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Degradation of a Signal Peptide by Protease IV and Oligopeptidase A

PAUL NOVAK AND INDERJIT K. DEV*

Department of Microbiology, Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709

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The degradation of the prolipoprotein signal peptide in vitro by membranes, cytoplasmic fraction, and two purified major signal peptide peptidases from *Escherichia coli* was followed by reverse-phase liquid chromatography (RPLC). The cytoplasmic fraction hydrolyzed the signal peptide completely into amino acids. In contrast, many peptide fragments accumulated as final products during the cleavage by a membrane fraction. Most of the peptides were similar to the peptides formed during the cleavage of the signal peptide by the purified membrane-bound signal peptide peptidase, protease IV. Peptide fragments generated during the cleavage of the signal peptide by protease IV and a cytoplasmic enzyme, oligopeptidase A, were identified from their amino acid compositions, their retention times during RPLC, and knowledge of the amino acid sequence of the signal peptide. Both enzymes were endopeptidases, as neither dipeptides nor free amino acids were formed during the cleavage reactions. Protease IV cleaved the signal peptide predominantly in the hydrophobic segment (residues 7 to 14). Protease IV required substrates with hydrophobic amino acids at the primary and the adjacent substrate-binding sites, with a minimum of three amino acids on either side of the scissile bond. Oligopeptidase A cleaved peptides (minimally five residues) that had either alanine or glycine at the P'_1 (primary binding site) or at the P_1 (preceding P'_1) site of the substrate. These results support the hypothesis that protease IV is the major signal peptide peptidase in membranes that initiates the degradation of the signal peptide by making endoproteolytic cuts; oligopeptidase A and other cytoplasmic enzymes further degrade the partially degraded portions of the signal peptide that may be diffused or transported back into the cytoplasm from the membranes.

Polypeptides to be translocated across membranes in *Escherichia coli* are generally synthesized in a precursor form, with an amino-terminal signal sequence. These signal sequences share some structural similarity, even though their primary sequences differ considerably (26). Many studies have shown that the signal peptide is required for the export of proteins across the membranes (2, 26). Signal peptides, once removed by signal peptidase, do not accumulate but are rapidly degraded (10, 12, 17, 24, 33). The reasons for this rapid degradation are not clear. However, data from many laboratories (1, 4, 18, 31) suggest that the signal peptide itself may be involved in the regulation of the export process. The rapid removal and degradation of the cleaved signal peptide may be necessary to maintain proper export.

Three different peptidases capable of cleaving an intact signal peptide from *E. coli* have been identified. Ichihara et al. (13) have demonstrated that protease IV (21), a membrane-bound protease, can hydrolyze the prolipoprotein signal peptide. The gene for protease IV (*sppA*) has been cloned (14) and used to obtain deletion mutants (28). The digestion of prolipoprotein signal peptide in an isolated cell envelope fraction in a deletion mutant suggested that another membrane-bound protease is also capable of cleaving the signal peptide, although slowly. Novak et al. (20) have shown that there are two cytoplasmic enzymes that can degrade a signal peptide in vitro; the majority of the cytoplasmic signal peptide hydrolase activity is due to oligopeptidase A (29), and less than 10% of the activity is due to protease So (5).

The role of these peptidases in the degradation of signal peptide in vivo is unknown. The intact signal peptide is not accumulated in *E. coli* mutants lacking protease IV (28) or in mutants of *Salmonella typhimurium* deficient in oligopeptidase A (I. Dev, unpublished data). The data suggest that these two major peptidases alone are not solely responsible for the cleavage of an intact signal peptide. Three possibilities regarding the degradation of the signal peptide in vivo have been proposed (20, 24). The data in this paper support the hypothesis that protease IV initially cleaves the signal peptide by making endoproteolytic cuts and that the products of the initial cleavage that may be diffused or transported back into the cytoplasm are further degraded into amino acids by oligopeptidase A and other cytoplasmic enzymes.

MATERIALS AND METHODS

Purification of protease IV. Membranes were isolated from 200 g of frozen (−70°C) *E. coli* K-12 cells (grown in minimal medium and harvested at mid-log phase; Grain Processing Inc., Muscatine, Iowa) according to the procedure of Dev and Ray (7). The methods for the washing of membranes, extraction of proteins from membranes, and purification of protease IV were as described by Pacaud (21), except the concentration of sodium lauryl sulfate, due to insolubility in buffer A, was less than 0.25%. Protease IV, as judged by sodium dodecyl sulfate (SDS)-gel electrophoresis, was only 25% pure. The enzyme was further purified by gel permeation high-pressure liquid chromatography on a TSK G3000 column (7.5 by 600 mm) according to the method described by Palmer and St. John (22). The final enzyme preparation was better than 80% pure and free of contaminating proteases as judged by its inhibitor profile and substrate specificity for chromogenic substrates (14, 21). The molecular weight of protease IV during SDS-gel electrophoresis was about 67,000 (14). The enzyme was stored at −20°C in a buffer containing 10 mM sodium phosphate, 10% (vol/vol)

* Corresponding author.

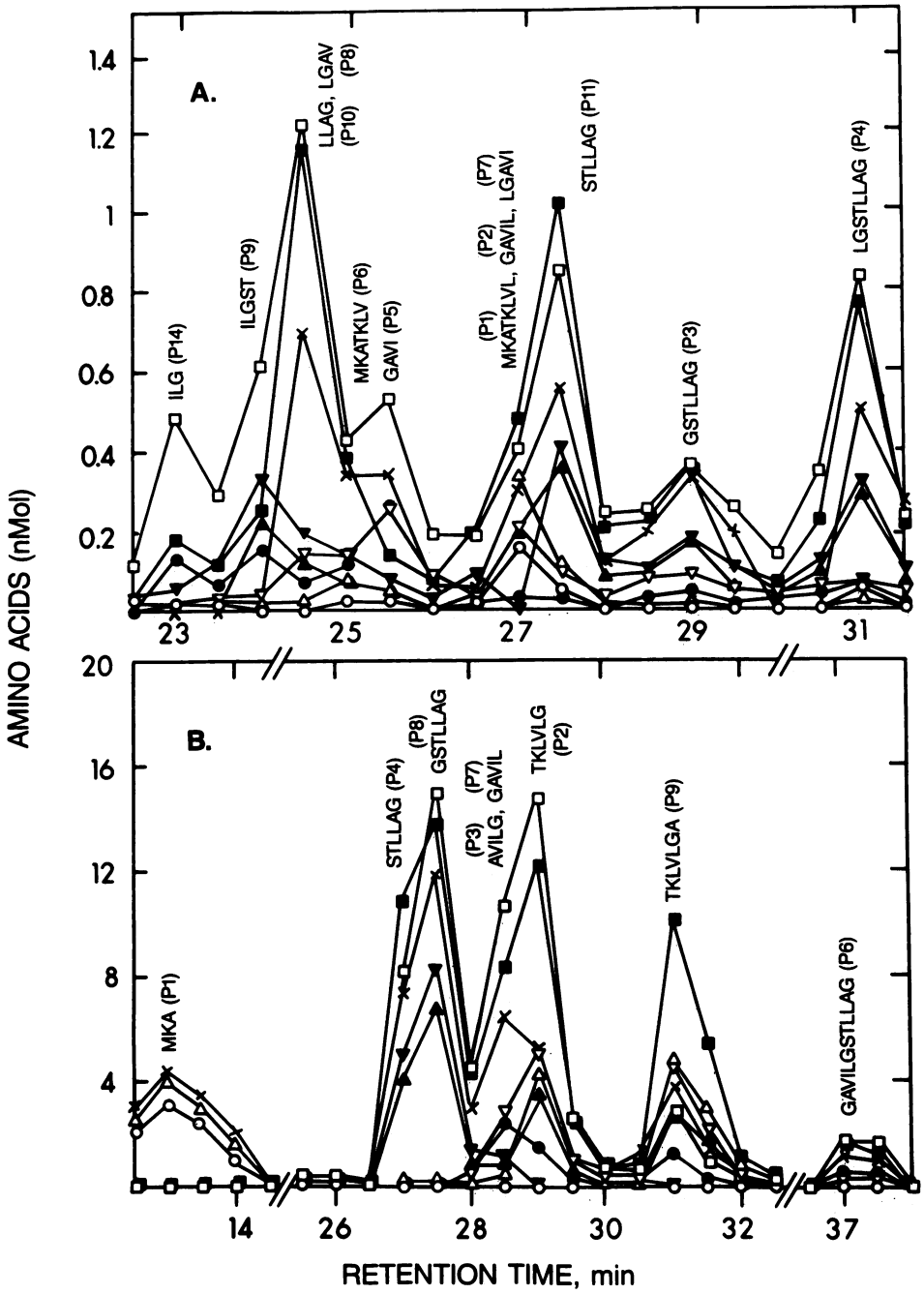

FIG. 1. Identification of peptide fragments from their amino acid compositions. The peptide fragments produced during the cleavage reactions were separated by RPLC with a linear gradient of 0 to 60% CH_3CN in 0.1% TFA for 50 min at a flow rate of 1.0 ml/min. Fractions of 0.5 min were collected, and the amino acid composition of each fraction was estimated and plotted as a function of retention time. The protease IV reaction (A) contained 135 nmol of signal peptide and 65 μg of protease IV. The peptide fragments were designated by the same numbers as in Fig. 3. Peptides P12 and P13 were not produced during this experiment. The oligopeptidase A reaction (B) contained 350 nmol of signal peptide and 2 μg of oligopeptidase A. The peptide fragments were designated by the same numbers as in Fig. 5. Peptides P5 and P10 were not produced during this experiment. Symbols: Leu (■), Gly (□), Ile (●), Met (○), Ala (×), Thr (▲), Lys (△), Ser (▼), and Val (▽).

glycerol, 0.05% Emulphogen BC-720 (Sigma), and 1 mM β-mercaptoethanol (pH 7.2). Under these conditions, protease IV was stable for more than 6 months.

Purification of oligopeptidase A. The methods for the preparation of cytoplasmic extracts free of membranes from *E. coli* K-12 and the purification of oligopeptidase A by conventional chromatography were as described by Novak et al. (20). However, these authors refer to this enzyme as 68,000-M_r signal peptide hydrolase. Oligopeptidase A (ca. 10 mg), after purification on a chromatofocusing column, was dialyzed and then concentrated on a DEAE column (0.25 g of DE-52; Pharmacia) as described before (20). The enzyme was eluted with 250 mM potassium phosphate, pH 8.0, and stored at 4°C. The enzyme was stable for at least 6 months

under these conditions. The final enzyme preparation was purified to near homogeneity, as judged by SDS-gel electrophoresis, and was free of contaminating proteases.

Enzyme assays. Signal peptide peptidase (hydrolase) activity due to both enzymes was measured by the hydrolysis of [^{35}S]methionine-labeled signal peptide (20). General proteolytic activity was assayed by the hydrolysis of casein and insulin (9). The activity of membrane proteases was estimated spectrophotometrically at 400 nm by the hydrolysis of *N*-acyl-amino acid-*p*-nitrophenol ester (Sigma) according to the procedure of Pacaud (21).

Isolation and purification of prolipoprotein signal peptide. Prolipoprotein labeled with [^{35}S]methionine or [^{3}H]leucine was prepared from *E. coli* B by using globomycin as described previously (7), except during labeling the growth medium was supplemented with an amino acid mix (10 μg/ml) without the labeled amino acid. The signal peptide was generated from prolipoprotein by purified signal peptidase and further purified by the methods of Novak et al. (20). A chemically synthesized prolipoprotein signal peptide was purchased from D. Klapper of the University of North Carolina at Chapel Hill and further purified by reverse-phase liquid chromatography (RPLC) on a C18 μBondapak column (0.39 by 30 cm; Waters Associates, Boston, Mass.). Approximately 2 mg of the signal peptide was applied and eluted with a linear gradient of 0 to 60% CH_3CN in 0.1% trifluoroacetic acid (TFA). The column eluate was monitored at 215 nm and collected in 0.5-ml fractions. Highly absorbing material, shown by amino acid composition to be prolipoprotein signal peptide, eluted at a CH_3CN concentration of about 50%. These fractions were pooled, dried in a Savant Speed Vac Concentrator (Savant Instruments), suspended in 0.25 M sodium phosphate, pH 8.0, and stored at −20°C.

Cleavage of signal peptide and isolation of peptide fragments. The hydrolysis of radiolabeled signal peptide was performed at 37°C as described previously (20). The chemically synthesized signal peptide (28 to 350 nmol) was hydrolyzed in a 1.0-ml reaction volume containing 0.25 M sodium phosphate (pH 8.0) and enzyme (65 μg of protease IV or 2 μg of oligopeptidase A). Reaction mixtures were centrifuged for 10 min in a 1.5-ml Eppendorf centrifuge tube, and the supernatant was chromatographed at 1 ml/min on a C18 μBondapak column. The reaction products were eluted with an appropriate CH_3CN gradient in 0.1% TFA. A mixture of standard amino acids (Pierce) was chromatographed to show that on a C18 μBondapak column free amino acids elute in 0.1% TFA without any added CH_3CN.

Estimation of amino acid composition of peptide fragments. Phenylisothiocyanate (PITC) reacts rapidly and quantitatively with amino acids. This property of PITC has been utilized by Henrikson and Meredith (11) to develop a sensitive and precise method of amino acid analysis. This method has been adapted by Waters Associates with some modifications (3). The fractions collected during RPLC were dried in Pyrex tubes (6 by 50 mm) with a Savant Speed-Vac Concentrator. The samples were hydrolyzed at 150°C for 60 min and derivatized with PITC, and the resulting phenylthiocarbamyl-amino acids (PTC-amino acids) were analyzed according to the operator's manual for the Pico-Tag amino acid analysis system of Waters Associates. A standard mixture of PTC-amino acids (500 pmol) was prepared and analyzed along with each set of peptide hydrolysate samples. Integration values (11) for each PTC-amino acid were estimated from the standards and used in the quantitation of amino acids in peptide hydrolysate samples.

Identification of peptide fragments from amino acid composition. Amino acid composition indexes have been used to assist in the identification of peptide fragments produced from a protein of known amino acid sequence (6). We have also used a similar approach to identify the peptide fragments generated from the cleavage of the signal peptide by protease IV and oligopeptidase A. The peptide fragments produced during these reactions were separated by RPLC with a linear gradient of CH_3CN in 0.1% TFA. The amino acid composition of each 0.5-min fraction was estimated and plotted as a function of retention time. Two such plots showing the identification of peptide fragments by these methods are shown in Fig. 1. Three major criteria were used to identify the peptide fragments.

(i) Shape of the peaks of amino acid profile. We assumed that if amino acids eluted together in a symmetrical peak, they probably originated from a common peptide. Based on this criterion, peptides MKA, ILG, TKLVLGA, GSTLLAG, LGSTLLAG, and GAVILGSTLLAG, which apparently eluted free of other peptide fragments, were identified (Fig. 1). However, many other peaks either lacked symmetry in the amino acid profiles or had amino acids present in nonstoichiometric amounts. This suggested that more than one peptide may have eluted together. A majority of the peptides in Fig. 1A were contaminated with other peptide fragments. For example, 10 peptides ranging from 200 to 600 pmol eluted between retention times of 24 and 28 min (Fig. 1A). We considered various possibilities concerning amino acid sequences for each peptide fragment. The identification of a peptide fragment that satisfied the next two criteria was considered final.

(ii) Knowledge of amino acid sequence of signal peptide. The molar ratio of each amino acid in a peptide was estimated and normalized to the nearest whole number. The contribution of a common amino acid present in closely eluting

TABLE 1. Comparison of observed and predicted retention times[a]

Peptide fragment	Retention time (min)	
	Predicted	Observed
MKA	5.64	13.0
ILG	23.27	23.0
ILGST	24.99	24.0
LLAG	23.57	24.5
LGAV	18.72	24.5
MKATKLV	21.92	25.0
GAVI	19.03	25.5
MKATKLVL	22.85	27.0
GAVIL[b]	25.24	27.0
GAVIL[c]	25.24	29.0
LGAVI	25.24	27.0
AVILG	25.24	29.0
STLLAG[b]	25.26	27.5
STLLAG[c]	25.26	27.0
GSTLLAG[b]	25.98	29.0
GSTLLAG[c]	25.98	27.5
TKLVLG	25.4	29.0
TKLVLGA	25.84	31.0
LGSTLLAG	29.94	31.0
GAVILGSTLLAG	34.01	37.0

[a] Retention times of peptide fragments on a C18 μBondapak column were predicted by using the equation in the text and the nonweighted constants for amino acids (25). Slope (A = 12.78) and intercept (C = −28.84) were estimated by analyzing the retention behavior of 15 known peptides under similar conditions. The observed values were obtained from the experiments described in the legend to Fig. 1.

[b] The observed values were from the experiment in Fig. 1A.

[c] The observed values were from the experiment in Fig. 1B.

peptides was estimated by subtracting the overlap. For glycine, a background value of 150 to 200 pmol was subtracted. The known sequence of the signal peptide was searched for the presence of these amino acids in a sequence. Most of the combinations of amino acids were unique and were only present in a certain sequence in signal peptide. However, a few ambiguities were also observed. For example, cleavage of signal peptide at the carboxyl termini of amino acids 7 and 12, 8 and 13, and 9 and 14 would result in peptide fragments with three different amino acid sequences (LGAVI, GAVIL, and AVILG) but the same amino acid composition. These and other ambiguities were resolved by reconstructing the known sequence of signal peptide from all the peptide fragments generated in an experiment.

(iii) Retention time during RPLC. The identification of a peptide was further confirmed by its retention time during RPLC. The retention time (t_{Ri}) of a peptide on a C18 column can be predicted from its amino acid composition (25). Retention times were predicted by using the equation

$$(t_{Ri} = A \ln(1 + \sum_j D'_j n_{ij}) + C$$

where D'_j is the nonweighted retention constant of amino acid j and n_{ij} is the number of residues of amino acid j in peptide i. Slope (A) and intercept (C) were estimated by analyzing the retention behavior of 15 known peptides under similar conditions. The predicted and observed retention times of the peptides identified by the above methods were compared (Table 1). The correlation coefficient was 0.92, and the mean percent deviation of retention time was 13%. These data suggested that there were no gross errors in the methods we used to identify the peptide fragments.

RESULTS

Cleavage of signal peptide by membrane and cytoplasmic extracts. The objective of this experiment was to investigate whether membranes or cytoplasmic extracts alone could hydrolyze the prolipoprotein signal peptide into free amino acids. The signal peptide is a 20-amino-acid peptide that has one methionine at its amino terminus and leucine at positions

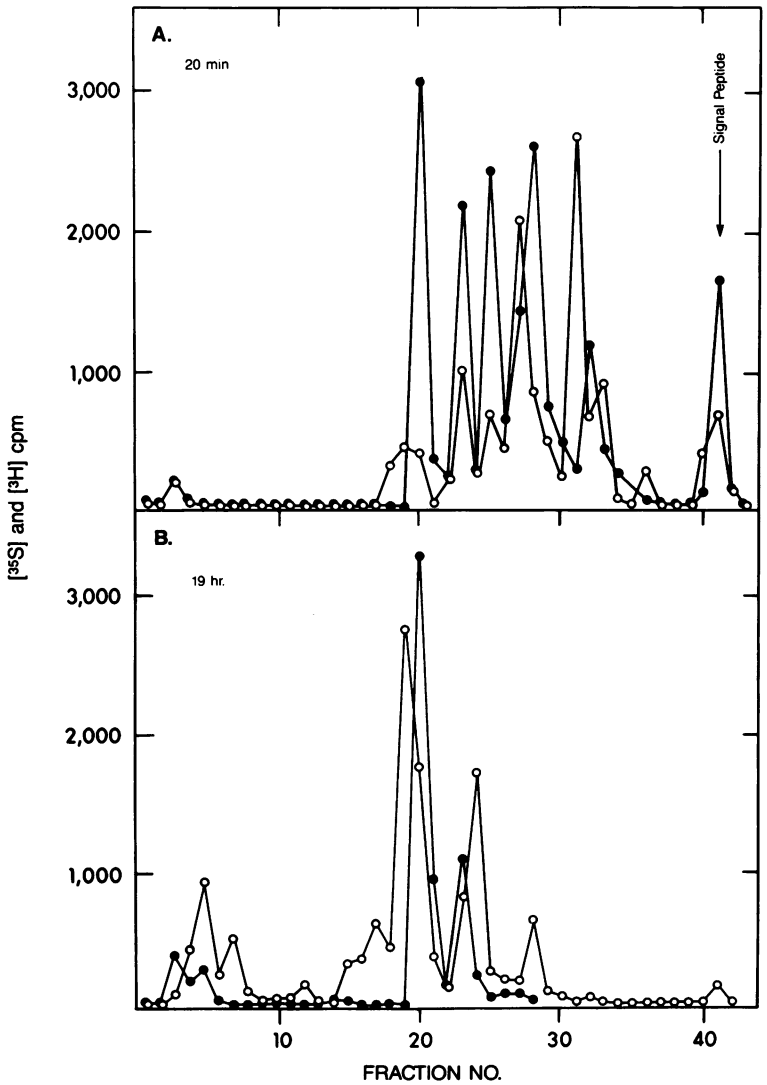

FIG. 2. Time course analysis of products during degradation of radiolabeled signal peptide by purified protease IV. Signal peptide labeled with [^{35}S]methionine (●) or [^{3}H]leucine (○) was hydrolyzed with protease IV for 0.33 h (A) or 19 h (B). The products were analyzed by RPLC with a linear gradient of 10 to 60% CH_3CN for 25 min at a flow rate of 1 ml/min. Fractions of 0.6 ml were collected, and the radioactivity was determined (7).

6, 8, 13, 17, and 18 (15). The native signal peptide was generated during the maturation of prolipoprotein by the purified prolipoprotein signal peptidase (7). The signal peptide isolated from prolipoprotein is intact and radiochemically pure, has a free amino terminus, and cochromatographs with the chemically synthesized signal peptide during SDS-gel electrophoresis and RPLC (7, 20; P. Novak and I. Dev, unpublished data). The signal peptide radiolabeled with [^{35}S]methionine or [^{3}H]leucine was cleaved by cell-free cytoplasmic fraction or Emulphogen extracts of membranes (21), and the cleavage products were analyzed by RPLC (data not shown). Cytoplasmic extracts completely hydrolyzed the purified signal peptide into free radiolabeled amino acids. Emulphogen extracts of membranes did not cleave the signal peptide into amino acids; however, many ^{35}S- and ^{3}H-radiolabeled peptide fragments accumulated as final products.

Cleavage of signal peptide by protease IV. The radiolabeled signal peptide was cleaved by protease IV, and the products were analyzed by RPLC. The data are shown in Fig. 2. Many ^{35}S- and ^{3}H-radiolabeled peptide fragments were generated as a function of time. The number and apparent sizes of radiolabeled peaks decreased with time. After 19 h of incubation, the radioactivity profile did not change after further addition of the enzyme or an increase in incubation time for the reaction. Only two major [^{35}S]methionine-labeled peaks accumulated as final products of the cleavages (Fig. 2B). In contrast, six different [^{3}H]leucine-labeled peaks were observed as final products of the reaction. The final products formed by the purified protease IV and the crude membrane extracts were compared. The retention times for most of the [^{3}H]leucine-labeled peaks were similar; however, their relative amounts were different. More significantly, neither [^{35}S]methionine-labeled peak that accumulated as a final product during protease IV cleavage was present in crude extracts. Apparently, these peptides were further fragmented into smaller peptides by another peptidase(s) in the crude membrane extracts.

The peptide fragments formed by the degradation of the radiolabeled native signal peptide were not identified because this substrate was only available in small amounts and was not chemically pure. A chemically synthesized signal peptide was used as a substrate to identify the peptide products (Fig. 1). The peptide fragments were separated by RPLC (Fig. 1) with a CH_3CN gradient different from that described in the legends to Fig. 2 and 4. Most of the products of cleavages by both the enzymes were not resolved on a C18 column and eluted together (Fig. 1). The change in the

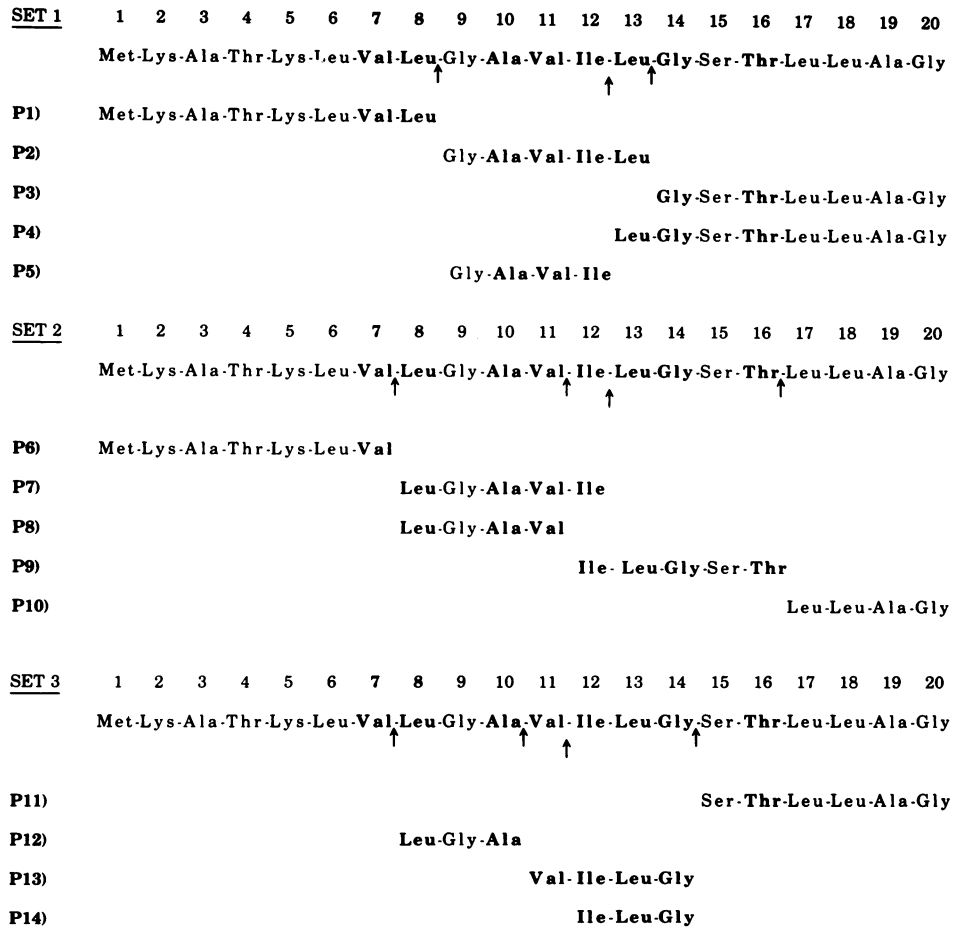

FIG. 3. Proposed cleavage sites for protease IV. Chemically synthesized signal peptide (28 and 135 nmol) was cleaved by 65 μg of protease IV for 19 h in two separate experiments. Fourteen products (P1 to P14) were identified as described in the legend to Fig. 1. The entire sequence of prolipoprotein signal peptide is depicted, and amino acid residues are numbered 1 to 20. A combination of cleavages by protease IV is proposed to account for all the products formed. The cleavages were separated into three sets to improve visual comprehension. The arrows indicate the proposed cleavage sites; the amino acid at the primary binding site of the substrate is shown in boldface letters.

gradient also did not alter the number of leucine- and methionine-containing peaks resolved during the chromatography. The peptide products for protease IV appeared as two methionine- and five to seven leucine-containing peaks during RPLC with either the native or the synthetic signal peptide as a substrate (compare Fig. 1A and 2B). Similarly, during oligopeptidase A reactions, only one methionine- and three to four leucine-containing peaks were observed with either signal peptide (compare Fig. 1B with 4B).

Two different amounts (28 and 135 nmol) of the synthetic signal peptide were cleaved by 65 μg of protease IV. Twelve peptides (0.19 to 0.61 nmol) were identified as products at higher substrate concentration (Fig. 1A). However, when the signal peptide was hydrolyzed more extensively at about five-times-higher enzyme-to-substrate ratio, two additional peptides (P12 and P13) were also identified (data not shown). All 14 peptide fragments (P1 to P14) identified as the products of protease IV cleavage in two different experiments are shown in Fig. 3. No free amino acids or dipeptides were detected during the cleavage reactions. Most of the products were tripeptides or larger (Fig. 3). Based on the peptide fragments produced, different combinations of cleavages for protease IV have been proposed and are depicted in Fig. 3.

Cleavage of signal peptide by oligopeptidase A. [^{35}S]methionine- and [^{3}H]leucine-labeled signal peptides were cleaved with oligopeptidase A for different times, and the products were isolated by RPLC (Fig. 4A and B). A major [^{35}S]methionine-labeled peak in fraction 3 with a small shoulder (Fig. 4B) accumulated as a final product of the cleavage reactions. In contrast, three [^{3}H]leucine-containing peptides accumulated as final products of oligopeptidase A cleavages.

Three different amounts of the chemically synthesized signal peptide (28 to 350 nmol) were cleaved by 2.0 μg of oligopeptidase A for 19 h. When 350 nmol of the signal peptide was hydrolyzed by oligopeptidase A, only eight peptide fragments were identified as products (Fig. 1B). Most of the peptides were present in about equal amounts (10 to 16 nmol). The only exception was the peptide designated P6 (1.1 nmol), which may be an intermediate for peptide fragments P7 and P8. The hydrolysis of signal peptide at a higher enzyme-to-substrate ratio resulted in the formation and identification of two more peptides, designated P5 and P10 (data not shown). All 10 peptide fragments (P1 to P10) identified as the products of oligopeptidase A cleavage during three different experiments are shown in Fig. 5. Only one peptide with an NH_2-terminal methionine (Met-Lys-Ala) and nine leucine-containing peptide fragments were identified. Neither free amino acids nor dipeptides were found. Based on the peptide fragments produced, many cleavage sites for oligopeptidase A have been proposed and are depicted in Fig. 5.

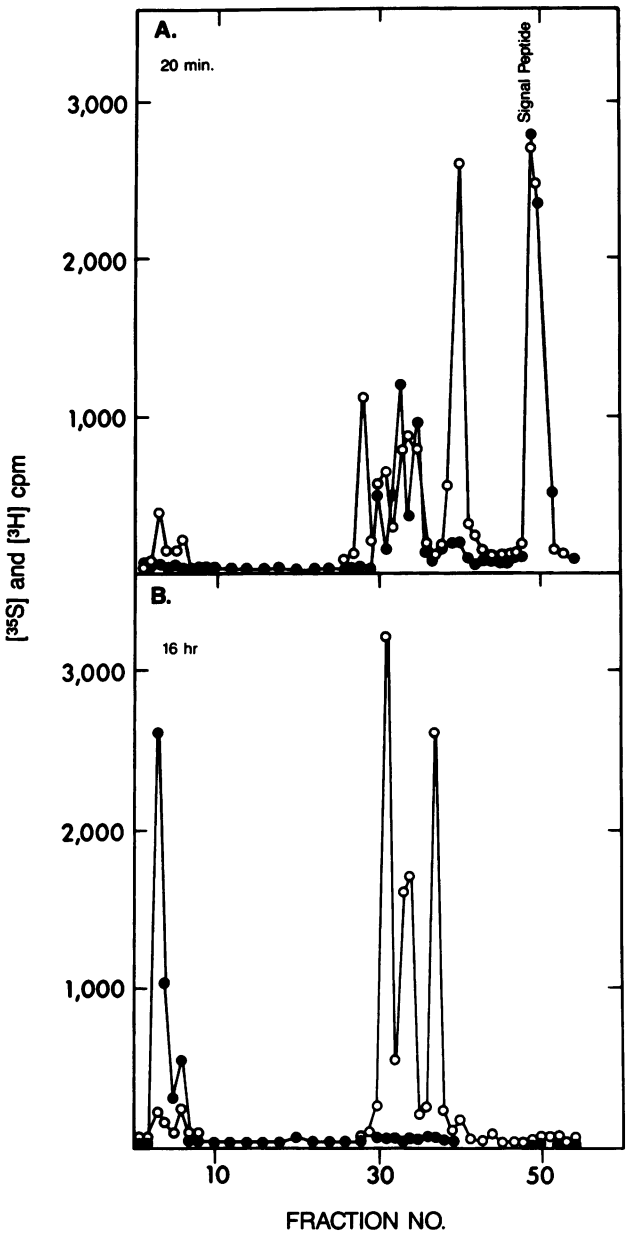

FIG. 4. Analysis of products formed during degradation of radiolabeled signal peptide by oligopeptidase A. Signal peptide labeled with [^{35}S]methionine (●) or [^{3}H]leucine (○) was hydrolyzed with oligopeptidase A for 0.33 h (A) or 16 h (B). The products were analyzed as described in the legend to Fig. 2.

DISCUSSION

We have used RPLC to follow the degradation of a synthetic and native prolipoprotein signal peptide by solubilized membranes, a cytoplasmic fraction, and two purified major signal peptide peptidases from *E. coli*. The cytoplasmic fraction hydrolyzed the signal peptide completely to amino acids (data not shown). Many cytoplasmic peptidases with different substrate specificities have been identified as part of the pathway of protein degradation in *E. coli* (9, 19, 32) and may be involved in complete degradation of the signal peptide. In contrast, many leucine-containing peptide fragments accumulated as final products during the cleavage by membrane fractions (data not shown). Most of these peptides had retention times identical to those of peptides formed during the cleavages of the signal peptide by the membrane-bound protease IV (Fig. 2B). However, two methionine-containing peptides that accumulated as final product during protease IV cleavages were degraded into smaller peptides by the crude extracts. This could be due to the presence of another signal peptide peptidase in the membranes, as suggested by Suzuki et al. (28), or to a contaminating cytoplasmic enzyme. These data suggest that protease IV is the major signal peptide peptidase that initiates the degradation of the signal peptide in the membranes.

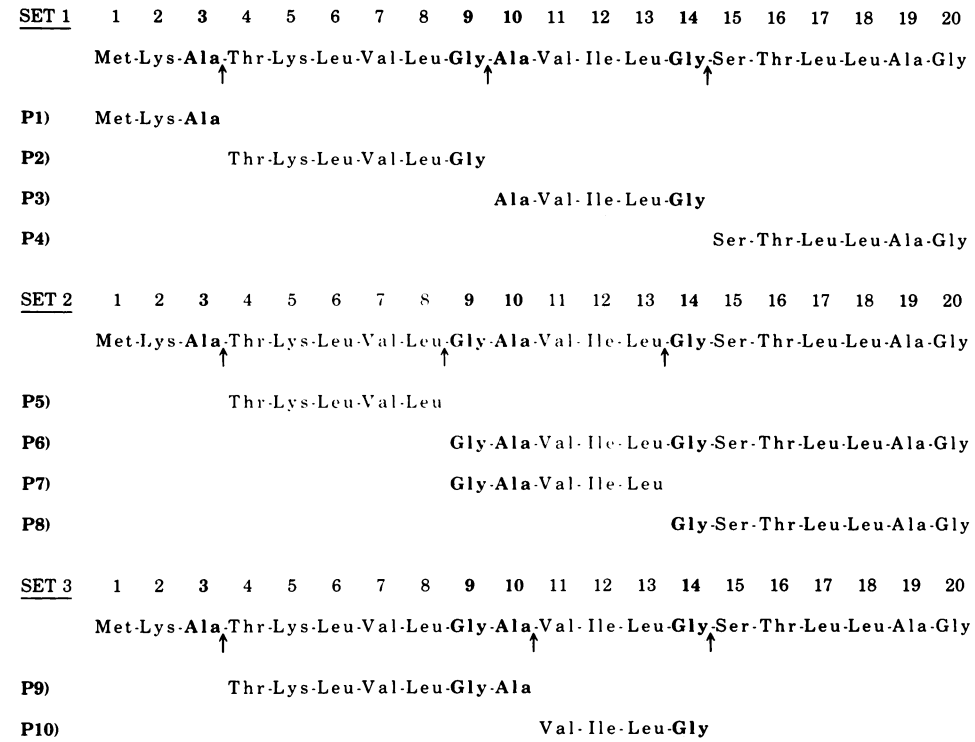

FIG. 5. Proposed cleavage sites for oligopeptidase A. Chemically synthesized signal peptide (28 to 350 nmol) was cleaved by 2.0 μg of oligopeptidase A for 19 h in three separate experiments. Ten products (P1 to P10) were identified as described in the legend to Fig. 1. The entire sequence of prolipoprotein signal peptide is depicted, and amino acid residues are numbered 1 to 20. A combination of cleavages by oligopeptidase A is proposed to account for the products formed. The cleavages were separated into three sets to improve visual comprehension. The arrows indicate the proposed cleavage sites. Alanine and glycine residues at the P'_1 (primary binding site) or P_1 (preceding P'_1) site of the substrate are shown in boldface letters.

The peptide fragments produced during the cleavage of the signal peptide by protease IV and oligopeptidase A were identified by an approach that used amino acid composition of peptides isolated during RPLC (Fig. 1). Amino acid composition indexes have been used to identify large peptide fragments produced during the autolysis of thermolysin (6). These approaches were useful not only when amino acid sequencing was not practical but also to identify peptide fragments that were not completely resolved by RPLC (Fig. 1A and B). However, this method of peptide identification requires knowledge of the complete sequence of the substrate and the ability to resolve products by column chromatography and perform many amino acid analyses rapidly and precisely.

The cleavage sites of protease IV were reconstructed from the peptide fragments identified and are shown in Fig. 3. Several interesting properties of the enzyme were noted. (i) The primary binding site of protease IV is specific for amino acids with hydrophobic side chains, e.g., Val, Leu, Ile, Ala, and Thr. Pacaud (21) had also observed esterase activity by protease IV against *N*-acyl-amino acid-*p*-nitrophenol esters containing Val, Leu, Phe, and Ala. (ii) The enzyme cleaved these residues in the hydrophobic segment of the signal peptide only. This suggested that protease IV requires hydrophobic amino acid residues at sites other than the primary binding site of the substrate. The lack of cleavages at Ala-3, Thr-4, and Leu-6 of signal peptide may be due to the presence of a charged Lys residue at P_2 or P'_2 of the substrate (by convention [8] the substrate residues towards the amino-terminal end of the scissile bond are labeled P_1, P_2, etc., and the residues towards the carboxyl-terminal end are labeled P'_1, P'_2, etc.). (iii) The tripeptides were the smallest products formed during protease IV reactions (Fig. 3). This observation suggests that the enzyme requires a substrate of at least six residues, with a minimum of three residues on either side of the scissile bond.

Vimr et al. (29) have investigated the substrate specificity of oligopeptidase A from *S. typhimurium* by using synthetic peptides. The identification of cleavage sites for oligopeptidase A on the signal peptide (Fig. 5) confirmed the following observations made by these workers. (i) The presence of Gly or Ala at the P_1 or P'_1 site of the substrate can result in the cleavage of a peptide bond by oligopeptidase A. As a consequence of this, cleavages at the amino-terminal end as well as at the carboxyl-terminal end of Gly and Ala were observed (Fig. 5). (ii) Oligopeptidase A cleaves peptides of at least five amino acid residues, with a minimum of two to three residues on either side of the scissile bond.

Oligopeptidase A and protease IV do not degrade the signal peptide while it is still attached to the mature protein (13, 20, 31). To explain this observation, it was suggested that these enzymes are carboxypeptidases and require the free carboxyl-terminal end of signal peptide for its degradation (13, 14, 20, 31). However, neither free amino acids nor dipeptides were generated during the cleavages of the signal peptide by either of the enzymes; the products were tripeptides or larger (Fig. 3 and 5). We conclude that oligopeptidase A and protease IV degrade the signal peptide by endopeptidic cleavages. Previous workers (21, 29) had also observed endopeptidase activity with both the enzymes. This raises the question of why the signal peptide is not cleaved by these enzymes while it is still attached to the

mature protein. We propose that in addition to the requirement of specific amino acid residues and minimum size of the peptides, these enzymes also recognize the altered conformation of a signal peptide after it is released from a precursor protein by signal peptidase.

The information concerning the substrate specificity of the peptidases can be used to define their role in the degradation of signal peptide in vivo. The localization of the signal peptide after cleavage from precursor protein is unknown. However, many different models of protein export (16, 23, 27, 30) suggest that signal peptide would initially be localized in the membrane, with the amino terminus being on the interior and the carboxyl terminus being on the exterior. Consistent with these models, we propose that the hydrophobic segment of signal peptide extends through the bilayer after its removal from the precursor protein and is initially cleaved by membrane-bound protease IV.

The major role of the cytoplasmic enzyme oligopeptidase A in the cell may be to degrade the products of the initial cleavage of protease IV that may be diffused or transported back into the cytoplasm. However, the substrate specificity of oligopeptidase A also suggested another role for this cytoplasmic enzyme. The models of protein export (16, 23, 27, 30) propose that the amino terminus of the signal peptide containing positively charged residues binds to some component of the inner membrane and is oriented toward the cytoplasm. If this view is correct, then oligopeptidase A may remove a Met-Lys-Ala peptide from the amino-terminal end of the signal peptide while it is still attached to the membranes.

The results presented in this paper are consistent with the proposal that protease IV initially cleaves the signal peptides by making endoproteolytic cuts and the products of the initial cleavage that may be diffused or transported back into the cytoplasm are further degraded into amino acids by oligopeptidase A and other cytoplasmic enzymes.

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