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Topology and quaternary structure of pro-sucrase/isomaltase and final-form sucrase/isomaltase

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Pig sucrase/isomaltase (EC 3.2.1.48/10) was purified from intestinal microvillar vesicles prepared from animals with and without pancreatic-duct ligation to obtain the single-chain pro form and the proteolytically cleaved final form respectively. The purified enzymes were re-incorporated into phosphatidylcholine vesicles and analysed by electron microscopy after negative staining. The two forms of the enzyme were observed as identical series of characteristic projected views that could be unified in a single dimeric model, containing two sucrase and two isomaltase units. This shows a homodimeric functional organization similar to that of other microvillar hydrolases. The bulk of the dimer was separated from the membrane by a maximal gap of 3.5 nm, representing a junctional segment connecting the intramembrane section of the anchor to the catalytically active domain of sucrase/isomaltase. The enzyme complex protrudes from the membrane for a distance of up to 17 nm. From charge-shift immunoelectrophoresic studies of hydrophilic pro-sucrase/isomaltase and from electron microscopy of reconstituted pro-sucrase/isomaltase, there was no evidence to suggest the presence of anchoring sequences between the sucrase and isomaltase subunits.

INTRODUCTION

The microvillar membrane of enterocytes harbours a group of integral hydrolases, comprising glycosidases and peptidases, as major components (for reviews see Kenny & Maroux, 1982; Norén *et al.*, 1986). These are synthesized and transported to the microvillar surface in various precursor forms and are proteolytically cleaved to their final mature forms (for review see Danielsen *et al.*, 1984). Thus pig sucrase/isomaltase (EC 3.2.1.48/10), a single-chain stalked intrinsic microvillar membrane polypeptide of apparent M_r 265000 (Sjöström *et al.*, 1980) (commonly referred to as 'pro-SI'), is cleaved at the microvillar surface by intestinal proteinases into two subunits of M_r 150000 (isomaltase) and M_r 140000 (sucrase) remaining associated with each other, the whole complex known as final SI. The final SI enzyme complex is attached to the membrane via a single highly hydrophobic *N*-terminal anchor on the isomaltase subunit (Frank *et al.*, 1978; Brunner *et al.*, 1979). Both the pro and final forms can be isolated after detergent solubilization, pro SI being isolated either from rat foetal intestinal explants (Hauri *et al.*, 1980) or from pigs having had their pancreatic duct ligated before slaughter (Sjöström *et al.*, 1980). Intracellular transport and processing during biosynthesis have been studied in detail in various systems (for review see Danielsen *et al.*, 1984). Although models have been proposed for the translational insertion of pro-SI into the membrane (Semenza *et al.*, 1983), the question whether hydrophobic anchoring sequences additional to the *N*-terminal anchor exist in pro-SI has not yet been resolved.

In the present study the quaternary structure and membrane topology of pro-SI and final SI are analysed, with a view to further understanding of the biosynthesis and microvillar expression of SI.

MATERIALS AND METHODS

Unless stated otherwise, all procedures were carried out at 4 °C.

Materials

Phenyl-Sepharose was obtained from Pharmacia, Uppsala, Sweden. Egg-yolk L-α-phosphatidylcholine and β-octyl glucopyranoside were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Phosphatidyl[*N*-*Me*-^{14}C]choline was a product of The Radiochemical Centre, Amersham, Bucks, U.K., and Aqualuma Plus scintillant was purchased from Lumac BV, Schaesburg, The Netherlands. DE-52 DEAE-cellulose was obtained from Whatman Biochemicals, Maidstone, Kent, U.K. Sources of other chemicals were as previously reported (Sjöström *et al.*, 1978, 1980), and, if not otherwise stated, of analytical grade. Pig small intestines were kindly donated by the Department of Experimental Pathology, Rigshospitalet, Copenhagen, Denmark.

Purification of Triton X-100-solubilized final SI and pro-SI

Pig intestinal pro-SI and final SI were prepared by immunoadsorbent chromatography as earlier described, pro-SI being prepared from a pig whose pancreatic duct had been ligated 3 days before it was killed (Sjöström *et al.*, 1980). Aprotinin (2.8 μg/ml) was present throughout the purification. For electron microscopy and reconstitution studies, the eluted fractions from the anti-SI–Sepharose column were made 1 mM with respect to EDTA (sodium salt). This additional proteinase inhibitor was implemented following initial experiments, where the purified SI preparations were found to be unstable, with extensive conversion of the amphiphilic form into a hydrophilic form. This conversion was not inhibited by

Abbreviations used: SI, sucrase/isomaltase (EC 3.2.1.48/10); pro-SI, single-chain-polypeptide sucrase/isomaltase; final SI, final-form, proteolytically processed, sucrase/isomaltase.

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2.8 μg of aprotinin/ml, 1 mM-iodoacetamide or 0.1 mM-phenylmethanesulphonyl fluoride.

Preparation of hydrophilic pro-SI

Some preparations of Triton X-100-solubilized pro-SI (prepared in the absence of EDTA) were found by charge-shift electrophoresis to contain sizeable amounts of a hydrophilic form of the enzyme, in addition to the amphiphilic form. This spontaneously formed 'hydrophilic pro-SI' was purified as follows. To remove detergent, 113 nkat of such a pro-SI preparation (in 2 mM-Tris/HCl buffer, pH 8.0, containing 0.1% Triton X-100) was applied to a 1 ml column of DE-52 DEAE-cellulose, equilibrated in 50 mM-Tris/HCl buffer, pH 8.0. After washing of the column, the activity was eluted by the same buffer supplemented with 0.5 M-NaCl. Separation of amphiphilic and hydrophilic pro-SI was then carried out by applying the sample to a 1 ml phenyl-Sepharose column, equilibrated in 50 mM-Tris/HCl buffer, pH 8.0, containing 0.5 M-NaCl. After being washed, the column was eluted with 50 mM-Tris/HCl buffer, pH 8.0, containing 2% Triton X-100. Fractions containing peak sucrase activity from the wash (hydrophilic) and eluted (amphiphilic) fractions were analysed by charge-shift immunoelectrophoresis and SDS/polyacrylamide-gel electrophoresis as described in Figure legends.

Detergent exchange

Triton X-100 in the SI preparations was replaced by β-octyl glucopyranoside (Helenius *et al.*, 1977), by using density-gradient centrifugation. Purified SI (up to 400 μg) was applied on top of a linear sucrose gradient (10.5 ml, 0.46–0.8 M-sucrose in 50 mM-Tris/HCl buffer, pH 7.5, containing 30 mM-β-octyl glucopyranoside, and normally, 1 mM-EDTA) layered on a 500 μl cushion of 2.2 M-sucrose in the same buffer. After centrifugation (284000 $g_{max.}$ for 20 h at 4 °C, Beckman SW 40 rotor), fractions were collected from the bottom of the gradient and assayed for sucrase activity.

Reconstitution of SI

The β-octyl glucopyranoside method was used (Helenius *et al.*, 1977). Typically, a film of phosphatidylcholine (1000 μg) containing a trace of [^{14}C]phosphatidylcholine was dried under vacuum for 1 h to remove all traces of solvent, and then redissolved in 1.0 ml of 30 mM-β-octyl glucopyranoside in 50 mM-Tris/HCl buffer, pH 7.4. SI (up to 200 μg) in 50 mM-Tris/HCl buffer, pH 7.4, containing 30 mM-β-octyl glucopyranoside and 0.6 M-sucrose was then added and mixed thoroughly at room temperature, and made 1 mM with respect to both $MgCl_2$ and $CaCl_2$. The mixture was dialysed overnight against 50 mM-Tris/HCl buffer, pH 7.4, containing 292 mM (10%, w/v)-sucrose at 4 °C. The dialysis was continued for a further 24 h against the same buffer without sucrose.

Purification of reconstituted SI

A 5 ml or 10 ml linear sucrose gradient (0.1–1.58 M) in 25 mM- or 50 mM-Tris/HCl buffer, pH 7.4, was prepared, and the crude reconstituted material was layered on top, after concentration, if necessary, by ultrafiltration (Amicon PM-10 filter). The tubes were centrifuged in a Beckman SW 50.1 rotor (~273000 $g_{max.}$) for 18 h, or a Beckman SW 40 rotor (284000 $g_{max.}$) for 18 h at 4 °C. Fractions were collected from the bottom and analysed for sucrase activity, ^{14}C radioactivity and refractivity.

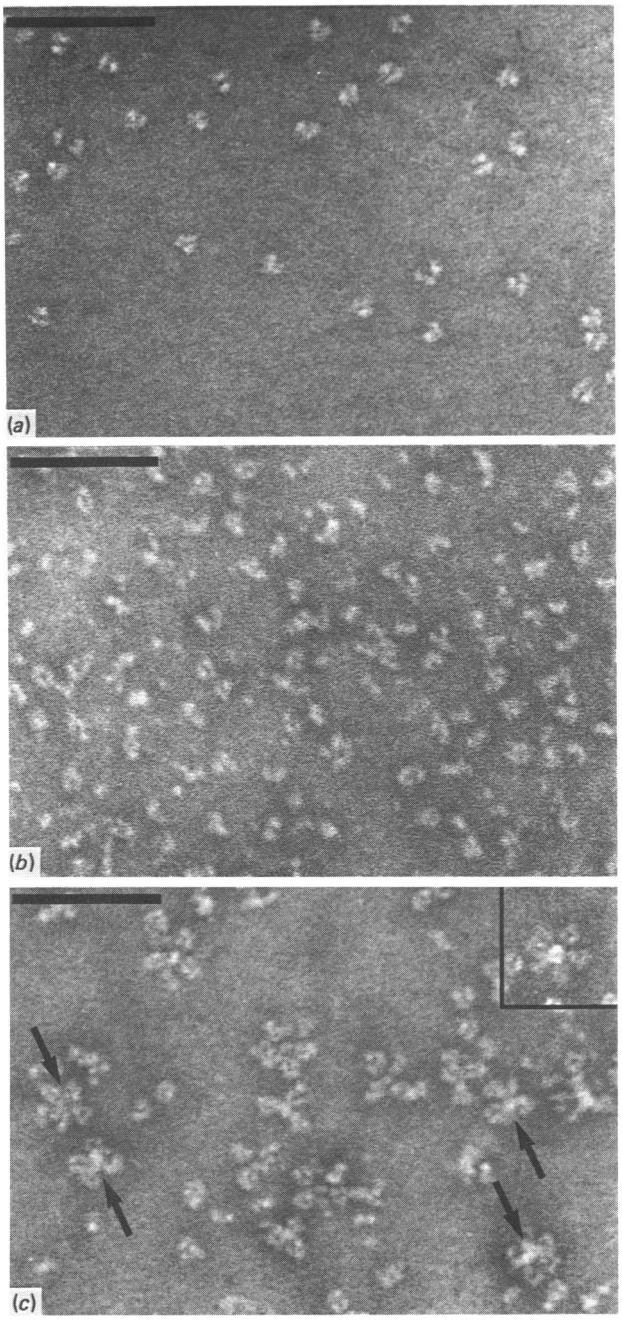

Fig. 1. Electron micrographs of negatively stained free pro-SI and final SI

(*a*) Free pro-SI seen in a crude reconstitution mixture. The protein was approx. 90% amphiphilic by charge-shift electrophoresis. Several apparently dimeric symmetric structures are seen. (*b*) Final SI solubilized by 1% Triton X-100. (*c*) Final SI freed of detergent by dialysis. Arrows and box indicate micellar structures composed of up to six units. Single copies of these units are seen scattered between the micelles. Sodium silicotungstate negative staining was used. Scale bars indicate 100 nm.

Fractions from the lipid peak were prepared for electron microscopy.

Electron microscopy

Samples for electron microscopy were freed of sucrose by dialysis, first against 50 mM-Tris/HCl buffer, pH 7.4,

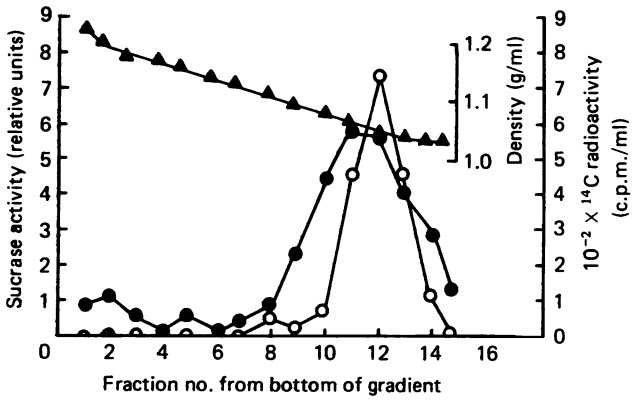

Fig. 2. Density-gradient-centrifugation analysis of reconstituted final SI

Experimental details are given in the text. ●, Sucrase activity; ○, [^{14}C]phosphatidylcholine radioactivity; ▲, density. Sucrase activity is expressed in relative units, rather than absolute activity, because of the presence of 25 mM-Tris in the gradient.

containing 30 mM-sucrose, then against 50 mM-Tris/HCl buffer, pH 7.4, containing 0.02% NaN_3. Samples were concentrated, if necessary, by ultrafiltration (Amicon PM 10 filter). Negative staining was carried out as described by Hussain *et al.* (1981). Specimens were examined at 80 kV in a JEOL 100-CX electron microscope equipped with an ultra-high-resolution objective-lens pole piece (coefficient of spherical aberration 0.7) using a 200 μm condenser and a 60 μm objective-lens aperture. Images at 60000–80000 times primary magnification were recorded on Agfa 23D56 emulsion at radiation doses of 35–60 $e^-/Å^2$ of specimen with the use of a minimum-dose technique.

Preparation of SI quaternary-structure model

A three-dimensional model of SI at scale $1:2 \times 10^6$ was shaped in transparent grey-toned Perspex rod, softened in a hot air stream. The model was constructed to give best fit with the range of random projections of SI molecules seen in negative staining of reconstituted SI. The model was photographed submerged in dibutyl phthalate, which has precisely the refractive index of Perspex. Surface reflections are thereby avoided, and the photographic image represents the projected mass thicknesses in the model. After contrast reversal of the negative film, prints were made at a reduced scale to match the negative staining images in dimensions and contrast.

Other methods

Sucrase activity was assayed with sucrose as substrate as described previously (Dahlqvist, 1968; Sjöström *et al.*, 1980). Polyacrylamide-gel electrophoresis was carried out on 11.6% slab gels containing 0.1% SDS by the procedure of Laemmli (1970), and stained with Coomassie Brilliant Blue R-250. ^{14}C radioactivity was measured in a Nuclear-Chicago mark II scintillation counter with Aqualuma Plus as scintillant. Density of fractions from sucrose gradients was determined by refractometry. Protein determinations were performed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

RESULTS

Electron microscopy of free and reconstituted SI

Electron micrographs of negatively stained preparations of free pro-SI and final SI solubilized by detergent are shown in Figs. 1(*a*) and 1(*b*). The preparations appear at first to be quite heterogeneous, because the molecules are seen in random projections. However, several of the profiles are repeated and some of them suggest a symmetric dimeric structure. When detergent was removed through dialysis of lipid-free preparations of the enzyme, discrete micellar protein structures formed for both forms of the enzyme, as shown for final SI in Fig. 1(*c*). The micelles were composed of up to six structural units, each of which closely resembled the dimeric enzyme complexes identified after membrane reconstitution of the enzyme, as described below.

A sucrose-density-gradient analysis of reconstituted final SI is shown in Fig. 2. In this particular experiment more than 90% of the sucrase activity accompanied the lipid peak; the remainder sedimented in the bottom fractions, not associated with appreciable amounts of lipid. In other experiments the reconstitutions were less quantitative, and sometimes only 25–30% of the enzyme activity accompanied the lipid peak, even though the initial lipid/protein ratio was the same, and amphiphilicity of the enzyme was ascertained before and after the reconstitution procedure. Similar unexplained variations in effectiveness of reconstitution were found between different preparations of pro-SI. A moderate downward displacement of the enzyme activity peak relative to the lipid peak was commonly observed, and this is explained by the electron-microscopical observation of marked varations in the number of enzyme molecules incorporated into liposomes even of the same size. The hydrophilic forms of SI could not be reconstituted into liposome (results not shown).

Electron micrographs of a preparation of negatively stained reconstituted pro-SI, recovered from the lipid peak fractions of a sucrose gradient, are shown in Fig. 3. The liposomes covered a size range from 40 nm up to 200 nm, the larger liposomes often appearing ruptured by the staining procedure. The incorporated proteins accumulated along the edges of the liposomes that are flattened during staining. The majority of the protein structures projected to a distance of 15–17 nm from the membrane edge. These 15–17 nm projections presented a spectrum of profiles ranging from 9 nm-broad rod-like profiles, predominating at sites where the proteins were lined up in close stacking, to 14 nm-broad U- and ring-shaped profiles, predominating where the proteins were more spaced along the liposomal edges. Between these extremes a number of consistently repeated intermediate forms were found. The spectrum of profiles of the 15–17 nm projections is shown in Figs. 4(*a*) and 4(*c*) for pro-SI and final SI respectively. The two forms of the enzyme presented basically the same profiles. Apart from their outer contours, each of the various profiles was characterized by a substructure, represented in the micrographs by different relative contrast densities corresponding to differences in thickness of the projected mass.

The visible mass of the membrane-bound SI dimers was consistently separated from the liposomal membrane by a stain-filled gap. This gap can be assumed to represent an extramembranous junctional segment of the SI

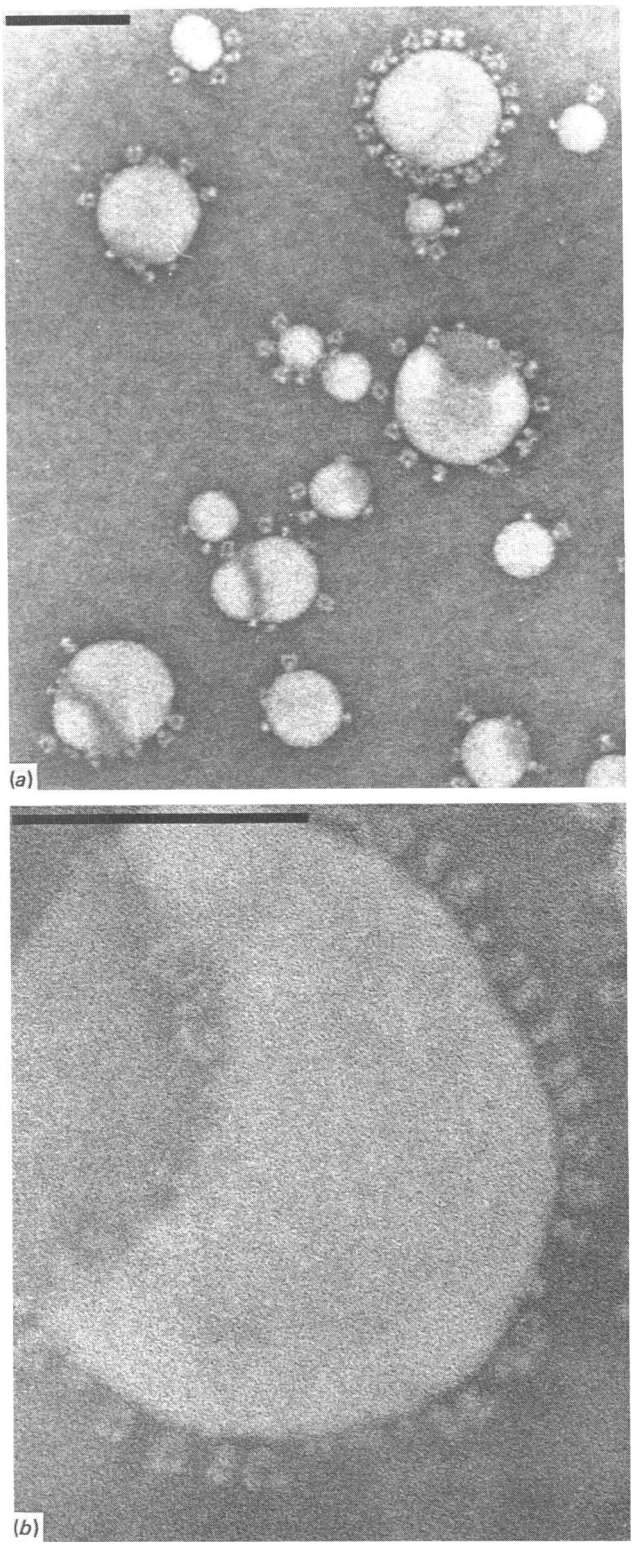

Fig. 3. Electron micrographs of reconstituted pro-SI

Pro-SI, recovered from the lipid peak of a sucrose-density-gradient centrifugation. The reconstituted enzyme presents a spectrum of profiles along the rim of the liposomal membranes. Where closely spaced, as to the right in (*b*), the profiles are narrower than when they are spaced out, as towards the bottom of (*b*). Sodium silicotungstate negative staining was used. Scale bars indicate 100 nm.

anchoring peptide. The gap distances for both pro-SI and final SI were in the range 0–3.5 nm. The broadest gaps can be taken to provide the best estimate of the true lengths of the junctional segment, because part of the segment may be shielded by co-projection with the rim of the liposomal membrane (Hussain, 1985).

Analysis of hydrophilic pro-SI

Preparations of pro-SI, in which both amphiphilic and hydrophilic forms were present, showed only two very closely spaced high-M_r bands (M_r approx. 260000) on SDS/polyacrylamide-gel-electrophoretic analysis. These two polypeptides could be separated by chromatography on phenyl-Sepharose after removal of Triton X-100, as monitored by sucrase activity measurements (results not shown). Fig. 5 shows charge-shift immunoelectrophoresis of the non-adsorbed and the Triton X-100-eluted peaks, demonstrating that they were hydrophilic and amphiphilic respectively. Fig. 6 shows SDS/polyacrylamide-gel-electrophoretic analysis of the same fractions from such a chromatography, demonstrating that the two forms have a very similar high M_r (approx. 260000), with the hydrophilic form (lane a) having a slightly greater mobility. The existence of a hydrophilic form of pro-SI having only a slightly lower M_r than the amphiphilic form indicates that the hydrophobic segment(s) of the amphiphilic form that are capable of binding the detergent micelles are small and located at or near the termini, excluding the possibility of a functional anchoring segment accessible to the detergent micelles between the I and S domains.

DISCUSSION

Both pro-SI and final SI are present predominantly as dimers in the membrane, and have the same topology

A model accounting for all the characteristics of the observed profiles of reconstituted SI was considered. A screw-symmetric structure consisting of two identical curved rods, 20 nm long and 4–5 nm in diameter, twisted about 45° and joined at one end (Fig. 7) was found to account satisfactorily for all the symmetric 15–17 nm projections observed. Fig. 4(*b*) shows this model rotated through 180° in 15° steps for comparison with the spectrum of enzyme profiles in Figs. 4(*a*) and 4(*c*). By using a partial specific volume of 0.739 for the protein, as determined by Mosimann *et al.* (1973) for rabbit SI, the dimensions of the rods correspond to an M_r in the range 215000–340000 for each of the rods, for minimum and maximum dimensions. These determinations are not used to give a precise M_r value, but to give a range of M_r values to exclude the possibility that the individual rods consist of only one S or I subunit (M_r about 140000) or that the single rods represent a dimer of SI (M_r about 560000). The proposed model therefore implies that each of the 15–17 nm projections accommodates two SI units joined in a dimeric entity.

In preparations of reconstituted pro-SI 75% of all the incorporated protein profiles conformed to the model (analysed on 100 liposomes), and for final SI 70%. Some of the remaining profiles may represent monomeric SI rods, others may be isolated I subunits attached to the membrane (Skovbjerg *et al.*, 1979) or degradation products of SI. A collection of such 'aberrant' profiles found in preparations of reconstituted pro-SI and final SI is shown in Figs. 4(*d*) and 4(*e*) respectively. In

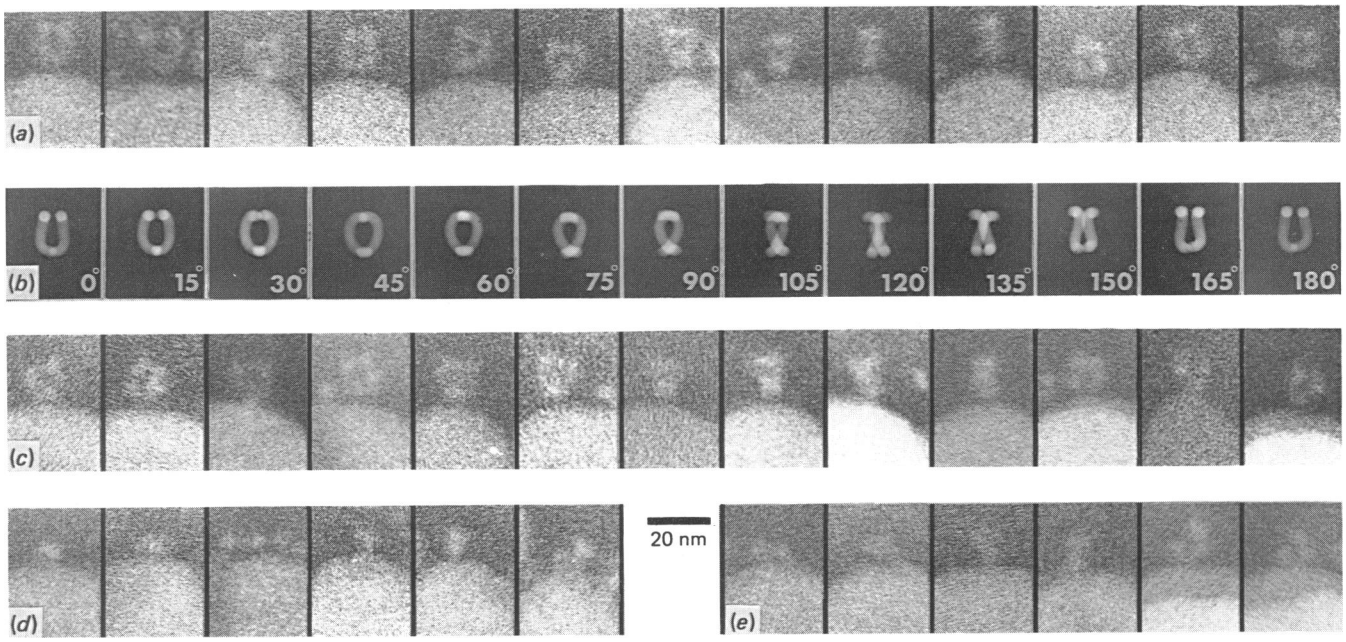

Fig. 4. Molecular profiles of reconstituted SI

(*a*) and (*c*) show the spectrum of molecular profiles seen in preparations of reconstituted pro-SI and final SI respectively. Basically the same profiles are found in either of the preparations. (*b*) shows projected views of the suggested model, rotated through 180° in 15° steps. The model was shaped in transparent toned Perspex to allow comparison with projected mass thicknesses in the negative stainings. (*d*) and (*e*) show the major types of 'aberrant' profiles in preparations of reconstituted pro-SI and final SI respectively. Such profiles accounted for 25–30% of the total number of profiles in the preparations. Sodium silicotungstate negative staining was used.

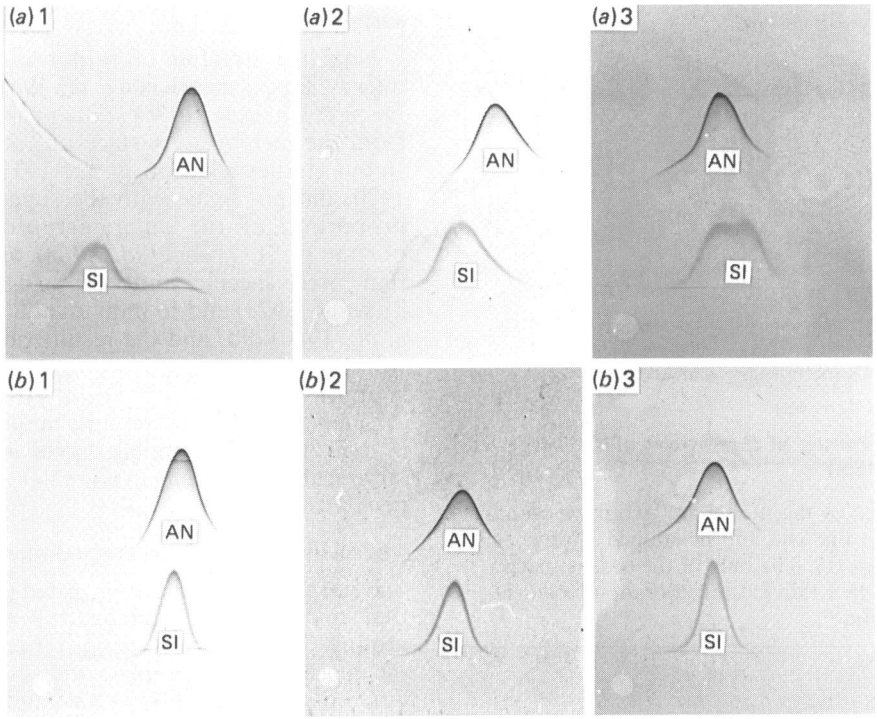

Fig. 5. Charge-shift crossed immunoelectrophoresis of fractions from phenyl-Sepharose chromatography

Charge-shift immunoelectrophoresis was carried out by the procedure of Bhakdi *et al.* (1977). A 1 μg portion of SI from (*a*) Triton X-100-eluted peak fraction, or (*b*) non-adsorbed peak fraction, was applied on each plate. To all samples 0.3 μg of hydrophilic aminopeptidase N (AN) (Sjöström *et al.*, 1978) was added as an internal reference (Sørensen *et al.*, 1982). The detergents used were as follows: 1, 0.0125% cetyltrimethylammonium bromide plus 0.1% Triton X-100; 2, 0.1% Triton X-100; 3, 0.2% sodium deoxycholate plus 0.1% Triton X-100.

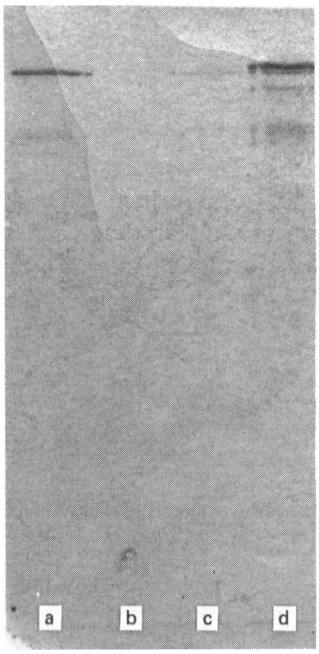

Fig. 6. SDS/polyacrylamide-gel electrophoresis of amphiphilic and hydrophilic pro-SI

Lane a, non-adsorbed fraction from phenyl-Sepharose chromatography, corresponding to series (*b*) in Fig. 5. Lanes b and c, wash fractions. Lane d, Triton X-100-eluted fraction, corresponding to series (*a*) in Fig. 5.

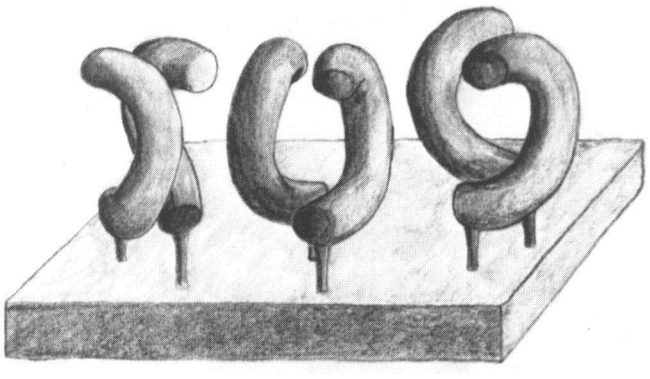

Fig. 7. Diagrammatic drawing of three views of the suggested model for dimeric SI

The principal features of the model are two curved and twisted rods joined at one end and positioned relative to a plane of screw symmetry. The bulk of the rods is spaced from the membrane by a thin extramembranous segment of the anchoring peptide.

particular for preparations of final SI, profiles suggestive of degraded forms were common, like the two frames to the right in Fig. 4(*e*), which may represent SI dimers that had lost the outer half of one of the rods.

Dimerization was not dependent on protein concentration in the membrane, as liposomes containing only one complex showed the dimeric enzyme structure. Previous hydrodynamic studies of reconstituted final rabbit SI suggested that monomers were largely present in the membrane, together with some dimers (Spiess *et al.*, 1981). The dimeric structure of the free, papain-treated, final SI (lacking the hydrophobic anchor) has previously been indicated by electron microscopy, the extent of dimerization depending on protein concentration (Nishi *et al.*, 1968). Mosimann *et al.* (1973) also inferred that some interaction exists between two papain-solubilized SI units, and hydrophilic pig SI has been shown to have a tendency to dimerize, having a larger M_r than expected (Sjöström *et al.*, 1983).

Our studies show that reconstituted pig pro-SI and final SI have basically the same quaternary structure, being indistinguishable by electron microscopy. For final-form, processed, SI, the proposed model can also be taken to represent the situation in the microvillar membrane *in vivo*. For pro-SI, however, extrapolation of the model to the situation *in vivo* is dependent on the absence of additional anchoring segments, as these would impose constraints *in vivo* during co-translational insertion into the endoplasmic reticulum. However, as shown here, there is no positive evidence for an anchoring sequence positioned between the two subunits. In addition, no 'twinned' liposomes, with a single complex spanning two liposomes, i.e. with two sites of membrane insertion per complex, or aggregates of liposomes were seen. This would be expected in the presence of free *C*-terminal hydrophobic anchors. The model shown in Fig. 7 is thus probably true both for newly synthesized pro-SI in the intracellular membranes, undergoing final processing, and for pro-SI at the microvillar surface.

Final SI and pro-SI project outwards from the membrane surface

Negative staining of rabbit and hamster intestinal microvillar vesicles (Nishi *et al.*, 1968; Benson *et al.*, 1971; Nishi & Takesue, 1978) show projections extending out from the membrane surface up to a distance of 17 nm. Initial studies (Benson *et al.*, 1971; Gitzelmann *et al.*, 1970) did not agree with the suggestion that at least a proportion of the outermost projections represent a portion of SI (Nishi *et al.*, 1968). However, later studies that used specific antibodies to rabbit SI (Nishi & Takesue, 1978) and to individual S and I subunits (Nishi *et al.*, 1980) did, and the results obtained in the present work, for reconstituted pro-SI and final SI, lend further support.

Stacking of S and I subunits in this economical manner is logical in an organelle involved in digestion on a large scale, and results in a greater number of active sites per unit membrane area.

Microvillar enzymes are homodimers

It has previously been suggested (Sjöström *et al.*, 1983) that microvillar hydrolases can be divided into two groups. The SI type is characterized as heterodimers in the sense of being dimers of two discrete functionally different enzyme units, i.e. asymmetric with respect to their hydrophilic domains, carrying two different active sites and with an anchor on only one subunit. The other group is represented by aminopeptidase N, characterized as a homodimer that is symmetric, with only one type of active site, and with an anchor on both subunits (Hussain *et al.*, 1981). Other proteins now classified in the latter group include dipeptidyl peptidase IV (EC 3.4.14.5)

(Hussain, 1985), endopeptidase 24.11 (EC 3.4.24.11) (Kenny *et al.*, 1983) and aminopeptidase W (Gee & Kenny, 1985). Our present results suggest that SI is in fact very similarly organized to aminopeptidase N, as a true dimer of two SI entities.

That pro-SI is present as a symmetric dimer may be of biosynthetic importance. For microvillar enzymes, membrane insertion occurs well before the newly synthesized enzymes appear in the microvillus (for review see Danielsen *et al.*, 1984), as first demonstrated by Hauri *et al.* (1979), and the enzymes are ultimately transported to the apical surface of this polarized cell in a membrane-bound state. After insertion into the endoplasmic reticulum, the enzymes could cluster within a specific membrane compartment before budding off to form transport vesicles, with the aid of receptor molecules. This process may be facilitated by the newly synthesized protein having two equal receptor-binding sites. It might be hypothesized that formation of a symmetric dimer is a general phenomenon for the microvillar hydrolases.

Note added in proof (received 16 May 1986)

The data presented in this paper suggesting the absence of an anchoring sequence between the S and I subunits and, indirectly, the absence of a *C*-terminal anchor in pro-SI are in agreement with results obtained from sequencing of the full-length cDNA clone for rabbit SI (Hunziker *et al.*, 1986).

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