A stretch of 10 consecutive dipeptides with the sequence -X-Ala- or -X-Pro-, possible cleavage sites for dipeptidyl aminopeptidase (DPAPase) activity, are located in the prepro-region of the alkaline extracellular protease (AEP) beginning at Led4. Evidence for DPAPase processing of this dipeptide stretch was obtained by characterizing the polypeptide secreted by a strain carrying a xpr6 mutation. The secreted polypeptide reacted with antibodies specific for AEP and was essentially identical to the 52-kilodalton intracellular AEP precursor based on mobility during sodium dodecyl sulfate-polyacrylamide gel electrophoresis, content of N-linked carbohydrate, and peptide mapping. Amino-terminal amino acid sequencing of this secreted precursor revealed that it consisted of at least three major polypeptides. One began at the end of the stretch of dipeptides, and two of the others began two and four amino acids upstream. These results confirm that DPAPase activity is involved in the formation of the 52-kilodalton AEP precursor. In other reported cases of DPAPase processing, the dipeptides are located directly upstream of the mature polypeptide. For AEP, the dipeptide stretch is located over 120 amino acids upstream from the N terminus of mature AEP. The novel location of the dipeptide stretch may provide a mechanism for preventing premature activation of AEP in the secretory pathway.

DPAPases \(^1\) cleave dipeptides from the amino end of polypeptides \((1, 2)\). Two of the four types of DPAPases show a significant preference for substrates with alanine or proline in the second position \((2)\). In the few cases where there is strong evidence for DPAPase processing of specific polypeptides (honeybee prepromelittin \((3)\), yeast \(\alpha\)-mating factor \((4)\), frog skin secretions \((5)\), and *Streptomyces plicatus* endoglycosidase \(H\) \((6)\)) the dipeptides that are removed are located directly upstream of the amino-terminal end of the mature polypeptide, and DPAPase processing is a late reaction in the processing of the polypeptide precursors. The presence of a stretch of -X-Ala- and/or -X-Pro- dipeptides directly upstream of other mature polypeptides suggests that DPAPase may have a role in their processing \((4, 5)\). There is a stretch of ten -X-Ala- or -X-Pro- dipeptides in the prepro-region of AEP of *Yarrowia lipolytica*, and this study was done to determine whether or not these dipeptides are sites for DPAPase processing.

*Y. lipolytica* in not closely related to *Saccharomyces cerevisiae* \((7, 8)\), but many of the biochemical and genetic manipulations possible with *S. cerevisiae* are also possible with *Y. lipolytica*. *Y. lipolytica* has potential as a host for secretion of foreign proteins (European Patent Office Application 0220864, European Patent Bulletin 1987/19), and recently a small ribonucleoprotein homologous to signal recognition particle involved in targeting of secreted proteins to the endoplasmic reticulum has been isolated from *Y. lipolytica* and the putative signal recognition particle RNA cloned and sequenced \((9)\). *Y. lipolytica* secretes AEP into the extracellular medium at levels of 1-2% of total cell protein \((10)\). The XPR gene coding for AEP from *Y. lipolytica* has been sequenced \((11, 12)\), and the intracellular precursors of AEP have been identified by immunoprecipitation of radiolabeled cell extracts \((12)\). These results suggest that AEP is originally synthesized with a prepro-region of 157 amino acids and that its processing is complex involving addition of N-linked carbohydrate, at least one KEX2-like cleavage \((13)\) after a Lys-Arg pair of basic amino acids, and possibly signal peptide cleavage and additional proteolytic cleavages \((12)\). Beginning with Leu \(^4\) there is a stretch of 10 consecutive dipeptides with the sequence -X-Ala- or -X-Pro- in the AEP prepro-region. The location of this putative DPAPase processing site is novel in that instead of being located directly upstream of the amino terminus of mature AEP, it is over 120 amino acids further upstream.

In this study, we confirm that this dipeptide stretch is a substrate in *vivo* for DPAPase processing, and we identify the intracellular AEP precursor that results directly from this DPAPase processing. The finding that a strain containing an xpr6 mutation secreted an AEP precursor, which was essentially identical to one of the intracellular AEP precursors, made it possible to obtain sufficient quantities of this polypeptide to obtain these results. For AEP, DPAPase processing is not the last processing event. It occurs prior to the KEX2-like cleavage between the end of the pro-region and the beginning of mature AEP. The novel location of the DPAPase processing site may reflect a novel role for DPAPase in AEP processing. Unlike the case for honeybee prepromelittin \((3)\) and yeast \(\alpha\)-mating factor \((4)\), removal of the dipeptides does not result in activation of AEP. We speculate that further processing and activation of AEP are inhibited until the dipeptides are removed and that this is a mechanism for preventing premature activation of AEP in the secretory pathway.
The 52-kDa AEP Precursor Is Secreted by D0613—In a previous study, intracellular AEP-related polypeptides of 55, 52, 44, and 36 kDa and the mature 32-kDa AEP were detected by immunoprecipitation (12). Whether or not the 55-kDa intracellular AEP precursor contains the signal peptide is not known (12). However, because of (i) the location of the dipeptide stretch, (ii) the relatively late appearance of the 52-kDa precursor in pulse-chase experiments, and (iii) the rather late occurrence of DPAPase processing in the secretory pathway in *S. cerevisiae* (20, 21), the 52-kDa intracellular AEP precursor (the second largest precursor detected) was most likely the precursor which resulted directly from DPAPase processing. If this hypothesis was correct, then the 52-kDa precursor would be expected to begin with Glu34 at the end of the dipeptide stretch (Fig. 1).

Determining if the 52-kDa AEP precursor results directly from DPAPase processing would be difficult to do by radiosequencing of immunoprecipitates from cell extracts because this precursor is present in small amounts and it is difficult to obtain free of the 55-kDa precursor which is present in much larger amounts (12). Strain D0613 secretes a large polypeptide instead of AEP (12). Therefore, we decided to investigate whether or not this secreted polypeptide was the 52-kDa AEP precursor.

Strains D0613 and D0625 which contain the xpr6-13 and xpr6-25 mutations were isolated based on their lack of a zones of clearing on skim milk plates (14). The xpr6 mutations are unlinked to the XPR2 structural gene for AEP (14, 22). Based on skim milk plate assays and casein hydrolysis assays, the strains produce little alkaline protease activity (14). Pulse-chase immunoprecipitation experiments on both strains revealed that AEP processing was slowed significantly and that the major polypeptide secreted was not AEP but a larger polypeptide of over 50 kDa (data not shown). The secreted polypeptide was antigenically related to AEP. On protein blots it reacted with mouse monoclonal antibody (1:1000 dilution) prepared against purified AEP. Cells were resuspended in glycerol/casein medium at a cell density of 1000 Klett units as in an immunoprecipitation experiment (12). After 20 min phenyldimethylsulfonyl fluoride was added to 2 mm final concentration. Ten minutes later the supernatant medium was collected by centrifugation and protein precipitated with trichloroacetic acid. Nonadjacent lanes from the same blot are shown. B, relatedness by peptide mapping. [3H]Leucine-labeled samples were prepared by trichloroacetic acid precipitation, SDS-PAGE, and electrolution. Lane 1, electroeluted 52-kDa polypeptide from D0613 supernatant medium (16,500 counts); lane 2, electroeluted mature AEP from wild type supernatant medium (13,000 counts). The samples were digested for 30 min at 37 °C with chymotrypsin, 2.5 µg for the 52-kDa sample and 0.25 µg for mature AEP. The digested samples were analyzed by SDS-PAGE on a 10–20% gel and fluorography. Film exposure was for 9 days.

In addition to having similar mobilities on SDS-PAGE (Fig. 3) and similar levels of N-linked oligosaccharides (Fig. 4), the secreted polypeptide and the intracellular 52-kDa AEP precursors had quite similar mobility of 55 kDa and the mature 32-kDa AEP (data not shown). The secreted polypeptide was antigenically related to AEP. On protein blots it reacted with mouse monoclonal antibody (1:1000 dilution) prepared against purified AEP. Cells were resuspended in glycerol/casein medium at a cell density of 1000 Klett units as in an immunoprecipitation experiment (12). After 20 min phenyldimethylsulfonyl fluoride was added to 2 mm final concentration. Ten minutes later the supernatant medium was collected by centrifugation and protein precipitated with trichloroacetic acid. Nonadjacent lanes from the same blot are shown. B, relatedness by peptide mapping. [3H]Leucine-labeled samples were prepared by trichloroacetic acid precipitation, SDS-PAGE, and electrolution. Lane 1, electroeluted 52-kDa polypeptide from D0613 supernatant medium (16,500 counts); lane 2, electroeluted mature AEP from wild type supernatant medium (13,000 counts). The samples were digested for 30 min at 37 °C with chymotrypsin, 2.5 µg for the 52-kDa sample and 0.25 µg for mature AEP. The digested samples were analyzed by SDS-PAGE on a 10–20% gel and fluorography. Film exposure was for 9 days.

In the Xpr6 precursor, the amino-terminal peptides are predicted to be the smaller amino-terminal peptides expected for the 52-kDa AEP precursor. The presence of the upper band in the secreted polypeptide (labeled 513) sample is more difficult to explain. Perhaps, a small amount of material larger than 52 kDa is secreted, although it cannot be seen in Fig. 3. The ratio of the upper band to the lower band is much less for the secreted polypeptide than for the 52-kDa AEP precursor, and the upper band is almost completely absent in the peptide map in Fig. 5B. Again the lower band is predicted to be the smaller amino-terminal peptides expected for the secreted polypeptide. If these lower band do contain the amino-terminal peptides, then their identical mobilities in the secreted polypeptide and the 52-kDa AEP precursor samples strongly suggest that the two polypeptides have essentially identical amino-terminal peptides.

The reason for the band in the 52-kDa AEP precursor sample that runs just above the starred region that does not appear in the other two samples is unclear. The samples present in greater amounts would be the smaller amino-terminal peptide expected for the 52-kDa AEP precursor. The presence of the upper band in the secreted polypeptide (labeled 613) sample is more difficult to explain. Perhaps, a small amount of material larger than 52 kDa is secreted, although it cannot be seen in Fig. 3. The ratio of the upper band to the lower band is much less for the secreted polypeptide than for the 52-kDa AEP precursor, and the upper band is almost completely absent in the map in Fig. 5B. Again the lower band is predicted to be the smaller amino-terminal peptides expected for the secreted polypeptide. If these lower band do contain the amino-terminal peptides, then their identical mobilities in the secreted polypeptide and the 52-kDa AEP precursor samples strongly suggest that the two polypeptides have essentially identical amino-terminal peptides.

The reason for the band in the 52-kDa AEP precursor sample that runs just above the starred region and that does not appear in the other two samples is unclear. The samples

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2 Portions of this paper (including "Experimental Procedures," part of "Results," and Figs. 3, 4, and 6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
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FIG. 5. Peptide maps of [3H]leucine-labeled 55- and 52-kDa AEP intracellular precursors and AEP precursor secreted by strain D0613. The stars indicate the positions of fragments which may contain the amino-terminal peptide. The bands were located by fluorography and cut out of the dried gel. Gel pieces with about 5,000 counts were loaded in each lane. No enzyme was added for the three left lanes in A. Ten μl of S. aureus V8 protease at 1 μg/ml was added to the middle three lanes in A and to the three lanes in B; 10 μl of protease at 2.5 μg/ml was added to the right three lanes in A. The gels were run at 20 mA for 40 min, and the power was then shut off for 30 min. The gel was then run at 20 mA until the dye front reached the top of the separating gel, and the power was shut off again for 30 min. The gels were then run at 20 mA for 7 h, dried, and fluorographed. For A, a 10-20% gel was used, and it was exposed for 12 days. For B, a 17.5% gel was used, and it was exposed for 3 days.

contain similar levels of radioactivity but different levels of protein, and they may be digested to different extents. We conclude that D0613 secretes a partially processed AEP precursor which has essentially the same amino-terminal end as the intracellular 52-kDa AEP precursor.

DPAPase Processing of the Secreted 52-kDa AEP Precursor—The amino-terminal amino acid sequence of the secreted polypeptide (Fig. 6) was determined by automated sequential Edman degradation. Several amino acids were detected in each cycle, but when the data was plotted it was clear that the sample contained three predominant polypeptides (Fig. 7). For example, there is only one tyrosine in this region, and it is at position 41 which is 8 amino acids after the end of the dipeptide stretch. Strong signals for tyrosine were obtained in cycles 8, 10, and 12 which suggests the presence of three polypeptides. One begins at the end of the dipeptide stretch at Glu34, the second begins two amino acids further upstream at Val35, and the third begins two additional amino acids further upstream at Ala36 (Fig. 7). These data strongly support a role for DPAPase activity in AEP processing. The last cleavage site was at the end of the stretch of 10 dipeptides of the sequence -X-Ala- or -X-Pro-. This suggests that the -Glu-Gly- dipeptide was not removed. If a significant proportion of molecules beginning at Pro36 or Ala36 were present, then tyrosine should have been detected in cycles 4 and 6 and isoleucine in cycles 7 and 9 and they were not. Molecules beginning at Ala30 and Val32 are expected if DPAPase processing is incomplete. Significant proportions of molecules beginning at Asp35 and Ala38 were not present based on the absence of aspartic acid in cycle 1, the first case and on the absence of alanine in cycle 3, valine in cycle 5, proline in cycle 6, glutamic acid in cycle 7, glycine in cycle 8, and proline in cycle 9 in the second case.

DISCUSSION

The major finding of this investigation was that the 10 consecutive -X-Ala- or -X-Pro- dipeptides located over 100 amino acids upstream of the amino-terminal of mature AEP were subject to DPAPase processing in vivo. Another significant finding was that complete processing of the AEP precursors was not essential for secretion. Also the results strongly

FIG. 7. Amino-terminal amino acid sequence of the AEP precursor secreted by strain D0613. (The sample is shown in Fig. 6, lanes 3 and 4). The raw data for nanomoles recovered for each of the nine amino acids present in significant amounts in one or more of the sequencing cycles is plotted. The amino-terminal amino acid sequences for the three major AEP precursors in the sample are at the top of the figure. (The abbreviations are as in Fig. 1.) The gray bars indicate that one of the AEP precursors would be expected to yield the given amino acid in that sequencing cycle. The black bars indicate that two of the AEP precursors would be expected to yield the given amino acid. The white bars indicate that the amino acid was not expected in that sequencing cycle.
suggest that the 52-kDa intracellular AEP precursor processing results directly from DPAPase processing.

Obtaining sufficient quantities of purified intracellular precursor to confirm DPAPase processing would have been quite difficult. Fortunately, strain D0613 containing the xpr6-13 mutation secreted an AEP precursor indistinguishable by several criteria from the 52-kDa intracellular AEP precursor. However, only radiosequencing of the amino-terminus of the 52-kDa intracellular precursor will directly confirm this identity, and this information will be important to obtain in the future. We believe that these results, especially the peptide mapping results, essentially eliminate the formal possibility that the secreted and intracellular 52-kDa AEP precursor, although of similar mobility, might have resulted from differing processing, i.e. carboxyl-terminal versus amino-terminal processing. In Fig. 2, peptides with identical mobilities almost as large as mature AEP were present in both the secreted polypeptide and mature AEP samples. Possibly these peptides resulted from internal cleavages near both the amino- and carboxyl-terminal ends of mature AEP. However, it is much more likely that they resulted from a single cleavage. Because the amino-terminal ends of these polypeptides are known to differ, the carboxyl-terminal ends must be similar for a single cleavage to yield large peptides of identical mobility. In Fig. 5, the results suggested directly that the amino-terminal peptides were identical. Also, the difference in relative molecular mass between the secreted precursor and the 55-kDa AEP precursor as determined by SDS-PAGE was 2.6 kDa (12); this underestimated the predicted difference based on removal of 29-33 amino acids or slightly overestimates the difference if the 55-kDa AEP precursor does not contain the 15 amino acids of the signal peptide (see below). In either case, these results suggest that the secreted precursor has not undergone extensive carboxyl-terminal processing compared to the 55-kDa AEP precursor. The relative molecular mass for mature AEP is 1.4 kDa greater than the molecular mass calculated from the deduced amino acid sequence (12), and this also suggests the absence of processing at the carboxyl terminus.

Besides being present in much greater amounts and being much easier to purify than the intracellular precursor, the secreted precursor offered another advantage for this study in that it was secreted from intact cells. Evidence for DPAPase processing obtained from radiosequencing of an intracellular precursor would always be subject to the criticism that the DPAPase processing was an artifact caused by breaking open the cells and exposing the AEP precursor to DPAPase activity that it would never encounter in intact cells.

DPAPase requires a free amino-terminal dipeptide in the correct "reading frame" to initiate hydrolysis (3). The first suitable dipeptide would be Leu14 and Ala15 (Fig. 1), and this would require a prior cleavage between Val13 and Leu14. However, based on preliminary radiosequencing data and on von Heijne's method for predicting signal sequence cleavage sites (34), the likely cleavage site is between Ala15 and Ala16, and this would allow initiation of DPAPase action.

Considering that the dipeptide stretch consists entirely of -X-Ala- and -X-Pro- dipeptides and assuming that the DPAPase(s) involved in AEP processing is similar to the DPAPase (the STE13 gene product) involved in alpha-factor processing in S. cerevisiae (4), the Y. lipolytica DPAPase is probably a type IV DPAPase (2, 4). A DPAPase from the skin secretions of Xenopus laevis has recently been purified and characterized which cleaves after -X-Gly- as well as after -X-Pro- and -X-Ala- (5). Following the stretch of 10 dipeptides in AEP is the sequence -Glu-Gly-Pro-Ala-Ala-Ala-. If the Y. lipolytica DPAPase cleaves after -X-Gly-, then the dipeptide stretch would be extended from 10 to 13 dipeptides. There was no evidence for cleavage after -X-Gly- in the amino acid sequencing data. If this processing occurred, then tyrosine should have been detected in cycles 2, 4, and/or 6, and it was not (Fig. 7). A low rate of -X-Gly- cleavage cannot be excluded for the Y. lipolytica DPAPase, but it may be unlikely because a proline follows the glycine, and at least for porcine kidney DPAPase IV there is evidence that dipeptides bonded to the imino group of proline cannot be removed (see Ref. 2).

The three major polypeptides that were detected by amino-terminal sequencing came from only the bottom third of the protein band obtained from preparative gel electrophoresis, and almost certainly larger polypeptides beginning at Ala28, Asp26, etc. are present in the remainder of the band. The frayed ends of the secreted AEP precursor raise the possibility that the xpr6 mutation reduces the level of DPAPase processing. If DPAPase processing is required before AEP can be processed further (see below), then this would explain why the large AEP precursor was secreted by D0613. For two reasons, we do not believe that the xpr6 mutation affects DPAPase processing. First, there is evidence for frayed ends in the dipeptide stretch in the wild type strain. The 52-kDa intracellular AEP precursor band does not appear to be dif fuse. However, wild type strains secrete mature AEP and a 19-kDa polypeptide derived from the AEP pro-region (12). The upper portion of this 19-kDa band is fuzzy and in some cases actually appears at the upper band of a doublet (12). This suggests that the dipeptide stretch is not completely removed in wild type strains and that complete removal is not required for secretion. Consistent with this interpretation is the fact that DPAPase activity seems to be rate-limiting for the maturation of alpha-factor in S. cerevisiae (4). Second, preliminary results indicate that cell extracts of strains D0613 and D0625 contain significantly less than wild type levels of KEX2-like processing activity assayed using the chromogenic peptide benzoyloxycarbonyl-Tyr-Lys-Arg-4-nitroanilide (data not shown).

What is the function of the novel location of the DPAPase processing site in AEP? We hypothesize that it provides a mechanism for avoiding premature activation of AEP in the secretory pathway. Many hormones, toxins, and potentially hazardous enzymes are synthesized as inactive proproteins which are activated by proteolytic processing. The logical hypothesis is that this protects the cell from stimulation by the hormones, action of the toxins, and activity of the enzymes in inappropriate cellular locations (36).

Honeybee melittin has strong lytic properties, and the DPAPase activation of promelittin occurs after its secretion into the chlorinated venom sac (1, 3). This prevents interaction of the lytic peptide with the bee's own phospholipid membranes. DPAPase processing activates yeast alpha-factor (4), and possibly adverse effects of high internal concentrations of the activated pheromone on cell growth may be a reason why it is advantageous for alpha-factor activation to occur late in the secretory pathway (20).

The 52-kDa secreted AEP precursor is not proteolytically active so DPAPase processing does not result directly in AEP activation. Pulse-chase experiments showed that the rate of conversion of the largest AEP precursor (55-kDa) to mature AEP was low until the rate of conversion of 55- to 52-kDa AEP precursor increased (12). 55-kDa→52-kDa→mature AEP was shown to be possibly the major pathway for AEP processing (12). This suggested that DPAPase processing must occur before KEX2-like processing (and AEP activation) could proceed at a significant rate.

AEP is an alkaline protease, but it still has activity at
neutral pH and below (10). The pH of the endoplasmic reticulum is thought to be near neutrality and the secretory pathway becomes progressively more acidic (37). We assume that the AEP precursor encounters KEX2-like processing activity earlier than DPAPase activity in the secretory pathway of Y. lipolytica by analogy to the situation in S. cerevisiae where KEX2 processing of α-factor precedes DPAPase processing (20). We speculate that for AEP the dipeptide stretch inhibits KEX2-like processing. Delaying AEP processing and activation until DPAPase activity is encountered (and presumably the secretory pathway is acidic enough so that AEP will not cause damage) would protect the cell. This model assumes that the KEX2-like and DPAPase activities overlap to some extent in the Y. lipolytica secretory pathway. This would be somewhat different from S. cerevisiae where the Kex2 protease is presumably located in a late Golgi compartment and the DPAPase seems to be located in a different secretory compartment (21). We will be determining if the presence of the dipeptide stretch does inhibit KEX2-like processing by altering the DPAPase processing sites and determining how these changes affect overall AEP processing.

Finally, Y. lipolytica provides a system in which milligram quantities of purified native forms of a large precursor (32 kDa) and the pro-region (19 kDa) and mature AEP (22 kDa) which results after processing can be obtained. Structural studies of these polypeptides could lead to interesting insights into protein processing and protease activation.

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Continued on next page.
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Supplemental Material to
A Novel Location for Dipeptidyl Aminopeptidase Processing
Sites in the Alkaline Extracellular Protease of
Toromos lipolytica

Sam Matashe and David M. Goyer

EXPERIMENTAL PROCEDURES

Strains, Growth Conditions, and Materials - Strain DO613 and DO625 were isolated from Y. lipolytica strain CL1-18 (ATCC 32338); wild type for this strain, which was obtained from the Carlsberg and R. Horten (ID. C. Selley). The organisms were maintained on MM medium (15).

GPP buffer: concentrated phosphate buffer. Cell densities were estimated with 10% trichloroacetic acid precipitation.

Preparation of labeled supernatant samples - [3H]Leucine labeled supernatant samples were obtained from cultures precipitated with 10% trichloroacetic acid and ammonium sulfate.

Preparation of intracellular precursors - Intracellular samples of AEP precursors were obtained by immunoprecipitation of [%leucine labeled cell extracts with rabbit anti-AEP polyclonal antibody and immunoprecipitated samples were phosphorylated with 10% trichloroacetic acid precipitation.

Preparation of the secreted 52 kDa AEP precursor - Strain DO613 was grown in GM medium (15). Cells were harvested by centrifugation at 4,000 x g for 10 min at 4°C. Following centrifugation, the pellet was lysed with digitonin and the supernatant was subjected to automated Edman degradation on a Sequon PPS-1890 1890A. The phenylthiohydantoin derivatives were identified and quantified by reverse-phase high-pressure liquid chromatography on two systems - a Waters Model 6000 gradient system and a Waters Model 6000 gradient system.

Amino-terminal sequencing - The sample was subjected to automated Edman degradation on a Sequon PPS-1890 1890A. The phenylthiohydantoin derivatives were identified and quantified by reverse-phase high-pressure liquid chromatography on two systems - a Waters Model 6000 gradient system and a Waters Model 6000 gradient system.

Proteins of the secreted 52 kDa precursor - Moieties of the polypeptide secreted by DO613 and the immunoprecipitated 52 kDa intracellular precursor were indistinguishable on SDS-PAGE (Fig. 3). Samples were mixed (Fig. 4) and treated with endo H. The labeling of polypeptides was analyzed by SDS-PAGE.

Preparation of the secreted 52 kDa precursor - In initial experiments, recovery of secreted AEP precursor was at least 10-fold lower than in our initial preparations. To improve the recovery of AEP precursor, the pH was adjusted from 6.8 to 7.8 to increase the amount of AEP precursor secreted and its stability.

Fig. 3 The AEP precursor secreted by strain DO613 and the 52 kDa intracellular AEP precursor have similar mobilities on SDS-PAGE. [%] Leucine labeled samples were obtained from supernatant medium from strain DO613 and from immunoprecipitates of cell extract from wild type. The samples were subjected to electrophoresis and fluorography. The migrating bands were excised and eluted. The eluted polypeptides (5,000 counts per minute per lane) were analyzed by SDS-PAGE and fluorography. Film exposure was for 3 days. Lane 1, DO613 secreted precursor; Lane 2, 52 kDa intracellular precursor; Lane 3, 52 kDa secreted precursor; Lane 4, DO613 and 52 kDa precursors; Lane 5, DO613 and 55 kDa precursors; Lane 6, 52 kDa and 55 kDa precursors.

Fig. 4. The AEP precursor secreted by strain DO613 contains N-linked carbohydrate. Cars were labeled with [%] leucine and supernatant samples obtained by trichloroacetic acid precipitation. The samples were analyzed by SDS-PAGE and fluorography. The migrating bands were excised and eluted. The eluted polypeptides (5,000 counts per minute per lane) were analyzed by SDS-PAGE and fluorography. Film exposure was for 12 days.

RESULTS

Properties of the secreted 52 kDa precursor - Moieties of the polypeptide secreted by DO613 and the immunoprecipitated 52 kDa intracellular precursor were indistinguishable on SDS-PAGE (Fig. 3). Samples were mixed (Fig. 4) and treated with endo H. The labeling of polypeptides was analyzed by SDS-PAGE.

Preparation of the secreted 52 kDa precursor - In initial experiments, recovery of secreted AEP precursor was at least 10-fold lower than in our initial preparations. To improve the recovery of AEP precursor, the pH was adjusted from 6.8 to 7.8 to increase the amount of AEP precursor secreted and its stability.

Ultrafiltration, ammonium sulfate precipitation, and trichloroacetic acid precipitation were examined as methods for concentration of the supernatant medium. Gel filtration chromatography and preparative PAGE were examined as methods for further purification of the AEP precursor. The methods were chosen because these methods gave the highest yields of intact 52 kDa secreted AEP precursor. In order to prepare a sample for N-terminal amino acid sequencing, 90% of supernatant medium from a DO613 culture (collected at a cell density of 5 x 10^8 cells per liter) was precipitated with trichloroacetic acid and a portion examined by SDS-PAGE. The secreted AEP precursor was the major protein present, but it probably constituted less than half of the total protein in the sample.
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Are there other polypeptides for which the DPAPase cleavage sites do not abut the N-terminal end of the mature polypeptide? In an attempt to identify other polypeptides for which the DPAPase cleavage sites were separated from the N-terminal end of the mature polypeptide, an attempt was made to search the available data base for polypeptide sequences with the sequences of the DPAPase cleavage sites. If no more such conserved cleavage sites were identified, almost 800 with 4 or more consecutive cleavage sites were examined. The sequences with 4 or more consecutive cleavage sites were examined.

Several cases where long X-Ala/Pro dipeptide stretches were present in the mature polypeptide were found. For example, the circumpolar protein from *N. goniophaera* has 4 repeats of -Glu-Val-Pro-Pro- intercalated with 3 repeats of -Glu-Glu-Glu-Pro-Pro- (33), and epidermin A1, and A4 catalytic light chains, and the *Corynebacterium* dipeptide stretches of 3 to 11 dipeptides containing only Ala and Pro (24-26). In most of these cases, the dipeptide stretches were not obviously involved in processing and these cases were excluded from further consideration. Also excluded were cases where the polypeptide was known to be localized in the cytoplasm or the nucleus (based on the assumption that DPAPase activity is localized in the secretory pathway).

There are several cases where the dipeptide stretch was in the proper region of a precursor polypeptide. There was no direct evidence for any of these cases of a precursor having the dipeptide stretch at the N-terminal end. If this was the case, then the dipeptide stretch did not overlap "in frame" with a known or presumed peptide signal cleavage site. If the evidence indicated that these polypeptides did not undergo further proteolytic processing, then these cases were not considered further. Two cases where the resulting polypeptides were processed further were found for procaryotes — dihydrolipase from *Pseudomonas* sp. (29), however, because of the fundamental differences in the secretory pathways of eucaryotes and procaryotes these cases are not considered analogous to APF processing.

For the eucaryotes, there were a few cases where potential DPAPase processing sites did not directly abut the N-terminal end of the mature polypeptide, but none of the cases were very convincing. For the eucaryotic transforming protein (int-1) the dipeptide stretch is from residues 19 to 21 (initiator methionine is residue 1) and the protein is possibly a growth factor that suggests it would undergo several proteolytic processing steps (32). The best prospect was the human cholecystokinin precursor, though no convincing cleavage sites were found. The computer search did not reveal any examples for which there was evidence for DPAPase processing that occurred in a location where the dipeptide stretch was not in the proper region of the mature polypeptide, nor did the search identify sequences which strongly suggest that this might be occurring.