Proteolytic Processing of Presecretory Proteins Is Required for Development of Biological Activities in Pancreatic Exocrine Proteins

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The biological activities of pancreatic presecretory and secretory proteins synthesized in vitro were compared in studies of (a) the binding of nascent amylase to its substrate, glycogen, (b) the binding of nascent trypsinogen 1, trypsinogen 2 + 3, and chymotrypsinogen 1 to Sepharose-bound soybean trypsin inhibitor, and (c) the activation of nascent trypsinogen by porcine enterokinase. Nascent secretory proteins synthesized in vitro using a mRNA-dependent gel-filtered reticulocyte lysate translation system supplemented with canine pancreas rough microsomes or canine pancreas mRNA and micrococcal nuclelease-treated microsomal membranes showed biological activities similar to authentic secretory proteins if oxidized glutathione was added during their synthesis. Proteins synthesized in the presence of membranes and the absence of glutathione showed significantly less biological activity due to incorrect development of conformation. Presecretory proteins synthesized in vitro with canine pancreas mRNA in the absence of microsomal membranes had little or no activity after translation in either the absence or presence of glutathione. These and previous findings (Scheele, G. A., and Jacoby, R. (1982) J. Biol. Chem. 257, 12277–12282) indicate that proteolytic removal of the NH2-terminal transport peptide is necessary to allow correct conformational development, including the formation of native disulfide bonds, which not only stabilizes the molecule but allows expression of authentic biological and probiological activity.

Pancreatic exocrine proteins are synthesized with short lived NH2-terminal extensions which provide for the translocation of nascent secretory proteins across the membrane of the rough endoplasmic reticulum (1, 2). During synthesis of these presecretory proteins, the peptide extensions, or transport peptides, are removed by a protease associated with the rough endoplasmic reticulum membrane (3, 4). In a study of 14 nonglycosylated pancreatic exocrine proteins, synthesized in vitro in the presence of canine pancreatic microsomal membranes, processing and segregation of proteins were complete and their translocation into the intracisternal space was an irreversible process (1). In order to determine whether the translocation mechanism observed in vitro represents the physiological mechanism which occurs in vivo, it is necessary to demonstrate the extent to which secretory proteins, segregated within microsomal vesicles, show conformational properties and biological activities similar to those synthesized in vitro. We have previously presented evidence that secretory proteins synthesized and translocated in vitro, in the presence of glutathione and an optimal redox potential, show conformational properties indistinguishable from those of authentic secretory proteins (5). In this report, we examine the extent to which presecretory and secretory proteins synthesized in vitro in the absence and presence of nuclelease-treated microsomal membranes, respectively, demonstrate biological activity.

MATERIALS AND METHODS

Proteins synthesized with [35S]methionine in dog pancreas tissue slices were isolated as previously described (1). [35S]Methionine-labeled presecretory proteins were translated in vitro using canine pancreas poly(A) mRNA and a nuclease-treated gel-filtered reticulocyte lysate supplemented with bovine liver tRNA, glucose-6-P, human placental RNase inhibitor, 3.25 mM dithiothreitol, and, when indicated, micrococcal nuclease-treated canine pancreas rough microsomes as described (1). Addition of oxidized glutathione to the translation mixture was penultimate just prior to the addition of mRNA. Radioactivity incorporated into protein was determined by liquid scintillation spectrometry after precipitation of samples in filter discs using 10% trichloroacetic acid.

Soybean trypsin inhibitor (Sigma type 1-S) was coupled to 1 g of cyanogen bromide-activated Sepharose 4B (Pharmacia) by the published procedure (7). Binding capacity for bovine trypsin in 0.02 M Tris-HCl, pH 7.5, and 0.5 M NaCl, as determined by trypt arginine methyl ester hydrolytic activity (8), was 3.0 mg/ml of resin for soybean trypsin inhibitor-Sepharose. On the basis of potential enzyme activity following activation with enterokinase, bovine trypsingen binding to soybean trypsin inhibitor-Sepharose was in the presence of 0.5% Triton X-100 was 17% compared to bovine trypsin. Binding of translation products to affinity beads was conducted as follows. Aliquot from each translation mixture containing equal quantities of radioactive labeled protein (trichloroacetic acid-precipitable 35S) were diluted to 160 μl with 0.02 M Tris-HCl, pH 7.5, and 0.5 M NaCl in 0.5 ml plastic vials. Triton X-100 was added at a final concentration of 0.5% to solubilize membranes. Soybean trypsin inhibitor-Sepharose equivalent to 12.5-μl bed volume was added and the resulting mixture was incubated at 0 °C with frequent mixing for 30 min. Sepharose beads were then sedimented at 8000 × g for 3 min in a microcentrifuge. The supernatant was discarded and the Sepharose beads were washed four times with 500 μl of 0.02 M Tris-HCl, pH 7.5, in 0.5 M NaCl. After the final wash, SDS gel sample buffer was added to the Sepharose beads to give 60 μl of a solution containing 5% SDS, 15% sucrose, 0.0025% bromphenol blue, 50 mM dithiothreitol, and Tris and glycine in concentrations identical with those in the electrode buffer. Samples contained in capped vials were heated in boiling water for 5 min and incubated at 40 °C for 15 min. Samples were carboxyamidomethylated with 100 mM iodoacetamide at 40 °C for 10 min, reheated in a boiling water bath, and transferred to gel slots with micropipette tips maintained under an infrared lamp at 55 °C. Samples which contained heated Sepharose beads gelled following their introduction into electrophoresis slots, maintained at room temperature. This procedure, which resulted in only slight reductions in electrophoretic mobilities, allowed quantitative analysis of proteins bound to the affinity beads.

The abbreviation used is: SDS, sodium dodecyl sulfate.

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2005
For the precipitation of amylase, aliquots of translation mixtures containing equal quantities of trichloroacetic acid-precipitable [35S] sulfur (25 μl) were diluted to 500 μl in 1.5-ml plastic vials with 10 mM phosphate buffer, pH 8.0. 100 units of unlabeled amylase (Worthington, 4340 units/ml, 5.1 mg/ml) and Triton X-100 at a final concentration of 0.6% were added. Amylase was quantitatively precipitated with shellfish glycogen as described by Schramm and Loyter (9). Amylase-glycogen precipitates were washed twice and prepared for SDS-gel electrophoresis as previously described (9).

Proteins were separated in one dimension by polyacrylamide gel electrophoresis in SDS (10) and in two dimensions by isoelectric focusing/SDS-gel electrophoresis as described by Bieger and Scheele (11), except that 0.1% Triton X-100 was added to the focusing gel. Radioactive proteins separated in one- and two-dimensional gels were detected by fluorography using DuPont-Cronex two-dimensional x-ray film after impregnation of gels with 2,5-diphenyloxazole.

RESULTS

Binding of Nascent Serine Protease Zymogens and Prezymogens to Protease Inhibitors—Soybean trypsin inhibitor binds not only to active pancreatic enzymes, trypsin, and chymotrypsin, but to their inactive zymogens, trypsinogen and chymotrypsinogen (this study), as well. Fig. 1 shows the extent to which nascent pancreatic zymogens and prezymogens bind to soybean trypsin inhibitor coupled to Sepharose beads. Serine protease zymogens synthesized in the presence of either reconstituted (tracks c and d) or authentic (tracks e and f) dog pancreas rough microsomes required the cotranslational presence of glutathione to bind selectively to immobilized soybean trypsin inhibitor in a manner similar to that of authentic zymogens (track g). Selective binding is indicated by a serine-protease zymogen to amylase ratio larger than that observed among translation products before binding. Prezymogens synthesized in the absence or presence of glutathione (tracks a and b) and zymogens synthesized in the absence of glutathione (tracks c and e) did not show selective binding. Apparent nonspecific binding under these conditions could be accounted for by the tendency of unstable (incorrectly folded) precursors and products to precipitate due to nonspecific protein-protein interactions (5).

In order to identify those canine serine protease zymogens which bound specifically to immobilized soybean trypsin inhibitor, radioactive proteins which remained attached to the Sepharose beads after three washes were solubilized in 8 M urea and separated by two-dimensional isoelectric focusing/SDS-gel electrophoresis, and analyzed by fluorography. Numbers on the upper abscissa indicate isoelectric points. Numbers on the right ordinate indicate molecular weight values \( \times 10^{-3} \) for nonreduced proteins. Radioactive proteins detected by this procedure are identified by co-migration with known canine pancreatic proteins, detected by Coomassie blue stain. T, trypsinogen; C, chymotrypsinogen. Numbers following abbreviations refer to individual canine pancreatic zymogen forms as described by Scheele et al. (1, 15). As previously described (1, 5, 14), nonreduced trypsinogen, analyzed by polyacrylamide gel electrophoresis in SDS migrates anomalously with a molecular weight of approximately 18,000.

![Fig. 2. Effect of precursor processing on binding of serine protease zymogens to soybean trypsin inhibitor-Sepharose.](http://www.jbc.org/content/101/2/338/F2.large.jpg)
residues) through cleavage at the Lys 6—Ile 7 bond. Concomitant with this endoproteolytic event, the substrate molecule develops enzymic activity. Fig. 3 shows the extent to which porcine enterokinase can activate (pre)trypsinogen present among translation products synthesized in the absence and presence of microsomal membranes and in the absence and presence of glutathione. No differences were observed in the electrophoretic distribution of presecretory proteins after they were incubated in the presence of enterokinase (Fig. 3, tracks e and g compared to f and h). Addition of enterokinase to secretory proteins synthesized in the presence of membranes, with or without glutathione (tracks a and c), resulted in the following changes: (a) the appearance of a discrete new product with apparent molecular weight 26,000 and (b) the simultaneous disappearance of higher molecular weight proteins and appearance of lower molecular weight degradation products. Based on its apparent molecular weight and the evidence of proteolysis, the 26,000-dalton protein is judged to be canine trypsin. Control studies indicated that bovine trypsinogen and trypsin were consistently resolved in our polyacrylamide gel system (data not shown). Enterokinase was not responsible for the appearance of low molecular weight degradation products since, in the absence of activatable trypsinogen, proteolytic degradation did not occur (tracks e and g).

**Binding of Substrate to Nascent Amylase and Preamylass.** Under the conditions described by Schramm and Loyter (9), shellfish glycogen will form an extensive lattice with amylase, resulting in precipitation of the enzyme-substrate complex. Fig. 4 shows the extent to which nascent preamylase or amylase, synthesized in the absence or presence of microsomal membranes, respectively, will selectively precipitate in the presence of glycogen. Amylase synthesized in the presence of microsomal membranes and glutathione (track d) showed selective precipitation with shellfish glycogen in a manner similar to that observed for authentic amylase synthesized in dog pancreas tissue slices (track e). Amylase synthesized in the presence of membranes but in the absence of glutathione (track c) showed only 35% precipitation with glycogen compared to that synthesized with glutathione (gravimetric peak weights 72 versus 205 mg, respectively). In contrast, preamylase did not show selectivity in precipitation with glycogen. The association of preamylase with the amylase-glycogen pellet could be entirely accounted for by the observation that pancreatic presecretory proteins tend to aggregate and precipitate due to nonspecific protein-protein interactions (5).

**DISCUSSION**

The present work demonstrates that four pancreatic secretory proteins, synthesized in vitro in the presence of microsomal membranes, glutathione, and an optimal redox potential, show biological activities similar to those of authentic secretory proteins synthesized in vivo. Nascent forms of trypsinogen 1, chymotrypsinogen 1, and trypsinogen 2+3 bound to immobilized soybean trypsin inhibitor in a manner similar to their authentic counterparts. Nascent amylase bound to its substrate glycogen and, due to bivalent binding and formation of an extensive substrate lattice, could be sedimented by low speed centrifugation. Nascent trypsinogen was proteolytically converted to trypsin upon the addition of enterokinase, and the appearance of trypsin resulted in evidence of proteolytic degradation. Direct enzyme assays on nascent proteins could not be carried out because canine pancreatic microsomal membranes contained levels of endogenous enzymes andzymogens in excess of those synthesized during our in vitro procedures. The efficiencies observed in segregation and processing of pancreatic polypeptide chains in our previous in vitro studies using canine pancreas mRNA and micrococcal nuclease-treated microsomal membranes (both processes carried to completion for 14 well defined secretory proteins, see Ref. 1) and the demonstration that segregated proteins express
conformational parameters (5) and biological activities (this study) indistinguishable from those of authentic counterparts indicate that the translocation mechanism observed with our in vitro system of reconstituted rough microsomes reproduces accurately the events which occur in vivo.

Little evidence has appeared in the literature to document the biological properties of secretory proteins translocated across membranes in vitro. Where reported, relatively small amounts of activity have been observed (17). In a previous study, we demonstrated that nascent secretory proteins, synthesized in a gel-filtered reticulocyte lysate (either in the absence or presence of 3.25 mM dihydrothreitol) and translocated into microsomal vesicles, had conformational properties distinctly different than those of authentic secretory proteins unless oxidized glutathione was added during translation (5). Addition of oxidized glutathione to the translation mixture served to oxidize exogenous thiols and re-establish necessary levels of glutathione, diminished during the gel filtration procedure. When synthesized in the presence of glutathione and an optimal redox potential, nascent secretory proteins showed conformational properties similar to those of proteins synthesized in vitro. As shown here, addition of glutathione was also critical for the determination of biological activities in nascent pancreatic proteins. Secretory proteins synthesized in the presence of membranes showed significantly increased levels of biological activity when oxidized glutathione was added during in vitro translation. The conditions optimal for determination of biological activity were identical with those previously described for the correct development of conformation, including formation of native disulfide bonds. Based on our previous conformational studies (5), the observation that individual pancreatic secretory proteins are processed similarly at the level of the rough endoplasmic reticulum and the observation that, with the exception of lipase (1), no further intracellular processing occurs with canine pancreatic secretory proteins, we can predict that all nascent pancreatic proteins segregated and processed by microsomal membrane show biological or probiological activity.

Pancreatic presecretory proteins, synthesized in the absence of microsomal membranes and either the absence or presence of oxidized glutathione, showed little or no biological activity. Preamylase and prezymogens (for trypsinogen 1, chymotrypsinogen 1, and trypsinogen 2-5) did not show selective binding to either glycogen or immobilized soybean trypsin inhibitor, respectively. Enterokinase was unable to activate pretrypsinogen by proteolytic reduction to trypsin. These findings appear in sharp contrast to those of previous investigators who reported enzyme activity associated with preribonuclease (18) and preamylase (19) synthesized in vitro with rat pancreatic mRNA. These workers, however, failed to compare their results for presecretory proteins with results of activities determined for secretory proteins processed by microsomal membranes. The finding of small amounts of biological activity associated with presecretory proteins in their studies is not entirely unexpected. Since our previous studies have indicated that uncleaved transport peptides inhibit the development of stable conformation, the ensemble of forms observed among unstable polypeptide chains will include some forms with biological activity. Based on the total number of unstable or unstructured conformational states, however, the amount of expressed biological activity should be relatively small.

Our findings, based on the measurement of biological activities associated with both presecretory (unprocessed) and secretory (processed) proteins, indicate that in the presence of a favorable redox potential and adequate quantities of glutathione, proteolytic removal of the transport peptide is a required step in the synthesis of biologically active pancreatic proteins. Taken together, our findings indicate that the sequence of steps required for synthesis of such proteins is as follows: synthesis → segregation → proteolytic processing → development of correct conformation including formation of native disulfide bonds → expression of biological or probiological activity. While initiation of each of these steps occurs in the sequence described, termination of individual steps may overlap, e.g. proteolytic processing occurs before synthesis and segregation are complete. Based on the findings reported here for pancreatic proteins, other secretory proteins with short-lived NH₂-terminal transport peptides should also show a similar sequence of synthetic events.

One secreted protein, ovalbumin, which contains an uncleaved transport peptide, can achieve the correct development of conformation and biological activity without proteolytic loss of its transport peptide (20, 21). Ovalbumin may be a vestige representing a class of secretory proteins which evolved prior to the evolutionary appearance of a transport peptidase. However, the finding that the majority of secretory proteins (39 out of 40) contain transport peptides which are cleaved during membrane translocation (22) indicates that after the evolutionary appearance of peptidases, activity, the development of secretory proteins with transient NH₂-termin al peptide extensions occurred with considerably higher efficiency. Such a proteolytic processing system confers considerable advantage since secretory proteins can evolve without the constraint imposed by the necessity to achieve a biologically active conformation in the presence of an uncleaved hydrophobic transport peptide. In addition, the presence of biological activity in nascent secretory proteins and its relative or complete absence in presecretory proteins suggest that the evolutionary development of secretory proteins and their precursor (transport) peptides is independent, converging only at the point that selective pressure determines the advantage associated with secretion of a specific protein. Consistent with this hypothesis is the finding that the majority of the transport peptide for preimmunoglobulin is coded by RNA representing a separate exon (23).

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G Scheele and R Jacoby

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