

Purification of a membrane-associated protein complex required for protein translocation across the endoplasmic reticulum

(*in vitro* protein synthesis/salt extract of microsomal membranes/hydrophobic chromatography/reconstituted translocation activity/*N*-ethylmaleimide labeling)

PETER WALTER AND GÜNTER BLOBEL

Laboratory of Cell Biology, The Rockefeller University, New York, New York 10021

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ABSTRACT The capacity of microsomal membranes to translocate nascent presecretory proteins across their lipid bilayer can be largely abolished by extracting them with high ionic strength buffers. It can be reconstituted by adding the salt extract back to the depleted membranes [Warren, G. & Dobberstein, B. (1978) *Nature (London)* 273, 569-571]. Utilizing hydrophobic chromatography, we purified to homogeneity a protein component of the salt extract that reconstitutes the translocation activity of the extracted membranes. This component behaves as a homogeneous species upon gel filtration, ion-exchange chromatography, adsorption chromatography, and sucrose-gradient centrifugation. When examined by polyacrylamide gel electrophoresis in NaDodSO₄, six polypeptides with apparent *M_r* of 72,000, 68,000, 54,000, 19,000, 14,000, and 9000 are observed in about equal and constant stoichiometry, suggesting that they are subunits of a complex. The sedimentation coefficient of 11S is in good agreement with the sum of the *M_r* of the subunits. The *M_r* 68,000 and 9000 subunits label intensely with *N*[³H]ethylmaleimide. Thus, the reported sulfhydryl group requirement of the translocation activity in the unfractionated extract [Jackson, R. C., Walter, P. & Blobel, G. (1980) *Nature (London)*, 286, 174-176] may be localized to either or both the *M_r* 68,000 and 9000 subunits of the purified complex.

The synthesis of secretory and several membrane proteins so far investigated is thought to be initiated on free ribosomes (1, 2). When the nascent polypeptide chain emerges from the ribosome, a specific section of it, the signal sequence, was proposed to interact with receptors in the endoplasmic reticulum (ER), triggering the formation of a ribosome-membrane junction and initiating the translocation process of the nascent chain into the cisternae of the ER (1, 2). In most cases the signal sequence is located at the NH₂-terminal portion of the nascent chain (2) and is cleaved by signal peptidase (3, 4), presumably on the luminal side of the ER (5, 6), concomitantly with elongation and translocation of the protein.

Little is known about the recognition and transport machinery that is involved in the translocation process. The translocation activity of the microsomal vesicles has been assayed directly, after extraction and reconstitution experiments. One approach used salt extraction (7); the other one used limited tryptic dissection (6) of the microsomes. In both cases, a water soluble fraction and a translocation-inactive membrane fraction were obtained that recombined to reconstitute the translocation activity. Both water soluble fractions have been shown to be functionally equivalent (8) (the trypsin-derived fraction reconstitutes salt-depleted membranes and vice versa) and both contain a sulfhydryl group, which is required for their activity (8).

We describe here the purification to homogeneity of the

active component of the salt extract that reconfers translocation activity to the salt-extracted microsomal membrane vesicles.

METHODS

All preparative procedures were carried out at 0-4°C. Optical absorbance determinations were done in 1% NaDodSO₄. All glassware was siliconized. The 4 M KOAc stock solution was adjusted to pH 7.5.

Preparation of Microsomal Membranes. Rough microsomal membranes were prepared from freshly excised dog pancreas (9) as described (10), with the following modifications: all buffers contained 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride. The rough microsomes were collected by centrifugation for 2.5 hr at 140,000 × *g*_{avg}. The resulting pellet of rough microsomes (RM) was resuspended by manual homogenization in a Dounce homogenizer (A-pestle) in buffer A (250 mM sucrose/50 mM triethanolamine-HOAc, pH 7.5 [(EtOH)₃N-HOAc]/1.0 mM dithiothreitol) to a concentration of 50 A₂₈₀ units/ml.

Adsorbed ribosomes and proteins were removed by passing the membranes through a Sepharose C1-2B column (11) in a low-salt buffer [50 mM (EtOH)₃N-HOAc/0.5 mM Mg(OAc)₂/1 mM dithiothreitol]. RM (20 ml) were loaded on a 200-ml column (upward flow, 15 ml/hr). The turbid fractions were pooled, and the membranes were collected by centrifugation (15 min at 50,000 × *g*_{avg}). The resulting washed RM were resuspended in 20 ml of buffer A.

Extraction of Washed Microsomal Membranes. Ten milliliters of an ice-cold salt solution (1.5 M KOAc/15 mM Mg(OAc)₂) was slowly added to 20 ml of washed RM. The mixture was incubated for 15 min on ice. The membranes were sedimented for 1 hr at 120,000 × *g*_{avg} through a cushion of 0.5 M sucrose in buffer B [50 mM (EtOH)₃N-HOAc/500 mM KOAc/5 mM Mg(OAc)₂/1 mM dithiothreitol] and the resulting pellet of salt-extracted microsomes was resuspended in 20 ml of buffer B containing 250 mM sucrose. The supernatant fraction, not including the cushion, was recentrifuged (3.5 hr at 200,000 × *g*_{avg}) to deplete it of ribosomes (postribosomal salt extract).

Fractionation of the Salt Extract by Hydrophobic Chromatography. A 2-ml column of ω-aminopentyl-agarose (12) (5.7 μmol of 1,5-diaminopentane coupled per ml) was pre-washed with 10 ml of 2 M KOAc and then equilibrated with 20 ml of buffer B. The postribosomal salt extract fraction (24 ml) was passed over the column (6 ml/hr). The column was then washed with 10 ml of buffer B and eluted with buffer C [50 mM (EtOH)₃N-HOAc/1 M KOAc/10 mM Mg(OAc)₂/1 mM dithiothreitol/0.05% Nikkol detergent]. As soon as buffer C ap-

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Abbreviations: ER, endoplasmic reticulum; RM, rough microsomes; (EtOH)₃N, triethanolamine; eq, equivalent; PRL, prolactin; prePRL, preprolactin.

peared in the eluent (detected by an abrupt change in drop size due to the presence of detergent), a 2-ml fraction was collected.

Cell-Free Protein Synthesis. Bovine pituitary RNA (10, 13) (0.2 A_{260} units per 25 μ l of translation mix) was translated in a staphylococcal nuclease-treated wheat germ system (14, 15) (6 μ l of wheat germ S23 per 25 μ l of translation mix). The ions that were added with the various membrane and salt extract fractions were taken into account and compensated for to yield a final ion concentration of 150 mM KOAc and 2 mM Mg(OAc)₂. Whenever detergent-containing fractions were assayed, the membranes were added last after all of the other components were mixed.

Polyacrylamide gel electrophoresis in NaDodSO₄ of the different fractions and translation products was performed on Cl₃CCOOH-precipitated, dithiothreitol-reduced, and iodoacetamide-alkylated samples as described (1). Polyacrylamide gradient (10–15%) slab gels were employed throughout.

Definitions. Percentage processing is defined as cpm in prolactin (PRL)/[cpm in PRL + cpm in preprolactin (prePRL)]. It is used as a measure of the translocation activity of membranes added in subsaturating amounts (6).

One equivalent (eq) is the amount of a fraction (supernatant or membrane) that is derived from 1 μ l of RM suspension at a concentration of 50 A_{260} units/ml. One eq is roughly derived from 1 mg of tissue (wet weight). One unit of translocation activity (U) (i) for a membrane fraction is the amount of membranes that gives the same amount of processing (i.e., translocation) as 1 eq of RM (which under the conditions of all of our assays yields 30% processing), and (ii) for a supernatant fraction is the amount of supernatant that has to be added back to 1 eq of the salt-extracted (i.e., inactive by itself) RM to restore activity to that of 1 eq of RM.

Materials. [³⁵S]Methionine (1000 Ci/mmol; 1 Ci = 3.7 × 10¹⁰ becquerels), [³H]OAc⁻ (140 mCi/mmol), and N-[³H]ethylmaleimide (525 mCi/mmol) were from New England Nuclear. Nikkol (octaethyleneglycol mono-*n*-dodecyl ether) was from Nikko Chemicals, Tokyo, Japan. ω -Aminoalkyl-agarose was from Sigma.

RESULTS

The translocation activity of microsomal membranes can be readily assayed in cell-free protein-synthesizing systems (6). If one translates bovine pituitary RNA in the absence of membranes, a larger precursor, prePRL, is synthesized (13). If one carries out the translation in the presence of microsomal membranes, the newly made secretory protein is translocated into the lumen of the vesicles, as judged by its resistance to externally added proteases. Coupled to the translocation process is the proteolytic processing by signal peptidase, presumably on the luminal side of the vesicles (6). Because this processing step is very efficient relative to translocation (no unprocessed prePRL is found inside the vesicles), the ratio of PRL to prePRL is a convenient measure of the translocation activity of the membranes (Fig. 1A).

Here we use the wheat germ cell-free translation system.

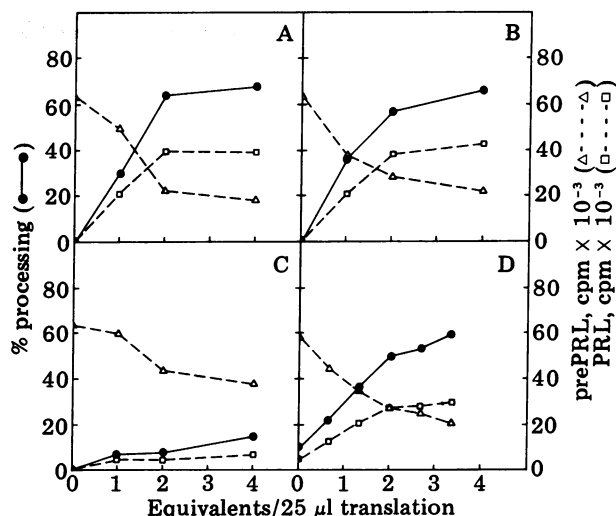


FIG. 1. Titration of membranes and crude salt extract in the translocation assay. Bovine pituitary RNA was translated in a 25- μ l wheat germ system with increasing amounts of RM, washed RM, or salt extract added. The translation products were separated by polyacrylamide gel electrophoresis in NaDodSO₄. Bands corresponding to prePRL and PRL were located by autoradiography and sliced from the dried gel, and the radioactivity was determined (6). From the cpm values (Δ , prePRL; \square , PRL) percentage processing was computed (\bullet). (A) RM. (B) Washed RM. (C) Salt-extracted RM. (D) Increasing amounts of salt extract assayed in the presence of 1 eq of salt-extracted RM. All translations contained a final concentration of 0.002% Nikkol.

Under our conditions, mammalian polysomes still attached to the RM preparation do not read out in the plant translation system and, therefore, no “stripping” or nuclease treatment of the microsomes to deplete them of their endogenous mRNA activity is necessary.

As a first step toward purification prior to salt extraction, we freed our starting preparation of RM of adsorbed proteins and ribosomes by passing the membrane suspension over a Sepharose C1-2B column in a low-salt buffer (11). Although this wash (Table 1) removed 40% of the A_{260} units (which otherwise would likely be coextracted by the high-salt buffer), it did not reduce the activity of the membrane fraction (Fig. 1A vs. 1B). Subsequent extraction by high-salt buffer depleted the membranes of most of their translocation activity (Fig. 1C). Recombining the salt extract with the depleted membranes restored their activity (Fig. 1D), as has been described (7, 8).

The activity of the salt extract turned out to be extremely unstable. It was rapidly lost upon attempts to fractionate it further. Moreover, the activity decreased upon storage in the cold, rapid freezing/thawing, or incubation at 37°C (Fig. 2, bar 9 vs. bar 11). Some preparations were more active than others (Fig. 2, bar 7 vs. bars 5 and 9), whereas some were completely inactive (Fig. 2, bar 3).

While testing whether small concentrations of the nonionic detergent Nikkol would be compatible with our assay system, we observed that the detergent increased the activity of crude

Table 1. Purification of the salt-extracted translocation activity

Fraction	Fig. 3, lane	ml	A_{260}/ml	A_{260}	Units/ml	Units	Units/ A_{260}	Recovery, %	Enrichment
RM	a	20	50	1000	1000	20,000	20	100	1
Washed RM	b	20	31	620	1000	20,000	32	100	1.6
Salt extract	e	30	2.4	72	650	19,000	270	98	13.5
Salt extract (postribosomal)	f	24	0.95	22.8	450	10,800	474	54	23.7
ω -Aminopentyl-agarose eluate	i	2	0.21	0.42	5500	11,000	26,190	55	1310

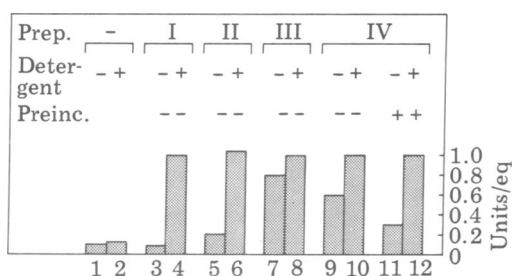


FIG. 2. Effect of low detergent concentrations on the activity of the salt extract. Translation (25 μ l) was performed in the presence of 1 eq of salt-extracted washed RM. Crude salt extracts from four different preparations (I-IV) were added in increasing amounts. An aliquot of the salt extract (IV) was preincubated for 1 hr at 37°C in the absence or presence of 0.01% Nikkol. The dilution of the salt extract into the translation mix decreased the detergent concentration to 0.002%. Translations were carried out in the absence (-detergent) or presence (+detergent) of 0.002% Nikkol. The activity (units/eq) was computed from the percentage processing values.

salt extracts up to the theoretically expected level of 1 unit/eq (Fig. 2, bar 7 to bar 8 and bar 9 to bar 10). The detergent also stabilized the activity during an incubation for 1 hr at 37°C (Fig. 2, bar 10 to bar 12) and even reactivated preparations previously considered inactive (Fig. 2, bar 3 to bar 4 and bar 5 to bar 6). At the concentration employed (0.002%), the detergent by itself does not disrupt the lipid bilayer (Fig. 2, bar 2). Its concentration is one order of magnitude below the concentration needed to expose signal peptidase. PRL molecules translocated in the presence of 0.002% Nikkol are still completely protected from added proteases (data not shown).

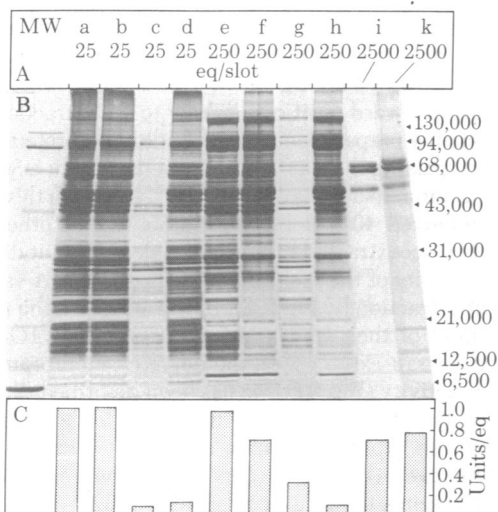


FIG. 3. Purification of the translocation activity monitored by polyacrylamide gel electrophoresis in NaDodSO₄. Fractions obtained during the purification of a typical preparation (lanes a-i) were Cl₃CCOOH-precipitated and their polypeptide composition visualized by Coomassie blue staining after electrophoresis in NaDodSO₄. (A) In lanes e-h 10 times more sample and in lanes i and k 100 times more sample (as defined in eq) is loaded relative to lanes a-d. (B) Lanes: MW, molecular weight standards; a, RM; b, washed RM; c, included fraction of the Sepharose C1-2B column; d, salt-extracted washed RM; e, salt extract; f, salt extract (ribosome-depleted); g, ribosomal pellet; h, fraction not bound to the ω -aminopentyl-agarose; i, eluate of the ω -aminopentyl-agarose; k, eluate of the ω -aminopentyl-agarose of another preparation of salt extract. (C) Activity of the fraction quantitated as described in Fig. 2. Membranous fractions (bars a, b, and d) were assayed directly, and soluble fractions (bars c and e-k) were assayed for their ability to reconstitute 1 eq of salt-extracted RM. Translations were carried out at a final concentration of 0.002% Nikkol.

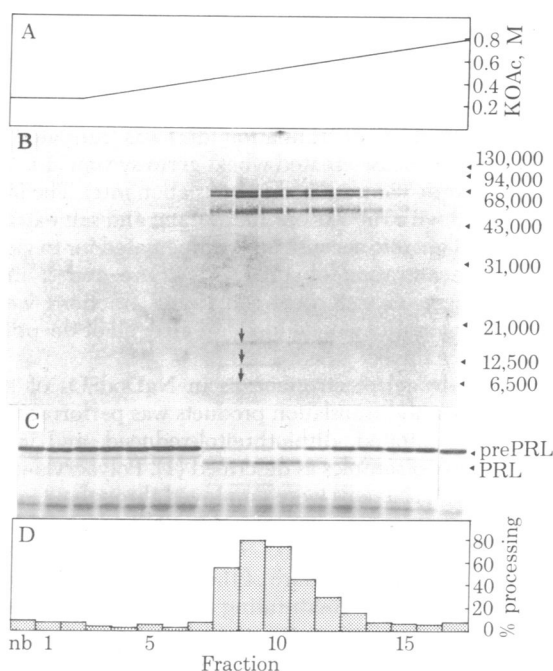


FIG. 4. DEAE-Sepharose ion-exchange chromatography of the ω -aminopentyl-agarose eluate. ω -Aminopentyl-agarose eluate (1.6 ml) was added to 4.8 ml of 50 mM (EtOH)₃N-HOAc/1 mM dithiothreitol. The sample was applied to a 1.5-ml column of DEAE-Sepharose C1-6B equilibrated in buffer D [50 mM (EtOH)₃N-HOAc/250 mM KOAc/2.5 mM Mg(OAc)₂/1 mM dithiothreitol/0.01% Nikkol] and a fraction of material not bound (nb) was collected. After loading, the column was washed with 4.5 ml of buffer D and eluted with 15 ml of a linear salt gradient of buffer D to buffer C (except that buffer C contained 0.01% Nikkol); 750- μ l fractions were collected. (A) Addition of [³H]OAc⁻ to the gradient buffers allowed determination of the precise ion composition of each fraction. To 10 μ l of each fraction, 5 μ l of salt solution was added to yield a final ion concentration of 500 mM KOAc and 5 mM Mg(OAc)₂; 5 μ l of these compensated fractions were assayed for activity (see Fig. 2). (B) Each fraction (300 μ l) was Cl₃CCOOH-precipitated, and the proteins were visualized by Coomassie blue staining after electrophoresis in NaDodSO₄. Arrows are used to indicate the position of the fainter bands of lower M_r. (C) Translation products. (D) Quantitative analysis of C.

Furthermore, the detergent does not interfere with the translocation activity of RM or washed RM preparations (data not shown).

These findings indicated that the rapid loss of activity was not an irreversible process but was merely caused by conversion of activity from a patent to a latent state. Moreover, the ability of nonionic detergent to restore activity to the theoretically expected level provided important clues concerning the properties of the active component and, thereby, opened the way to its complete and rapid purification.

To enrich the active component, the crude salt extract (without the addition of Nikkol) was centrifuged to remove ribosomes and ribosomal subunits. The activity present in both the supernatant and the ribosomal pellet was then assayed in the presence of Nikkol. From the total activity in the crude salt extract (Fig. 3C, bar e) and its quantitative partition (after centrifugation) into ribosome pellet (Fig. 3C, bar g) and into ribosome-depleted supernatant (Fig. 3C, bar f), it is apparent that about 30% of the activity sedimented into the ribosome pellet and that about 70% remained in the ribosome-depleted supernatant. Sedimentation of the activity with ribosomes is most likely because of its relatively high sedimentation coefficient (see below).

On the premise that the detergent stabilization of the active component might be caused by amphiphilic interactions, we

decided to use hydrophobic chromatography as a means of exploiting this peculiar property of the activity. We investigated the behavior of the activity on ω -aminoalkyl-agarose columns (12). The activity was not bound to ω -aminopentyl- and ω -aminobutyl-agarose but bound to ω -aminopentyl- and ω -aminohexyl-agarose in buffer B (data not shown). Elution of the activity by raising the salt concentration and including small concentrations of detergent in the buffer allowed 100% recovery from the ω -aminopentyl-agarose but only poor recovery from the ω -aminohexyl-agarose. Because most of the applied proteins were found not to interact with the ω -aminopentyl-agarose (Fig. 3B, lane h) a 55-fold purification was obtained in this step (see Table 1).

Three polypeptides of high M_r (72,000, 68,000, and 54,000) and three polypeptides of low M_r (19,000, 14,000, and 9000) were reproducibly obtained in the eluate (Fig. 3B, lane i). Some preparations contained contaminating bands (Fig. 3B, lane k) that did not appear consistently (Fig. 3B, lane i) and did not copurify with the activity upon subsequent sucrose gradient centrifugation, ion-exchange chromatography, or adsorption chromatography. The staining intensity with Coomassie blue of the three low M_r bands was slightly variable from gel to gel.

The substantially purified fraction obtained after hydrophobic chromatography was subjected to further fractionation using gel filtration on Sepharose C1-6B (data not shown), ion-exchange chromatography on DEAE-Sepharose C1-6B (Fig. 4), and adsorption chromatography on hydroxyapatite (Fig. 5). All six polypeptides consistently cochromatographed with

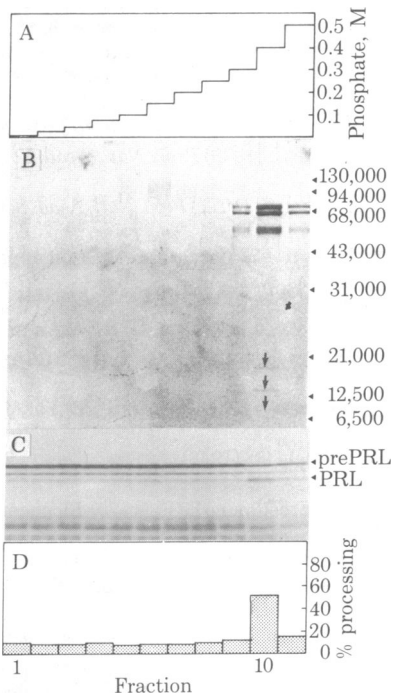


FIG. 5. Adsorption chromatography of the ω -aminopentyl-agarose eluate on hydroxyapatite. ω -Aminopentyl-agarose eluate (0.5 ml) was salt-exchanged on a 2.5-ml Sephadex G-25 column into a 10 mM sodium phosphate buffer (pH 6.5) containing 0.01% Nikkol and was applied to a 0.5-ml hydroxyapatite column. (A) The column was eluted with a step gradient of increasing phosphate concentration (1 ml per step). The phosphate buffer of each fraction collected was exchanged for buffer B (containing 0.01% Nikkol) on a 5-ml Sephadex G-25 column. (B) Each fraction (840 μ l) was Cl_3CCOOH -precipitated, and the proteins were visualized by Coomassie blue staining after electrophoresis in NaDodSO_4 . (C) Each fraction (5 μ l) was then assayed for translocation activity (see Fig. 2). (D) Quantitative analysis of the translation products in C.

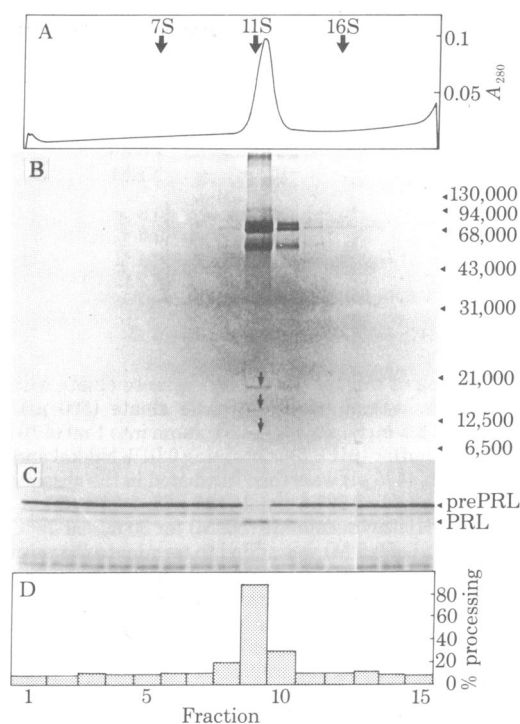


FIG. 6. Sucrose gradient centrifugation of the ω -aminopentyl-agarose eluate. ω -Aminopentyl-agarose eluate (250 μ l) was diluted with an equal volume of water, was layered on top of a 5–20% sucrose gradient (13 ml) in buffer B containing 0.01% Nikkol, and was centrifuged for 20 hr at 4°C at 40,000 rpm in the Beckman SW 40 rotor. Fifteen fractions were collected using an ISCO gradient fractionator. (A) The A_{280} profile of the gradient with the positions of standards human IgG (7S), catalase (11S), and β -galactosidase (16S). (B) The remainder of each fraction was Cl_3CCOOH -precipitated, and the proteins were visualized by Coomassie blue staining after electrophoresis in NaDodSO_4 . (C) Each fraction (5 μ l) was assayed for translocation activity (see Fig. 2). (D) Quantitative analysis of the translation products in C.

the activity, which strongly suggests that the activity consists of a complex containing six subunits.

When centrifuged on a sucrose gradient, the activity (containing all six bands) was found to sediment at about 11S (Fig. 6). This sedimentation rate indicates a molecular weight of about 250,000, in good agreement with the sum of the apparent M_r of the six subunits (236,000) as estimated by electrophoresis in NaDodSO_4 /polyacrylamide gels.

The only biochemical characterization of the translocation activity that had previously been achieved is the observation that it can be inactivated by *N*-ethylmaleimide and, therefore, most likely requires a sulfhydryl group(s) for activity (8). We inactivated our purified preparation with *N*-[^3H]ethylmaleimide under conditions that had previously been shown (8) to inactivate the salt- or trypsin-extracted activity (Fig. 7, bar D vs. bar E). An autoradiograph of the inactivated preparation reveals that the M_r 68,000 and the 9000 bands were labeled intensely, the M_r 54,000 band was labeled less efficiently, and the M_r 19,000 band was labeled to trace amount. No label was detected in the M_r 72,000 and 14,000 bands.

DISCUSSION

We have described here the purification of a protein component involved in the cotranslational translocation of nascent presecretory proteins across the membrane of the ER. This component is associated with the ER membrane of dog pancreas and can be extracted from the membranes by high ionic strength buffers (7, 8), thereby largely depleting the vesicles of their

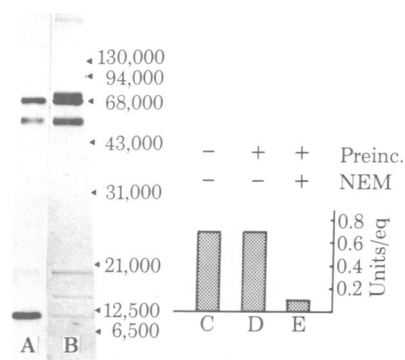


FIG. 7. Labeling of the ω -aminopentyl-agarose eluate with N - ^{3}H ethylmaleimide. ω -Aminopentyl-agarose eluate (500 μl) was salt-exchanged on a 2.5-ml Sephadex G-25 column into 1 ml of 10 mM sodium phosphate buffer (pH 6.5) containing 0.01% Nikkol and 150 mM KOAc. Aliquots (475 μl) were then incubated in the absence (-) and in the presence (+) of 237.5 nmol (125 μCi ; 1 Ci = 3.7×10^{10} becquerels) of N - ^{3}H ethylmaleimide (NEM) for 30 min at 25°C (8). Dithiothreitol (9.5 μl of 0.5 M) was added to terminate the reaction. Aliquots (50 μl) were then salt-exchanged on 250- μl Sephadex G-25 superfine columns into buffer B (containing 0.01% Nikkol) and were assayed for translocation activity (see Fig. 2) (bars C, D, and E). Aliquots (425 μl) of the incubated samples were Cl_3CCOOH -precipitated and subjected to electrophoresis in NaDodSO_4 . The gel lane containing the N - ^{3}H ethylmaleimide-treated aliquot was fluorographed (lane A), whereas the gel lane containing the nontreated aliquot was stained with Coomassie blue (lane B).

translocation activity. Reconstitution of the translocation activity by adding salt extract back to the extracted vesicles (7, 8) provided a convenient assay to monitor the purification of the active component present in the extract. We found that hydrophobic chromatography of the ribosome-depleted salt extract allowed essentially a one-step purification of the active protein component.

When analyzed by polyacrylamide gel electrophoresis in NaDodSO_4 , the purified protein was composed of six polypeptides. Because these six polypeptides were not separated from each other by several nondenaturing chromatographic procedures, they most probably form a complex with a molecular weight of about 250,000, as judged by sedimentation analysis and summation of the estimated size of the individual subunits. However, we cannot rule out the possibility that one or more of the six polypeptides become associated with the activity during isolation.

The mode of interaction of the complex with the membrane and its relationship to a functionally equivalent activity that can be extracted (even at low salt concentration) after limited proteolysis of the membranes (6, 8) remain to be established. However, a few conjectures on its possible mode of action can be made, based on its quantitatively estimated relationship to the number of translocated chains, its labeling behavior with its inhibitor N -ethylmaleimide, and its observed interactions with hydrophobic moieties.

We have estimated (data not shown) that 3 to 10 fmol of PRL are translocated per round of translation in 25 μl of our *in vitro*

assay containing 1 eq of RM. We furthermore estimated (from the Coomassie blue staining intensity) that 1 eq of purified complex consists of about 20 fmol. Therefore, these estimates suggest a stoichiometric relationship within one order of magnitude between the number of complexes and the number of translocated chains. Consequently, the complex is likely to be required stoichiometrically rather than catalytically for each chain translocation event.

Incubation with N - ^{3}H ethylmaleimide labeled two of the six subunits intensely under conditions that abolish its activity. Because a sulfhydryl group requirement for the activity of the unfractionated salt extract has been demonstrated (8), these results suggest that the sulfhydryl group(s) in question locate to either one or two subunits of the complex.

The purification was facilitated by the observation that the activity can be stabilized and, in fact, can be reactivated by low concentrations of nonionic detergent. This permitted quantitation of the activity at each step of the purification. If one takes into consideration that the complex is essentially the only component of the salt extract that interacted with the hydrophobic matrix of the ω -aminopentyl-agarose, it is conceivable that the exposed hydrophobic region of the complex is important in the recognition of the hydrophobic portion of the signal sequences or, alternatively, in anchoring of the complex to the hydrophobic core of the lipid bilayer.

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