Tronrud, D. E., Holden, H. M., & Matthews, B. W. (1987) *Science (Washington, D.C.) 235*, 571–574.
Weaver, L. H., Kester, W. R., & Matthews, B. W. (1977) *J. Mol. Biol. 114*, 119–132.
Westerik, J. A., & Wolfenden, R. (1972) *J. Biol. Chem. 247*, 8195–8197.
Williams, J., & Morrison, J. (1979) *Methods Enzymol. 63*, 437.

Purification and Characterization of Hen Oviduct Microsomal Signal Peptidase†

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ABSTRACT: Hen oviduct signal peptidase requires only two proteins for proteolysis of fully synthesized secretory precursor proteins in vitro: one with a molecular mass of 19 kilodaltons (kDa) and one which is a glycoprotein whose mass varies from 22 to 24 kDa depending on the extent of glycosylation. Purified signal peptidase has been analyzed both as part of an active catalytic unit and after electroelution of the individual proteins out of a preparative polyacrylamide gel. The multiple forms of the glycoprotein component of signal peptidase bind to concanavalin A and are shown to be derived from the same polypeptide backbone. Removal of their oligosaccharides by digestion with *N*-glycanase converts these proteins to a single 19.5-kDa polypeptide. The glycoproteins all exhibit very similar profiles following individual digestion with trypsin and separation of the resulting peptides by reverse-phase high-performance liquid chromatography. In addition, sequence analysis of selected peptides from corresponding regions in chromatograms representing each form of the glycoprotein reveals the same amino acid sequences. The 19-kDa signal peptidase protein does not bind concanavalin A, has a distinct tryptic peptide map from that of the glycoprotein, and appears to share no amino acid sequences in common with the glycoprotein. Its copurification on a concanavalin A–Sepharose column indicates that it must interact directly with the glycoprotein subunit.

The current model for translocation of secretory proteins across the lipid bilayer of the endoplasmic reticulum is founded on a sequence of recognition events which are initiated by an amino-terminal extension peptide of the nascent protein (Blobel & Dobberstein, 1975a; Walter et al., 1984; Hortsch & Meyer, 1986; Walter & Lingappa, 1986). The role of this signal peptide is transient, and its removal is catalyzed by a highly specific endoproteinase located on the lumenal side of the endoplasmic reticulum (Blobel & Dobberstein, 1975b). Hen oviduct signal peptidase (HOSP)[1] belongs to this unique class of enzymes which are integral membrane proteins (Jackson & Blobel, 1977; Lively & Walsh, 1983; Mollay et al., 1982; Fujimoto et al., 1984) requiring a phospholipid environment for activity in vitro (Jackson & White, 1981; Baker et al., 1986).

Two distinct signal peptidases have been identified in *Escherichia coli* (Wolfe et al., 1983; Innis et al., 1984). Each of these enzymes is a single-chain molecule which requires detergent for solubilization, yet, unlike the eukaryotic enzyme, neither appears to require phospholipid for activity. In contrast to the apparently simple prokaryotic enzymes, purified microsomal signal peptidase from canine pancreas is associated with a glycosylated complex of from four to six polypeptides (Evans et al., 1986a). It has not yet been determined which of the proteins of this complex are absolutely required for signal peptide cleavage.

It is not yet known to which proteolytic enzyme family, if any, the signal peptidases may belong, and information regarding their mechanisms of action is very limited. No clear pattern of inhibition of these enzymes has emerged, and additional studies of the purified proteases are required to delineate the nature of their reaction mechanisms. In an effort to further our understanding of the enzymology of signal peptidase and its role in the translocation process, we have purified the enzyme from tubular gland cells from the magnum region of hen oviduct using affinity chromatography on concanavalin A–Sepharose. The purified enzyme is composed of polypeptides of 24, 23, 22, and 19 kDa. The 24-, 23-, and 22-kDa proteins are differentially glycosylated forms of a single 19.5-kDa polypeptide. The 19-kDa protein is not a glycoprotein and is distinct from the glycosylated polypeptides. We demonstrate that HOSP requires no more than two proteins for cleavage of full-length secretory precursor proteins in vitro.

Experimental Procedures

Materials. DEAE-cellulose and CM-cellulose were obtained from Whatman (Clifton, NJ). Phosphatidylcholine (egg, L-α-lecithin) was purchased from Avanti Polar-Lipids (Birmingham, AL). Hydroxylapatite (Bio-Gel HTP) and Affi-Gel 15 are products of Bio-Rad (Richmond, CA). Con A–Se-

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[1] Abbreviations: HOSP, hen oviduct signal peptidase; DEAE, diethylaminoethyl; CM, carboxymethyl; HA, hydroxylapatite; NP-40, Nonidet P-40; Con A, concanavalin A; HRP, horseradish peroxidase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PC, phosphatidylcholine; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; endo H, endo-β-*N*-acetylglucosaminidase H; TPCK–trypsin, L-1-(*p*-tosylamido)-2-phenylethyl chloromethyl ketone treated trypsin; HPLC, high-performance liquid chromatography; kDa, kilodalton(s); Tris, tris(hydroxymethyl)aminomethane; PTH, phenylthiohydantoin; ER, endoplasmic reticulum.

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pharose was purchased from Pharmacia (Piscataway, NJ). TPCK–trypsin was obtained from Sigma (St. Louis, MO). HRP-labeled Con A and antibodies specific for Con A were also obtained from Sigma and dissolved to protein concentrations of 1 and 13.7 mg/mL, respectively, prior to use. Electroblotting was performed by using a 0.2-μm nitrocellulose filter from Schleicher & Schuell (Keene, NH). *N*-Glycanase was acquired from Genzyme (Boston, MA) and had a specific activity of 260 units/mL. The apparatus for electroelution of proteins out of SDS–polyacrylamide gels was obtained from C.B.S. Scientific Co. (Del Mar, CA).

Preparation of Oviduct Microsomes, Solubilization with NP-40, DEAE-cellulose Chromatography, and CM-cellulose Chromatography. The preparation of carbonate-treated microsomes (Fujiki et al., 1982), solubilization of the lipid bilayers with 2.5% (w/v) NP-40, and chromatography on DEAE- and CM-cellulose were performed on a larger scale than previously described (Baker et al., 1986) but in an otherwise identical manner. Two hundred sixty-three milliliters of a suspension of rough microsomes (A_{280} = 57) was treated with 12.5 volumes of 0.1 M Na_2CO_3, and the recovered lipid bilayers were solubilized at 15 °C in 2.5% (w/v) NP-40. The solubilized enzyme (80 mL) was dialyzed and the clarified dialysate (115 mL) applied to a column (4.8 × 30 cm) of DEAE-cellulose. Fractions (25 mL) containing signal peptidase activity were pooled (230 mL).

CM-cellulose chromatography was performed exactly as previously described. Protein concentration was determined according to the method of Peterson (1977), and SDS–PAGE was performed in the presence of 2-mercaptoethanol according to Laemmli (1970). All steps were performed at 4 °C unless otherwise indicated.

Hydroxylapatite Chromatography. The pool obtained from CM-cellulose chromatography (58 mL) was dialyzed against two 4-L changes of HA buffer [1 mM sodium phosphate, pH 6.8, 10% (v/v) glycerol, and 0.5% (w/v) NP-40]. The solution was filtered (Gelman 0.45-μm Acrodisk) to remove a small quantity of precipitate which formed during dialysis and then applied at a flow rate of 10 mL/h to a column (1.5 × 4.5 cm) of hydroxylapatite equilibrated in HA buffer. The column was washed with buffer [1 mM sodium phosphate, pH 6.8, 10% (w/v) glycerol, 5 mM CHAPS, and 0.08 mg/mL PC] until the absorbance at 280 nm achieved a steady base line. HOSP was eluted with 200 mM sodium phosphate, pH 6.8, in Con A buffer [10 mM Tris, pH 8, 250 mM NaCl, 10% (w/v) glycerol, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM CHAPS, and 0.08 mg/mL PC], and fractions (1.1 mL) comprising the peak of the absorbance were pooled (4.6 mL).

Sephadex G-25 Chromatography. The HA pool was applied to a column (1.6 × 20 cm) of Sephadex G-25 equilibrated in Con A buffer, and the fractions (2.4 mL) comprising the peak of the absorbance at 280 nm were pooled for lectin affinity chromatography (7.3 mL).

Preparation of Anti-Con A Affinity Column. Two milliliters of Affi-Gel 15 was washed with 10 mL of isopropyl alcohol followed by 10 mL of deionized water. The gel was immediately combined with 1 mL of 0.2 M $NaHCO_3$, pH 8, and 1.1 mL of Con A antibodies (15 mg of protein). The suspension was mixed overnight at 4 °C. Remaining *N*-hydroxysuccinimide esters were reacted by the addition of 0.1 mL of 1 M ethanolamine, pH 8. The gel was packed into a column and thoroughly washed with Con A buffer containing 0.1 M methyl α-D-mannopyranoside.

Con A Chromatography. The G-25 pool was applied at a flow rate of 10 mL/h to a column (1 × 17 cm) of Con A–Sepharose previously equilibrated in Con A buffer. The flow was stopped after the entire sample was applied, and the column was allowed to stand for 3 h. The column was washed with 100 mL of Con A buffer, and then the bound protein was eluted with 0.25 M methyl α-D-mannopyranoside in Con A buffer. Fractions (2.3 mL) containing signal peptidase activity were pooled (14 mL), passed through the 2-mL immunoaffinity column containing immobilized antibodies against Con A, and then concentrated to a final volume of 3.7 mL of dialysis against solid poly(ethylene glycol) 20 000.

Signal Peptidase Assay. A translocation-independent assay (Jackson, 1983) was used and enzymatic activity quantified according to Baker and co-workers (Baker et al., 1986) with the exception that human preplacental lactogen was synthesized in the absence of an antibody specific for mature placental lactogen. Aliquots of the HA pool and Con A pool were diluted with Con A buffer prior to initiation of the reaction by addition of substrate. All of the assays contained 20 mM dithiothreitol during incubation which was found to enhance the detection of HOSP activity.

Purification of Polypeptides by Electroelution. The protein in 2 mL of HA pool (2.8 mg) was precipitated in 10% (w/v) trichloroacetic acid and the pellet washed with 2 mL of acetone. The proteins were separated by SDS–PAGE in a 12.5% polyacrylamide slab gel (0.75 mm thick). Following electrophoresis, the gel was rapidly stained and destained with Coomassie blue, and the individual protein bands were excised with a sharp razor blade. The gel slices were processed and the proteins electroeluted according to Hunkapiller et al. (1983). The estimated yield of each purified polypeptide was more than 2 nmol.

Identification of Glycoproteins. Approximately 4 μg of the Con A fraction and 2 μg of each purified polypeptide were separated by SDS–PAGE in a 10–15% linear gradient polyacrylamide gel and electrophoretically transferred to nitrocellulose according to Burnette (1981). The nitrocellulose was probed with a 1:2500 dilution of Con A conjugated to HRP and visualized with the substrate 3,3′-dimethoxybenzidine dihydrochloride as described (Evans et al., 1986a).

Peptide:N-Glycosidase F (N-Glycanase) Digestion. Approximately 40 μg of the Con A fraction was treated with *N*-glycanase (60 milliunits/mL) as described (Tarentino et al., 1985), and the reaction products were separated by SDS–PAGE in a 12.5% polyacrylamide gel.

Trypsin Digestion. The purified polypeptides obtained by electroelution were precipitated overnight with 9 volumes of ethanol at −20 °C. The protein pellets were dissolved in 90 μL of 2 M urea/10 mM NH_4HCO_3 and digested overnight at 37 °C with 4 μg of TPCK–trypsin. The resulting peptides were separated by HPLC on a SynChropak RP-P (C18) column using 0.1% (w/v) trifluoroacetic acid as the mobile phase and 0.08% (w/v) trifluoroacetic acid in 70% (v/v) acetonitrile as the mobile phase modifier (Mahoney & Hermodson, 1980).

Amino Acid Sequence Analysis. Tryptic peptides were subjected to automated Edman degradation using an Applied Biosystems Model 470A protein sequencer (Hewick et al., 1980). PTH-amino acids were identified by reverse-phase HPLC as described (Hunkapillar & Hood, 1983).

Results

Microsomal signal peptidase has been purified to near homogeneity from the tubular gland cells of the magnum region of hen oviducts. The purification protocol requires four major steps: (1) treatment of microsomes with carbonate at pH 11.5; (2) solubilization of the membrane-bound protein with 2.5%

Table I: Summary of the Purification of Oviduct Signal Peptidase

	volume (mL)	protein (mg)	total act. (units × 10^3)[a]	sp act. (units/mg × 10^3)
rough microsomes	263	4720	N.D.[b]	N.D.
carbonate-treated microsomes	67	990	N.D.	N.D.
solubilized HOSP before dialysis	80	371	80	0.22
solubilized HOSP after dialysis	115	327	72	0.22
DEAE fraction	230	28	193	6.90
CM fraction	58	10.4	4830	460
HA fraction	4.6	6.4	1420	219
Con A fraction[c]	3.7	0.6	463	785

[a] One unit of activity is defined as the amount of enzyme required for 20% conversion of preHPL to HPL. [b] N.D., not determined. Signal peptidase is latent in intact microsomes and was assayed only after solubilization with detergent. [c] Values obtained following passage through the anti-Con A column and concentration with poly(ethylene glycol) 20 000.

NP-40; (3) ion-exchange chromatography on DEAE-cellulose and CM-cellulose; (4) and affinity chromatography on Con A–Sepharose. A summary of a typical purification is given in Table I, and the polypeptide profiles of the final two stages of purification are shown in Figure 1A. The purified enzyme is composed of four proteins revealed by staining with Coomassie blue after SDS–PAGE. Their relative masses are 24, 23, 22, and 19 kDa. Figure 1B shows that the purified enzyme remains active in the translocation-independent assay for cleavage of human preplacental lactogen.

Carbonate Treatment, Detergent Solubilization, and Ion-Exchange Chromatography. The initial stages of the purification take advantage of the immobilized nature of HOSP within sedimentable lipid bilayers (Lively & Walsh, 1983) followed by the removal of more than 90% of contaminating proteins which bind a column of DEAE-cellulose at pH 8.2 (Baker et al., 1986). These steps combined with chromatography on CM-cellulose result in the removal of greater than 99% of the protein present in crude microsomes without apparent loss of signal peptidase activity (Table I). However, the dramatic increase in signal peptidase activity in the fractions following DEAE chromatography is unexplained. Because of the semiquantitative nature of the enzyme assay used, values obtained for activity units must be interpreted with caution (Baker et al., 1986). We have recently determined that the detection of enzyme activity is enhanced when the assay is performed in the presence of 20 mM dithiothreitol and the resulting specific activities of catalysis are significantly higher than we have reported previously (Baker et al., 1986). HOSP in the CM fraction has been shown to be dependent upon phospholipid for activity which is maximal in the presence of 2.0 mg/mL PC. For this reason, exogenous PC is included in all buffers and enzyme assays following CM chromatography.

Hydroxylapatite and Con A Chromatography. The CM fraction is applied to a column of hydroxylapatite primarily to concentrate the protein and exchange NP-40 for CHAPS, a mild, zwitterionic detergent which permits monitoring of the absorbance at 280 nm during chromatography. The HA fraction is then desalted on a Sephadex G-25 column to equilibrate the enzyme in an alkaline buffer required for binding to Con A (Hawkes, 1982).

HOSP in the G-25 fraction is applied to a column of Con A–Sepharose and allowed to stand on the column for 3 h to assure maximal binding. However, signal peptidase binding to the Con A column is not quantitative (Table I), and a significant amount of the enzyme is recovered in the column wash. Only a small percentage of the HOSP in the unbound fraction, including the glycosylated subunits (see below), is capable of binding to fresh Con A (data not shown). The bound enzyme is eluted from Con A–Sepharose with methyl α-D-mannopyranoside and is subsequently passed through an anti-Con A affinity column to remove fragments of Con A which leach from the lectin column in the presence of detergent (Campbell & MacLennan, 1981; Lotan et al., 1977). Analysis of purified signal peptidase by SDS–PAGE reveals only four proteins with relative molecular masses of 24, 23, 22, and 19 kDa (Figure 1, lane 3). The mobilities of these proteins are not changed by electrophoresis under nonreducing conditions (data not shown).

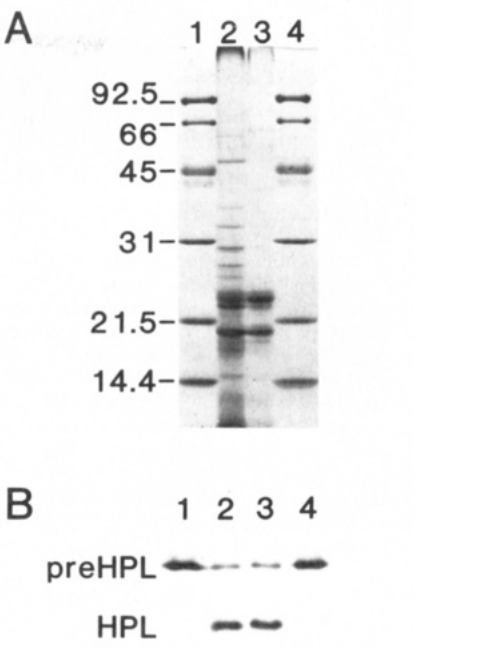

FIGURE 1: (A) Polypeptide profile of purified HOSP. An aliquot of the hydroxylapatite pool containing approximately 56 μg of protein (lane 2) and of purified HOSP containing 40 μg of protein (lane 3) was separated by SDS–PAGE in a 12.5% polyacrylamide gel and visualized with Coomassie blue R-250. Lanes 1 and 4 indicate the relative positions of molecular mass standards in kilodaltons. (B) Signal peptidase posttranslational assay. Three microliters of human preplacental lactogen (preHPL) was incubated for 60 min with either 20 μL of a 1:40 dilution of HA pool (lane 2) or 20 μL of a 1:20 dilution of purified HOSP (lane 3). The reaction products were immunoprecipitated with antibodies against HPL and then separated by SDS–PAGE in a 12.5% polyacrylamide gel. The dried gel was exposed to X-ray film for autoradiography. Lanes 1 and 4 contain preHPL without added signal peptidase.

Characterization of Glycosylated Proteins. When the CM fraction was separated by SDS–PAGE and then stained using a Schiff–periodate method specific for carbohydrates (Zacharius et al., 1969), two proteins at approximately 23 kDa were found to react with the stain (data not shown). The 19-kDa protein was unreactive with the Schiff–periodate stain. In order to confirm the identity of the glycoproteins present in purified HOSP, an aliquot of the enzyme was subjected to SDS–PAGE, electrophoretically transferred to nitrocellulose, and probed with Con A conjugated to HRP. Only two polypeptides of the Con A fraction bound tightly to Con A under these conditions (Figure 2, lane 1). These two bands correspond to the 24- and 23-kDa proteins. The assignments of the relative molecular masses of the bands stained by the Con A–HRP conjugate were confirmed by purification of each band by preparative SDS–PAGE and electroelution according

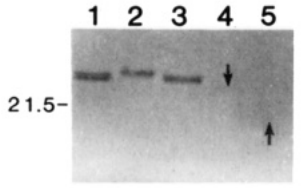

FIGURE 2: Identification of the glycosylated polypeptides present in HOSP. Approximately 4 μg of purified HOSP and 2 μg of each of the individual subunits purified by electroelution were subjected to SDS–PAGE in a 12.5% gel. The proteins were electrophoretically transferred to nitrocellulose and probed with horseradish peroxidase conjugated to Con A as described under Experimental Procedures. Glycosylated polypeptides were identified by using the substrate 3,3′-dimethoxybenzidine dihydrochloride. Lane 1 contains purified HOSP, and lanes 2–5 contain the individual 24-, 23-, 22-, and 19-kDa proteins, respectively. The downward arrow in lane 4 indicates the position of the 22-kDa protein which is not easily visible in this photograph of the blot. The upward arrow in lane 5 indicates the relative position of the 19-kDa protein which does not stain with the conjugate. The relative position of the 21.5-kDa molecular mass marker is also indicated.

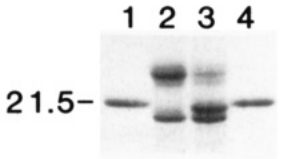

FIGURE 3: Treatment of purified signal peptidase with *N*-glycanase. Purified HOSP (40 μg) was either untreated (lane 2) or digested with *N*-glycanase (lane 3), then separated by SDS–PAGE in a 12.5% gel, and stained with Coomassie brilliant blue R-250. Lanes 1 and 4 indicate the relative position of the 21.5-kDa molecular mass standard.

to Hunkapiller and co-workers (Hunkapiller et al., 1983). Each purified band was then examined separately by analytical SDS–PAGE and electroblotting as described above (Figure 2, lanes 2–5). The mobility of each band remained the same as the region of the preparative gel from which it was excised, and the Con A–HRP bound to the diffuse 24-kDa band and the 23-kDa band most intensely. The 22-kDa band was faintly stained by the Con A–HRP, and this band was not easily visible in the photograph of the blot (Figure 2, lane 4). The 19-kDa band did not bind the conjugate.

Treatment of purified HOSP with *N*-glycanase, an enzyme which hydrolyzes all classes of asparagine-linked carbohydrates (Tarentino et al., 1985), resulted in the disappearance of more than 80% of the glycoproteins between 22 and 24 kDa and the appearance of a single polypeptide at 19.5 kDa (Figure 3, lane 3). The electrophoretic mobility of the 19-kDa HOSP polypeptide was unaffected by *N*-glycanase digestion. These results suggest that the glycoproteins may be the same protein with individual differences in glycosylation. The major species revealed by staining with Coomassie blue are at 22 and 23 kDa. Endo H digestion of purified HOSP also converted the glycoproteins to a single 19.5-kDa band (data not shown). However, the 22-kDa band was hydrolyzed slowly and incompletely by this enzyme which has a more restricted specificity than *N*-glycanase (Trimble & Maley, 1984; Tarentino et al., 1985).

Tryptic Peptide Analysis of Individual HOSP Proteins. In order to determine whether the signal peptidase glycoproteins have the same polypeptide backbone as suggested by *N*-glycanase treatment, the purified proteins obtained by electroelution from SDS–PAGE gels were digested with TPCK–trypsin, and the resulting peptides were separated by reverse-phase HPLC (Figure 4A–D). The tryptic profiles of the 24- and 23-kDa glycoproteins are nearly indistinguishable (compare panels A and B of Figure 4). These are the proteins

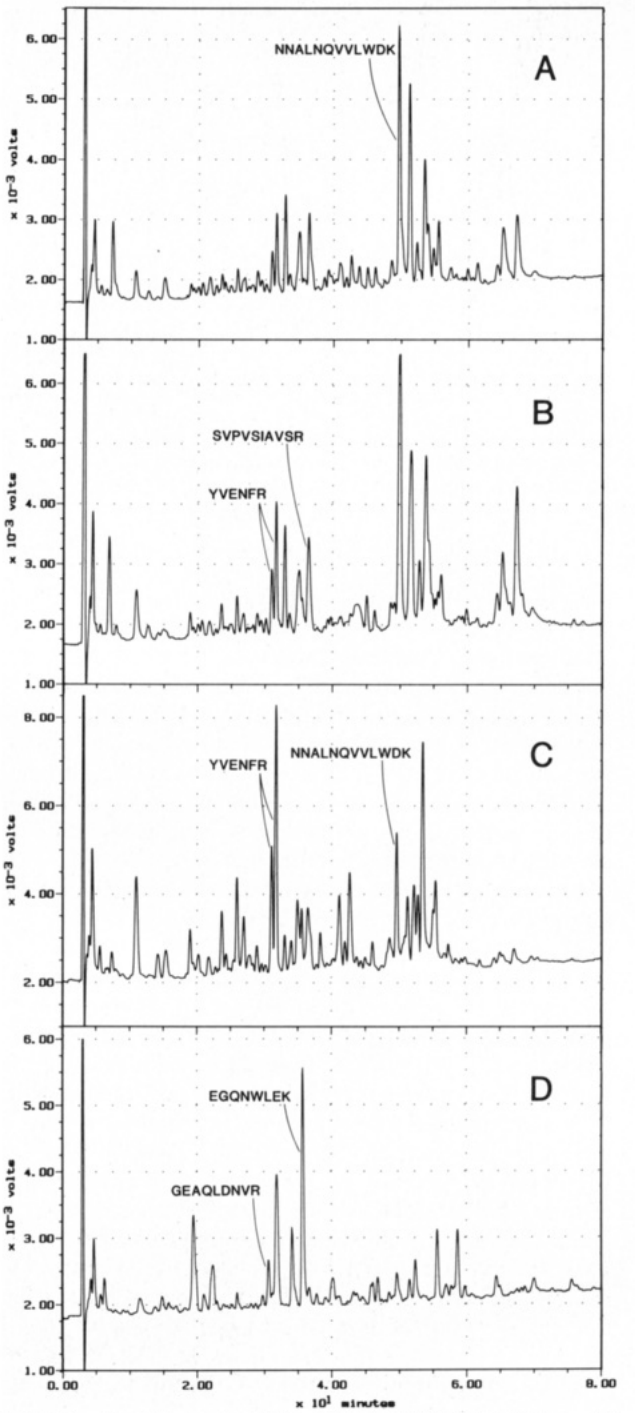

FIGURE 4: Tryptic maps of the individual HOSP proteins. Approximately 2 nmol of each of the individual HOSP proteins was purified by electroelution out of a preparative SDS–PAGE gel and then digested with 4 μg of TPCK–trypsin for 16 h at 37 °C. The resulting peptides were separated by reverse-phase HPLC using 0.1% (w/v) trifluoroacetic acid as the mobile phase and a 2-h linear gradient of 0.08% (w/v) trifluoroacetic acid in 70% (v/v) acetonitrile as the mobile phase modifier. Shown are computer-generated reproductions of the original chromatograms obtained by monitoring the absorbance at 220 nm and using the Maxima data acquisition system of the Dynamic Solutions Corp. (Ventura, CA). An absorbance of 0.02 is equal to an output of 1 mV in chromatograms A (24-kDa protein), B (23-kDa protein), and D (19-kDa protein). An absorbance of 0.01 is equal to an output of 1 mV in chromatogram C (22-kDa protein).

that bound the most Con A–HRP after electroblotting (Figure 2, lanes 2 and 3). The profile of the 22-kDa protein, which bound less Con A–HRP and was resistant to carbohydrate digestion by *N*-glycanase and endo H, is qualitatively very similar to those of the two higher molecular weight glyco-

proteins (compare Figure 4C with Figure 4A,B). Although the relative peak heights of the 22-kDa tryptic profile (Figure 4C) differ from the corresponding peaks seen in the 23-kDa (Figure 4B) and 24-kDa (Figure 4A) profiles, the overall pattern of peak groupings for each chromatogram is the same for all three. The 23- and 24-kDa profiles each have a group of peptides that elute between 60 and 70 min which are present at a much lower level in the 22-kDa profile. Similarly, a doublet at approximately 31 and 32 min is more prominent in the 22-kDa profile than in the 23- and 24-kDa profiles.

Sequence comparison of the 32-min peaks from the 22- and 23-kDa proteins revealed that they are the same peptide whose amino acid sequence is Tyr-Val-Glu-Asn-Phe-Arg. Similarly, tryptic peptides which elute at 49 min in the 22- and 24-kDa proteins have the identical amino acid sequence: Asn-Asn-Ala-Leu-Asn-Gln-Val-Val-Leu-Trp-Asp-Lys. Taken together, these data demonstrate that the 22-, 23-, and 24-kDa glycoproteins are differentially glycosylated forms of a single polypeptide. The differences in peak heights observed in the tryptic maps probably resulted because the differences in glycosylation could affect the reactivity of trypsin toward each protein. From these data, it is not possible to determine whether the differences in glycosylation are due to the addition and processing of core oligosaccharides at one or more distinct glycosylation sites or to further processing of the core oligosaccharide chain attached to a single site. Since digestion of HOSP yields a single, new protein at 19.5 kDa, it seems unlikely that the differences in molecular mass are due to differences in the polypeptide backbone.

The tryptic map of the 19-kDa protein (Figure 4D) is clearly different from the maps of the glycoproteins. Amino acid sequence analysis substantiates this difference. A tryptic peptide which elutes at 36 min in the 19-kDa protein has the sequence Glu-Gly-Gln-Asn-Trp-Leu-Glu-Lys while a corresponding peptide in the 23-kDa protein has the sequence Ser-Val-Pro-Val-Ser-Ile-Ala-Val-Ser-Arg. Comparison of additional tryptic peptides of the 19-kDa protein with peptides of the other proteins which elute at a similar retention time revealed no sequences in common with any of the glycoprotein tryptic peptides. These results clearly establish that the 19-kDa protein is distinct from the glycoprotein.

Discussion

The role of the signal peptide and of the signal recognition particle in the selection and targeting of nascent secretory proteins to synthesis sites on the endoplasmic reticulum has become better understood in recent years (Hortsch & Meyer, 1986; Walter & Lingappa, 1986). However, the actual mechanism by which the nascent chains are transported across the lipid bilayer of the endoplasmic reticulum remains unknown. At one extreme, mechanisms have been proposed that have the purely biophysical perspective that the signal peptide and its nascent protein contain all of the physical properties necessary for direct transfer into and across the bilayer without assistance from additional proteins (Engelman & Steitz, 1981; Von Heijne & Blomberg, 1979; Wickner, 1979). At the other extreme, it has been proposed that a complex of ER proteins termed the "translocon" (Walter & Lingappa, 1986) is required for the transport of nascent proteins through a proteinacious pore or tunnel into the lumen of the ER. Currently, evidence seems to be in favor of the participation of a number of different proteins in the translocation process for most secretory proteins (Perara et al., 1986; Gilmore & Blobel, 1985; Perara & Lingappa, 1985). It seems reasonable to assume that signal peptidase should be near to, if not actually a part of, such a complex because it is known that signal peptides are removed before the translocation process has been completed (Blobel & Dobberstein, 1975a) and because the enzyme is an integral membrane protein (Lively & Walsh, 1983) that appears to be exposed on the lumenal side of the ER (Walter et al., 1979).

The purification of canine pancreas signal peptidase as a complex of proteins (Evans et al., 1986a) appears to provide further support for the involvement of additional proteins in the translocation process and for the association of the peptidase with the putative translocon. Canine signal peptidase remained associated with a group of six proteins during many steps of purification including solubilization with detergent, anion- and cation-exchange chromatography, hydroxylapatite chromatography, and gel filtration. The six proteins also sediment together during sucrose gradient velocity centrifugation. These data suggest that the association of the six proteins is specific and that they are present as a native complex within the ER. However, it is important to recognize that these proteins are all very hydrophobic membrane proteins that require detergent for solubility. ER membrane proteins tend to self-associate even in the presence of detergent and phospholipid under the relatively mild conditions used in these studies. Such proteins are difficult to dissociate using conditions that retain biological activity, so it is difficult to be certain that the association of hydrophobic membrane proteins during purification is specific and, therefore, representative of the native state of the enzyme.

While there are unmistakable similarities between canine signal peptidase and oviduct signal peptidase, the oviduct enzyme has not been isolated as a complex of six proteins. Purified HOSP requires only two proteins for cleavage of full-length precursor molecules in vitro: a 19-kDa protein and a glycoprotein with three electrophoretically different forms with apparent molecular masses of 22, 23, and 24 kDa which appear to differ only in their carbohydrate content. These three glycoproteins are differentially processed forms of a single, parent polypeptide. Their tryptic maps are very similar, and amino acid sequence analysis of selected tryptic peptides reveals sequences common to all three forms (Figure 4A–C). The 22- and 23-kDa proteins of canine signal peptidase are also glycosylated and have been reported to have the same amino-terminal amino acid sequence (Evans et al., 1986b). It seems likely that the two sets of glycoproteins in the enzyme from these different species are related. Tryptic mapping and sequence analysis of selected tryptic peptides of the 19-kDa protein revealed a different tryptic map (Figure 4D) with no amino acid sequences in common with the glycoproteins. The corresponding protein in canine signal peptidase may be the 21-kDa band which, like the 19-kDa HOSP band, appears to stain intensely with Coomassie blue.

While HOSP requires only two proteins for catalysis in vitro, very little is currently known about the native enzyme in the lipid bilayer of the endoplasmic reticulum. The possibility remains that oviduct signal peptidase subunits analogous to the additional proteins purified with canine pancreas signal peptidase were dissociated during the purification procedure. The only significantly different purification step, in terms of ionic strength or buffer composition, that we used for the purification of HOSP is the initial treatment of oviduct microsomes with 0.1 M sodium carbonate, pH 11.5. This procedure effectively removes nonmembrane proteins from the lipid bilayers and allows the stripped membrane vesicles containing only membrane proteins to be recovered following centrifugation (Fujiki et al., 1982). While it is possible that this treatment at high pH could have caused the dissociation

of the putative oviduct signal peptidase complex, it seems unlikely because the lipid bilayers are recovered intact, containing active signal peptidase. If the additional subunits are integral membrane proteins, then one would expect them to remain associated with the lipid bilayer as with signal peptidase. Subsequent solubilization of the membrane vesicles should then release the intact complex. Since the conditions of the chromatographic steps following solubilization of HOSP are similar to those used in the isolation of the canine complex, it is difficult to explain how such an oviduct complex would then become dissociated.

The different electrophoretic migration of the three glycoproteins is reproducible, resulting from real differences in mass, and is not due to the typical diffuse electrophoretic migration observed with many glycoproteins analyzed by SDS–PAGE. Our data suggest that the altered electrophoretic mobility is the result of differential processing of one, or possibly two, core N-linked oligosaccharide. The 22-kDa form is a poor substrate for *N*-glycanase and appears not to be cleaved at all by endo H. While *N*-glycanase is able to cleave all classes of N-linked carbohydrates, endo H requires at least three α-mannosyl residues for effective binding to oligosaccharides (Ogata et al., 1975). Together, these data suggest that the oligosaccharides of the 24- and 23-kDa proteins retain properties of the high-mannose, core oligosaccharide while the 22-kDa protein has properties of a partially processed oligosaccharide. The resolution of multiple glycosylated forms of a single protein by SDS–PAGE has been observed with another integral membrane protein of the endoplasmic reticulum, ribophorin I (Rosenfeld et al., 1984).

The behavior of HOSP during chromatography on Con A–Sepharose supports the hypothesis that the 19-kDa protein is specifically associated with the glycoproteins. The 19-kDa protein is not glycosylated because it is not stained by a Schiff–periodate carbohydrate stain, it does not bind to Con A–HRP (Figure 2), and its electrophoretic mobility is not affected by treatment with either *N*-glycanase (Figure 3) or endo H (not shown). However, this nonglycosylated protein copurifies with the signal peptidase glycoproteins during chromatography on Con A–Sepharose. Since it does not bind to Con A by itself, the 19-kDa protein must bind to and specifically elute from the Con A column in association with the glycoproteins while several other membrane proteins are clearly removed at this step. We are currently not able to completely eliminate the possibility that the association of these two proteins is the result of nonspecific hydrophobic interactions and that only one of the proteins present in purified HOSP is responsible for catalytic activity.

The simplest arrangement of proteins consistent with our data would be a two-chain signal peptidase catalytic unit with a single 19-kDa subunit and a single glycosylated subunit. This enzyme would have a peptide mass of approximately 39 kDa with from 2500 to 4500 Da of carbohydrate (roughly estimated by SDS–PAGE). By comparison, purified *E. coli* leader peptidase has a similar mass in a 37-kDa single-chain protein (Wolfe et al., 1982). On the other hand, *E. coli* prolipoprotein signal peptidase is much smaller with a molecular mass of 17.8 kDa (Dev & Ray, 1984; Innis et al., 1984) which is just slightly smaller than either of the two proteins present in purified HOSP. It is possible that the two subunits of oviduct signal peptidase arise by proteolytic processing of a single gene product, a hypothesis that we are currently pursuing.

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REFERENCES

Baker, R. K., Bentivoglio, G. B., & Lively, M. O. (1986) *J. Cell. Biochem. 32*, 193.
Blobel, G., & Dobberstein, B. (1975a) *J. Cell Biol. 67*, 835.
Blobel, G., & Dobberstein, B. (1975b) *J. Cell Biol. 67*, 852.
Burnette, W. N. (1981) *Anal. Biochem. 112*, 195.
Campbell, K. P., & MacLennan, D. H. (1981) *J. Biol. Chem. 256*, 4626.
Dev, I. K., & Ray, P. H. (1984) *J. Biol. Chem. 259*, 11114.
Engelman, D. M., & Steitz, T. A. (1981) *Cell (Cambridge, Mass.) 23*, 411.
Evans, E. A., Gilmore, R., & Blobel, G. (1986a) *Proc. Natl. Acad. Sci. U.S.A. 83*, 581.
Evans, E. A., Shelness, G. S., & Blobel, G. (1986b) *J. Cell Biol. 103*, 291a.
Fujiki, Y., Hubbard, A. L., Fowler, S., & Lazarow, P. B. (1982) *J. Cell Biol. 93*, 97.
Fujimoto, Y., Watanabe, Y., Uchida, M., & Ozaki, M. (1984) *J. Biochem. (Tokyo) 96*, 1125.
Gilmore, R., & Blobel, G. (1985) *Cell (Cambridge, Mass.) 42*, 497.
Hawkes, R. (1982) *Anal. Biochem. 123*, 143.
Hewick, R. M., Hunkapillar, M. W., Hood, L. E., & Dreyer, W. J. (1981) *J. Biol. Chem. 256*, 7990.
Hortsch, M., & Meyer, D. I. (1986) *Int. Rev. Cytol. 102*, 215.
Hunkapillar, M. W., & Hood, L. E. (1983) *Methods Enzymol. 91*, 486.
Hunkapiller, M. W., Lujan, E., Ostrander, F., & Hood, L. E. (1983) *Methods Enzymol. 91*, 227.
Innis, M. A., Tokunaga, M., Williams, M. E., Loranger, J. M., Chang, S. Y., Chang, S., & Wu, H. C. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3708.
Jackson, R. C. (1983) *Methods Enzymol. 96*, 784.
Jackson, R. C., & Blobel, G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5598.
Jackson, R. C., & White, W. R. (1981) *J. Biol. Chem. 256*, 2545.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Lively, M. O., & Walsh, K. A. (1983) *J. Biol. Chem. 258*, 9488.
Lotan, R., Beattie, G., Hubbell, W., & Nicolson, G. (1977) *Biochemistry 16*, 1787.
Mahoney, W. C., & Hermodson, M. A. (1980) *J. Biol. Chem. 255*, 11199.
Mollay, C., Vilas, U., & Kreil, G. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2260.
Ogata, S., Muramatsu, T., & Kobata, A. (1975) *J. Biochem. (Tokyo) 78*, 687.
Perara, E., & Lingappa, V. R. (1985) *J. Cell Biol. 101*, 2292.
Perara, E., Rothman, R. E., & Lingappa, V. R. (1986) *Science (Washington, D.C.) 232*, 348.
Peterson, G. L. (1977) *Anal. Biochem. 83*, 346.
Rosenfeld, M. G., Marcantonio, E. E., Hakimi, J., Ort, V. M., Atkinson, P. H., Sabatini, D. D., & Kreibich, G. (1984) *J. Cell Biol. 99*, 1076.
Tarentino, A. L., Gomez, C. M., & Plummer, T. H., Jr. (1985) *Biochemistry 24*, 4665.
Trimble, R. B., & Maley, F. (1984) *Anal. Biochem. 141*, 515.
Von Heijne, G., & Blomberg, C. (1979) *Eur. J. Biochem. 97*, 175.
Walter, P., & Lingappa, V. R. (1986) *Annu. Rev. Cell Biol. 2*, 499.
Walter, P., Jackson, R. C., Marcus, M. M., Lingappa, V. R.,

& Blobel, G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1795.
Walter, P., Gilmore, R., & Blobel, G. (1984) *Cell (Cambridge, Mass.) 38*, 5.
Wickner, W. (1979) *Annu. Rev. Biochem. 48*, 23.
Wolfe, P. B., Silver, P., & Wickner, W. (1982) *J. Biol. Chem. 257*, 7898.
Wolfe, P. B., Wickner, W., & Goodman, J. M. (1983) *J. Biol. Chem. 258*, 12073.
Zacharius, R. M., Zell, T. E., Morrison, J. H., & Woodlock, J. J. (1969) *Anal. Biochem. 30*, 148.

Catalytic Oxidation of 2-Aminophenols and Ortho Hydroxylation of Aromatic Amines by Tyrosinase†

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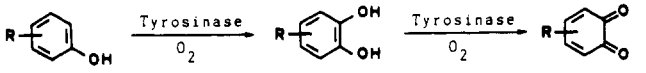
ABSTRACT: The usual substrates of tyrosinase, a copper-containing monooxygenase (EC 1.14.18.1), are monophenols and *o*-diphenols which are both converted to *o*-quinones. In this paper, we studied the reaction of this enzyme with two new classes of substrates: aromatic amines and *o*-aminophenols, structural analogues of monophenols and *o*-diphenols, respectively. They undergo the same catalytic reactions (ortho hydroxylation and oxidation), as documented by product analysis and kinetic studies. In the presence of tyrosinase, arylamines and *o*-aminophenols are converted to *o*-quinone imines, which are isolated as quinone anils or phenoxazones. As an example, in the presence of tyrosinase, 2-amino-3-hydroxybenzoic acid (an *o*-aminophenol) is converted to cinnabarinic acid, a well-known phenoxazone, while *p*-aminotoluene (an aromatic amine) gives rise to the formation of 5-amino-2-methyl-1,4-benzoquinone 1-(4-methylanil). Kinetic studies using an oxygen electrode show that arylamines and the corresponding monophenols exhibit similar Michaelis constants (K_m = 0.11–0.49 mM). In contrast, the reaction rates observed for aromatic amines are relatively slow (k_{cat} = 1–3 min^{-1}) as compared to monophenols (1320–6960 min^{-1}). The enzymatic conversion of arylamines by tyrosinase is different from the typical ones: N-oxidation and ring hydroxylation without further oxidation. This difference originates from the regiospecific hydroxylation (ortho position) and subsequent oxidation of the intermediate *o*-aminophenol to the corresponding *o*-quinone imine. Finally, the well-known monooxygenase activity of tyrosinase was also confirmed for the aromatic amine *p*-aminotoluene, with $^{18}O_2$. In the case of *o*-aminophenols, the kinetic studies indicate that the K_m values are rather similar to those of the corresponding *o*-diphenols. The oxidation rates, k_{cat}, for *o*-aminophenols are comparable to 2,3-dihydroxybenzoic acid (0.2–0.8 s^{-1}).

Tyrosinase is a copper-containing monooxygenase (EC 1.14.18.1) that catalyzes the ortho hydroxylation of monophenols and the oxidation of *o*-diphenols to *o*-quinones (Mason, 1957) (Scheme I). This enzyme is widely distributed in nature and is responsible for the formation of melanin pigments and other polyphenolic compounds (Lerch, 1981).

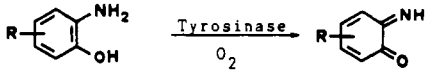
The active site of tyrosinase consists of a binuclear copper center where activation of molecular oxygen and substrate interaction take place (Schoot Uiterkamp & Mason, 1973; Lerch, 1983; Wilcox et al., 1985). As anticipated from the chemical knowledge of copper-catalyzed hydroxylations and oxidations (Nigh, 1973; Capdevielle & Maumy, 1987), the enzymatic activity of tyrosinase should not be only restricted to aromatic hydroxy compounds (monophenols and catechols).

It seemed therefore of interest to study the activity of tyrosinase on aromatic amines and *o*-aminophenols, which are analogues of monophenols and *o*-diphenols, respectively. These compounds are widespread in nature and have been shown to be metabolized under various conditions (Tomoda et al., 1984).

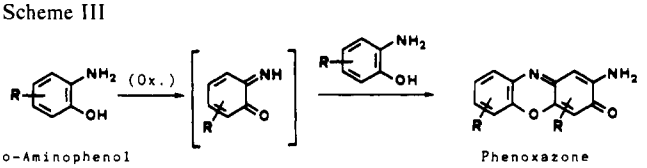
Scheme I



Scheme II



Scheme III



First, we observed that *o*-aminophenols undergo an oxidation reaction in the presence of tyrosinase leading to the formation of the corresponding *o*-quinone imines (Scheme II).

Different oxidizing agents, manganese dioxide (Prinz & Savage, 1977) and potassium ferricyanide or dichromate (Schäfer, 1964), and some metalloproteins, hemoglobin (To-

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