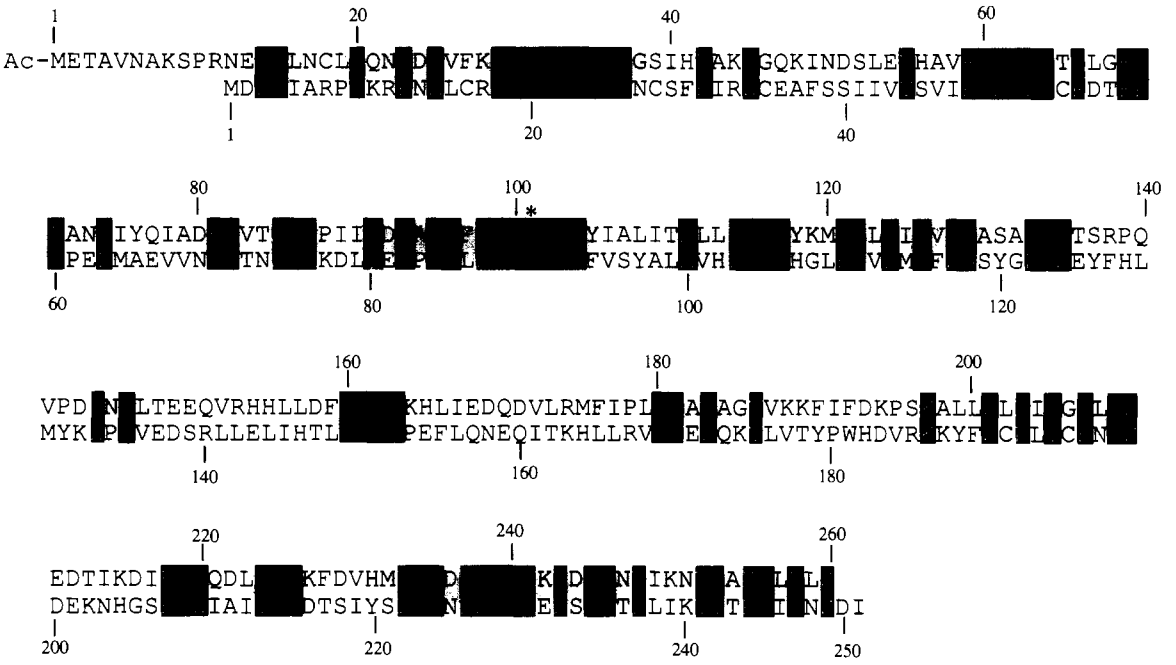

FIGURE 7: Comparison of the amino acid sequences of rat mammary (top line) and mallard uropygial (bottom line) thioesterase II enzymes. Identical residues are shown in black boxes. An acetyl moiety is tentatively presented as the amino-terminal blocking group of the rat enzyme; it is not known whether the amino-terminal residue of the uropygial enzyme is blocked. The active site serine is identified by an asterisk.

Table VII: Characterization of [^{14}C]Methionyl Tryptic Peptides Derived from Thioesterase II

[^{14}C]methionyl tryptic peptide (^{14}C-M)	^{14}C-M-1	^{14}C-M-2	^{14}C-M-3	^{14}C-M-4	^{14}C-M-5
residue no.	1–8	120–153	175–181	232–240	241–250
HPLC method [RT (min)][a]	A (6), B (35), C (30)	A (43), B (63)	A (34), B (49)	A (23), B (36)	A (9), C (40)
sequence	[B,T,Z,A(2),V,M[b],K][c]	M[b]EPL- -	M[b]FIP- -	M[b]LPGDHFYL	M[b]KPDNENFI(K)

[a] RT, retention time. [b] Radioactivity present in the peptides was associated exclusively with the methionine residue. [c] Ten nanomoles was subjected to automated Edman degradation, but no residues were released.

Table VIII: Rate of Release of Amino Acids from Thioesterase II by Carboxypeptidase Y

	amino acids released (mol/mol of peptide)			
residue	30 min	60 min	150 min	240 min
Ser	0.51	0.81	1.00	1.15
Leu	0.19	0.33	0.50	1.00

thioesterase from mallard uropygial gland was reported (Poulose et al., 1985). The rat and mallard enzymes exhibit 40% overall homology (Figure 7). Several regions exhibit lengthy, perfectly conserved sequences, 29–37, 59–64, and 98–104. This latter sequence has been shown to contain the active site serine residue (Randhawa et al., 1987). The carboxy-terminal 28 residues also show an exceptionally high degree of homology. Since birds and mammals diverged over 200 million years ago, the presence of this signficant degree of overall homology is highly indicative that the enzymes share a common evolutionary ancestry.

Thioesterase II contains an unusually high proportion of hydrophobic amino acids; Val, Phe, Ile, Leu, and Met account for 32% of the total residues in this enzyme but only 24% on average in other proteins (Dayhoff & Hunt, 1972). A particularly high concentration of hydrophobic residues is clustered in the region 82–114 (50% hydrophobic), which contains the active site serine. Since thioesterase II can hydrolyze acyl thio ester substrates containing from 8 to 20 atoms, it is tempting to speculate that the hydrophobic residues in this region may play a role in substrate binding. Evidence has been presented that a histidine residue and a cysteine residue may play critical roles in the hydrolysis of the acyl fatty acid synthetase substrate (Smith et al., 1986; Witkowski & Smith, 1985). We are presently attempting to identify the location of these residues.

A computer search has not identified any other proteins exhibiting significant overall homology with the medium-chain thioesterases. Although the thioesterases exhibit at their active site the motif Gly-X-Ser-X-Gly characteristic of serine esterases and have similar molecular weights to the serine proteases, the location of the active site at Ser-101 in the rat, Ser-90 in the mallard, is quite different from that of the serine proteases (Ser-195 in bovine chymotrypsinogen, Ser-183 in bovine trypsinogen, Ser-188 in porcine elastase; Barker & Dayhoff, 1972). It seems likely therefore that the chain-terminating thioesterases of the fatty acid biosynthetic pathway may represent a separate class of serine active site enzymes.

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Registry No. Thioesterase II, 69553-49-7; thioesterase II (rat mammary gland reduced), 106519-98-6.

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Structure and Dynamics of the Pf1 Filamentous Bacteriophage Coat Protein in Micelles†

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ABSTRACT: The major coat protein of filamentous bacteriophage adopts its membrane-bound conformation in detergent micelles. High-resolution ^1H and ^{15}N NMR experiments are used to characterize the structure and dynamics of residues 30–40 in the hydrophobic midsection of Pf1 coat protein in sodium dodecyl sulfate micelles. Uniform and specific-site ^{15}N labels enable the immobile backbone sites to be identified by their $^1H/^{15}N$ heteronuclear nuclear Overhauser effect and allow the assignment of ^1H and ^{15}N resonances. About one-third of the amide N–H protons in the protein undergo very slow exchange with solvent deuterons, which is indicative of sites in highly structured environments. The combination of results from $^1H/^{15}N$ heteronuclear correlation, ^1H homonuclear correlation, and ^1H homonuclear Overhauser effect experiments assigns the resonances to specific residues and demonstrates that residues 30–40 of the coat protein have a helical secondary structure.

The current level of understanding of biological processes involving membrane-bound proteins is not as detailed as for those involving cytoplasmic proteins. This is due, in large part, to the difficulty in applying methods capable of yielding structural information at atomic resolution to membrane-bound proteins (Eisenberg, 1984). Although considerable progress has been made with diffraction (Leifer & Henderson, 1983) and NMR (Keniry et al., 1984) studies on describing the structure of bacteriorhodpsin in purple membrane, and with diffraction on the structure of porin of *Escherichia coli* outer membrane (Kleffel et al., 1985), only the recent diffraction results on the photoreaction center (Deisenhofer et al., 1985) crystallized from detergent (Michel, 1982) approach the

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