Structural and Functional Correlates of Sucrase–α-Dextrinase in Intact Brush Border Membranes†

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ABSTRACT: The structure and catalytic function of rat intestinal sucrase–α-dextrinase (sucrase–isomaltase) were characterized in intact brush border membranes by differential denaturation in 1% SDS at 4, 37, 45, 55, and 100 °C, analysis by acrylamide electrophoresis, and subsequent renaturation by transfer to nitrocellulose and in situ analyses of immunoactivity and catalytic activity (immunoblotting and catalytic blotting). Both the sucrase and α-dextrinase activities were associated with two mature oligomers, with sucrase predominantly in a 250–260-kDa unit and dextrinase in a 330–350-kDa unit. While sucrase activity declined progressively in response to increasing temperature to 45 °C due to loss of active sites, α-dextrinase activity increased reciprocally (V_{max} +176%). Three principal monomeric products of postinsertional processing comprise the oligomers: α, 140 kDa, which carries the sucrase active site; β, 125 kDa, harboring the dextrinase active site; and γ, 110 kDa, produced by removal of 185 amino acid residues from the N-terminus of the α. Rather than being a simple hybrid dimer, membrane-associated sucrase–α-dextrinase appears to consist of two major oligomeric forms having complex structural associations that dramatically affect the availability of the active catalytic sites at the brush border membrane surface.

Sucrase–α-dextrinase (S–D),[1] a hybrid α-glucosidase expressed only in the brush border membrane of the small intestinal enterocyte, plays a key role in the final surface digestion of oligosaccharide nutrients in preparation for the assimilation of released monosaccharides (Conklin et al., 1975; Crane, 1977; Semenza, 1981; Kenny & Maroux, 1982). Initially synthesized as a large glycoprotein precursor, pro-sucrase–α-dextrinase (or P), in association with the endoplasmic reticulum and Golgi (Sjöström et al., 1980; Montgomery et al., 1981; Wacker et al., 1981; Danielsen, 1982; Naim et al., 1988) and directionally transported to the brush border surface membrane, the single-chain P undergoes postinsertional cleavage by luminal pancreatic proteases to a hybrid dimer with sucrase and α-dextrinase active sites residing on separate subunits (Quaroni et al., 1974; Gray et al., 1979; Sjöström et al., 1980; Hauri et al., 1980). The complex anchors in the membrane via an N-terminal hydrophobic segment of the larger (~140 kDa) α subunit (Spiess et al., 1982). The smaller (~125 kDa) β subunit, although lacking a transmembrane domain, remains noncovalently associated with its α partner on the luminal side of the brush border membrane.

Although the α subunit is usually assumed to carry the α-dextrinase active site and the β subunit the sucrase site (Brunner et al., 1979), this assignment is uncertain because it is based on separation of the subunits by stringent treatment of the native S–D hybrid after detergent solubilization, resulting in the loss of the vast majority of functional catalytic sites. Indeed, other studies have suggested that the larger α subunit carries the sucrase catalytic site and the β harbors the dextrinase site (Cogoli et al., 1973; Braun et al., 1975; Takesue et al., 1977). Also, despite the close association of the α and β subunits, each presumably carrying a single active site directed specifically at sucrose or the α-dextrin intraluminal substrates, the usual activity ratio of sucrase to dextrinase (S/D) of 1.0–1.2 in jejunum decreases appreciably in distal ileum to 0.4–0.6 (Goda et al., 1988) in association with a loss of the α subunit (S. J. Najjar, J.-S. Zhu, L. A. Schering, and G. M. Gray, unpublished results), a change that would have been expected to increase rather than decrease the S/D ratio, presuming the α subunit carries the dextrinase active site.

In addition to the uncertain assignment of the active sites, there are several structural and functional features that bring into question the presumed one-step postinsertional cleavage of the single-chain precursor and the subsequent dimeric reassociation of the released fragments. First of all, the mass sum of the α and β subunits (~265 kDa) is greater than that of their precursor after modification by pancreatic proteases in all mammalian species studied including rat, rabbit, pig, and human (Takesue et al., 1977; Sjöström et al., 1980; Hu et al., 1987; Naim et al., 1988). For instance, human S–D in organ culture displayed α and β subunits with a combined mass 20–30 kDa greater than the precursor, regardless of whether the native or deglycosylated enzyme was examined (Naim et al., 1988). Second, several transient molecular species intermediate in mass between the P and the final α and β products have been observed when the radioactive P in isolated brush border vesicles was converted to the authentic hybrid dimer under physiologic conditions by pancreatic proteases (Shapiro et al., 1991). Thus, a single-step postinsertional cleavage of the precursor to its hybrid dimer appears to be unlikely. Finally, in addition to the subunits comprising

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[1] Abbreviations: S–D, sucrase–α-dextrinase (also commonly called sucrase–isomaltase); P, single-chain precursor of S–D; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; PVDF, poly(vinylidene difluoride); NC, nitrocellulose membrane sheet; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; CV, coefficient of variation; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; PMSF, phenylmethanesulfonyl fluoride.

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the putative S–D dimer, a third smaller species, γ, has been observed in SDS electrophoresis in several laboratories (Montgomery et al., 1981; Hauri et al., 1982; Hu et al., 1987; Naim et al., 1988; Yeh et al., 1989). While the γ species may constitute a proteolytic artifact, its structure and relationship to the S–D oligomer have not been explored. Hence, the postinsertional processing of the S–D precursor as well as the structural and functional relationships of the final subunits (α, β, and possibly γ) to their precursor appears much more complex than generally assumed. Some of these findings may relate to artifacts created during the membrane protein solubilization, whether by exogenous proteases such as papain or by endogenous membrane proteases that may cleave domains uncovered by the nonionic detergents employed for the solubilization process.

To correlate the relationship of structure and catalytic specificity of the S–D complex, we have examined purified intact brush border membranes (not previously exposed to papain or nonionic detergents) by incubation in SDS at varying temperatures. This approach has allowed identification of molecular species both by immunolocalization and by a new catalytic blotting technique on the same electrophoretic blot. The results reveal that the S–D hybrid, rather than being a simple dimer, exists in two oligomeric forms consisting of combinations of the α, β, and γ subunits strategically interrelated so that the sucrase catalytic site, a component of the larger α subunit, appears to sterically regulate the availability of the α-dextrinase site on the β subunit. The γ subunit proved to be derived from near the N-terminus of the P precursor probably by modification of the α subunit.

Experimental Procedures

Chemicals and Reagents. Sucrose was obtained from Pfanstiehl. 3-Amino-9-ethylcarbazole, BSA, 4-chloro-1-naphthol, D-mannitol, D-sorbitol, glucose oxidase (type V), glycerol, peroxidase (type II), protease inhibitors (antipain, chymostatin, leupeptin, pepstatin A, ovomucoid, and PMSF), and Tris were from Sigma. Isomaltose was a gift of Dr. Allene Jeanes. Ultrapure urea and SDS were obtained from BDH. Agarose, ammonium persulfate, Coomassie Brilliant Blue R250, horseradish peroxidase labeled goat anti-rabbit antibody, nitrocellulose membranes (NC), polyacrylamide, and protein standards were from Bio-Rad. DTT was a product of Boehringer Mannheim Biochemicals. Acetone, acetic acid, calcium chloride, EDTA, hydrogen peroxide, and sodium acetate were from JT Baker. Emulphogene BC-$_{720}$ was a product of GAF.

Animals and Brush Border Membrane Preparation. Male adult Sprague-Dawley rats (250–300 g) were housed in a central animal facility and fed with a commercial lab rat chow. After being allowed only water for 10 h, rats were rendered unconscious in a CO_2 atmosphere and killed by a blow on the head. Jejunal mucosa (10–15-cm length) was scraped with 25 × 75 mm microslides, and the brush border membrane vesicles were purified by calcium precipitation in a protease inhibitor mixture (antipain, chymostatin, leupeptin, pepstatin A, and ovomucoid, each 25 μg/mL, and PMSF, 1 mM) or sorbitol gradient (25–60%) equilibrium centrifugation as detailed previously (Ahnen et al., 1982; Nguyen et al., 1987).

Enzyme Assays. Sucrase and α-dextrinase activities were determined in a reaction mixture of 100 μL as previously described (Conklin et al., 1975). For hydrolytic assays of S–D bound to nitrocellulose membranes, the reaction volume was increased to 200–300 μL.

Differential and Sequential Denaturation of S–D in Intact Brush Border Membrane in SDS. The pure, intact (nonsolubilized) brush border vesicles, prepared as detailed above, were taken up in 1% SDS, 63 mM Tris (pH 6.8), 10 mM DTT, 1.0 mM EDTA, 1.0 mM PMSF, and 30% sorbitol and incubated at various temperatures (4, 37, 45, or 55 °C), either individually or in sequence, for 30 or 60 min or fully denatured at 100 °C for 5 min. After in vivo intestinal labeling with [^{35}S]methionine, recovery of the brush border proteins from acrylamide gels was 69–81% of the radioactivity applied after prior exposure of the sample to SDS throughout the temperature range examined (Table I).

Table I: Recovery of ^{35}S-Labeled BB Proteins and Immunoreactive S–D Protein after Electrophoresis and Blotting

temp (°C)	time (min)	radioact.[a] % of added	radioact.[a] f of 4 °C	integral density of immunoblot[b] ×10^{-3}	integral density of immunoblot[b] f of 4 °C
4	30	69	1.00	71	1.00
37	30	75	1.07	66	0.93
37	60	NA[c]	NA	70	0.99
45	30	81	1.17	67	0.94
55	30	75	1.09	79	1.11
55	60	NA	NA	81	1.14
100	5	81	1.17	17	0.24
		CV = 0.06		CV = 0.09[d]	

[a] An equal quantity of [^{35}S]methionine-labeled intact BB membranes was differentially exposed to 1% SDS at the given temperature and time and subjected to SDS gel electrophoresis. After removal of the stacking gel, individual gel lanes were cut, and the radioactivity was determined. CV, coefficient of variation. [b] An equal quantity of intact BB membranes was differentially denatured in 1% SDS–30% sorbitol, subjected to gel electrophoresis, and transferred to an NC membrane sheet. S–D protein was detected by immunoblotting, and individual lanes for each different denaturation conditions were scanned by densitometry. [c] Not assayed. [d] CV for 4, 37, 45, and 55 °C samples.

Sucrase–α-Dextrinase Protein Purification. For purification of the individual α, β, and γ subunits, brush border membrane vesicles were solubilized by 2% Emulphogene BC-$_{720}$ in 0.01 M phosphate buffer, pH 6.1, or allowed to autolyze at 37 °C for 40 min. The 100 000 g × 1 h supernatant was applied to a DEAE-TSK-650M anion-exchange column (1 × 13 cm) that had been equilibrated in 0.005 M phosphate buffer (pH 6.5) containing 0.02% BC-$_{720}$. Brush border proteins were eluted in 1-mL fractions with a 50-mL linear gradient of 0–0.5 M NaCl and assayed for sucrase and aminooligopeptidase (AOP). The S–D peak was pooled and further purified by HPLC on a Zorbax (DuPont) GF-450 gel filtration column (0.94 × 25 cm) in 0.2 M phosphate, pH 6.5, containing 0.02% BC-$_{720}$ and 0.02% NaN_3. The final pure S–D was homogeneous on SDS–acrylamide gel electrophoresis after 100 °C × 5 min exposure to 1% SDS, revealing the α, β, and γ species (140, 125, and 110 kDa, respectively). Further evidence of homogeneity was obtained by sequencing 15–20 amino acid residues of each subunit blotted to PVDF membranes, revealing a single amino acid residue per cycle, identical N-terminal sequences for both detergent-solubilized and autolyzed β and γ subunits, and close agreement with the sequences previously reported for the rabbit and rat α and β subunits (cf. Results for details).

SDS–Polyacrylamide Gel Electrophoresis. SDS gel electrophoresis was performed as described by Laemmli (1976) with 6% polyacrylamide in the separation gel. A rapid Bio-Rad minigel (7.5 × 5.5 × 0.15 cm) electrophoretic system was used at 4 °C to minimize the exposure time of S–D protein to denaturants. When complete denaturation was desired, individual bands isolated from gels were exposed to 4 M urea and 1% SDS at 100 °C for 5 min prior to electrophoresis (Delacourte et al., 1982). Molecular weights were determined by linear regression analysis (Shapiro et al., 1967) of the relative migration (R_f) of the protein bands as a function of

the logarithm of the molecular mass of standard proteins (myosin, 200 kDa; β-galactosidase, 116 kDa; phosphorylase *b*, 97 kDa; BSA, 66 kDa; ovalbumin, 42 kDa).

Protein Electrotransfer, Catalytic Blotting, and Immunoblotting. After separation by SDS–acrylamide electrophoresis, brush border proteins were transferred from the gel slab to an NC membrane by a semidry electroblotter (E & K Scientific Products, Inc.) at 1.0 mA/cm^2 gel surface for 90 min (Towbin et al., 1979; Gershoni & Palade, 1983). After blocking of residual protein binding sites with 2% BSA, in situ hydrolytic activities of the various S–D protein species were identified on the NC sheet after denaturants had been removed by rinsing with 40 mL of PBS (0.01 M sodium, potassium phosphate and 0.15 M NaCl, pH 6.2) containing 0.5% BSA and 1.0 mM PMSF. A layer of 1.0% substrate-containing agarose gel was prepared by modifying Filipe's method (1987) as follows: 1.2% (w/v) agarose in 0.01 M PBS (pH 6.2), 58 mM sucrose or 12 mM isomaltose, 3.5 units/mL glucose oxidase, 25 mg/mL peroxidase and 4-chloro-1-naphthol (0.2 mL of a 4 mg/mL methanol stock solution). This agarose–substrate solution was overlaid on a Gelbond (FMC) support and allowed to solidify for 10 min. The NC electrophoretic blot was gently settled on the agarose–substrate gel surface and incubated at 37 °C for 2–24 h to allow maximal development of a blue–purple color. A control gel was prepared identically, except that sucrose or isomaltose substrates were not included in the gel. For detection of immunoreactive S–D, a monospecific polyvalent rabbit anti-rat S–D (capacity, 9 milliunits of sucrase/μL; Cézard et al., 1979) was diluted 1:1000 in 40 mL of 10 mM Tris (pH 7.5) and 150 mM NaCl and incubated with the NC blot at 22 °C for 12 h. The NC membrane sheet was then washed 3 times with 40 mL of 25 mM Tris and 150 mM NaCl (pH 8.0), and specifically bound antibody was localized by incubation with horseradish peroxidase conjugated goat anti-rabbit IgG antibody (1:3000) overlaid for 2 h at 22 °C. The sheet was then washed 3 times with 25 mM Tris, pH 8.0, and 150 NaCl containing 0.5% Tween 20 and the peroxidase reacted for 10 min in freshly prepared chromogen (4 mg of 3-amino-9-ethylcarbazole in 1 mL of cold acetone, diluted with 0.05 M sodium acetate buffer, pH 5.0, containing 0.003% hydrogen peroxide) and flushed with running distilled water to remove excess soluble chromogen. The recovery of immunoreactivity after blotting determined by reflectance scanning of the NC blot by a GS-300 scanning densitometer and integration by the GS-370 1-D data system (Hoefer Scientific Instruments) revealed a comparable signal after pretreatment at 4, 37, 45, and 55 °C with a CV of 0.09 (Table I). As might have been expected, there was considerable loss of the immunological S–D signal when membranes were completely denatured at 100 °C, probably because of incomplete renaturation.

Quantitative Catalytic Analysis of Hydrolases on Blots. After the proteins were transferred to NC, residual protein binding sites were blocked with 2% BSA, and individual lanes were cut out, washed, and stored in PBS (0.01 M, pH 6.2) containing 0.5% BSA and 1.0 mM PMSF. Repeated assays of fresh or stored strips for sucrase and α-dextrinase over a period of 1 month revealed reproducible catalytic values (CV = 0.045). Strips were used for routine assays and for kinetic analysis by determining the velocity of sucrose and isomaltose cleavage over a 10-fold range of substrate concentrations at 37 °C in a shaking water bath. Reaction velocity was expressed as nanomoles of substrate hydrolyzed per minute per strip, and kinetic parameters were computed from Eadie–Hofstee plots (Wong, 1975).

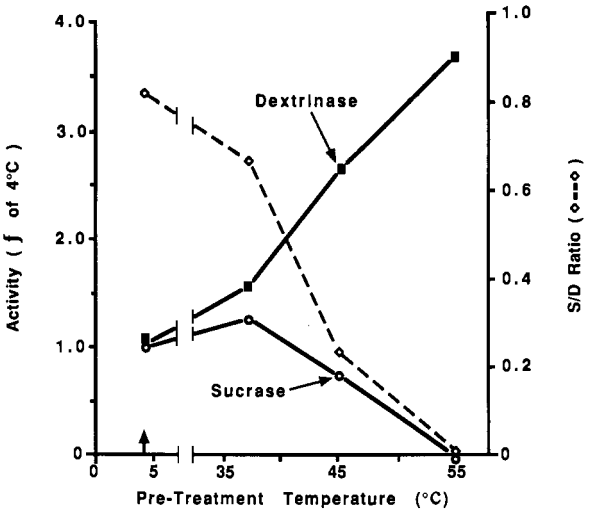

FIGURE 1: Alteration in activity of sucrase–α-dextrinase during differential denaturation. After exposure to 1% SDS–30% sorbitol for 30 min at the temperatures shown on the abscissa, equal quantities (13 milliunits) of purified brush border membrane vesicles were subjected to SDS–acrylamide electrophoresis on a 6% gel and transblotted to the NC sheet. Individual lanes were cut out, and the sucrase and α-dextrinase activities were quantified, as detailed under Experimental Procedures. Relative activities are expressed on the ordinate (left), where the activity of the 4 °C, 30-min pretreatment sample was taken as 1.0. Sucrase activity declined slightly at 45 °C and was entirely inactivated at 55 °C; α-dextrinase activity reciprocally increased by nearly 4-fold.

Protein Sequencing and Amino Acid Analysis. Protein sequencing was performed on an Applied Biosystems gas-phase sequencer (Model 470 A) with an on-line HPLC (Model 120). Samples were prepared for sequencing by transblotting onto PVDF sheets after separation on SDS gel electrophoresis (Matsudaira, 1987). Protein recoveries were determined by performing amino acid analysis on PVDF blots using a Beckman Instruments 6300 amino acid analyzer.

RESULTS

Reciprocal Alteration in α-Glucosidase Catalytic Activity of Sucrase and Dextrinase during Differential Denaturation. Equal quantities of the purified intact brush border membrane vesicles (13 milliunits of sucrase each) were pretreated for 30 min at various temperatures (4, 37, 45, and 55 °C) as detailed under Experimental Procedures and then subjected to 6% SDS–acrylamide electrophoresis. The separated brush border proteins were then transferred to the NC sheet at 45 mA for 90 min at 4 °C. After exposure of the NC sheet to 2% BSA overnight to block any residual protein binding sites, individual lanes were excised, and the catalytic activities for sucrase and α-dextrinase were quantified. Notably, after SDS treatment at 4 °C for 30 min, recovery of the sucrase and α-dextrinase activities was 40–60% of that originally assayed in brush border membrane starting material. This recovery was comparable to that found when samples were applied directly to the NC membrane. Figure 1 displays the dramatic changes in the activities of the two saccharidase components of S–D in response to the exposure to 1% SDS at various temperatures. While appreciable activity was recovered after incubation at 37 °C, sucrase was decreased somewhat by exposure to SDS at 45 °C and inactivated completely at 55 °C. In marked contrast, α-dextrinase reciprocally increased to nearly 4 times the activity present in the 4 °C control sample. As a result, the S/D activity ratio declined from 0.82 at 4 °C to 0.23 at 45 °C and eventually to 0 at 55 °C (Figure 1). These reciprocal changes suggested that there is an interdependence or even interconversion between the sucrase and the α-dex-

Table II: Recovery of S–D Activity after Sequential Exposure of Brush Borders to 1% SDS and 30% Sorbitol[a]

		sucrase		dextrinase		
temp (°C)	time (min)	milliunits	*f* of 4 °C	milliunits	*f* of 4 °C	S/D ratio
4	30	13	1.0	19	1.0	0.68
→37	10	17	1.3	21	1.1	0.81
→55	10	13	1.0	69	3.6	0.19
	30	6.8	0.5	102	5.4	0.07

[a] Intact brush border membrane vesicles were exposed to 1% SDS–30% sorbitol sequentially at 4, 37, and 55 °C, subjected to SDS gel electrophoresis, and transferred to an NC membrane sheet. The S–D (sucrase, 55 milliunits; dextrinase, 44 milliunits) was initially exposed at 4 °C and then in sequence at the temperature for the time shown. Aliquots were removed for electrophoresis and transblotting to NC. Individual lanes were then excised, and catalytic activity was assayed on the NC strips. Precision of duplicate assays was ±5% of the mean values shown. The recovery from combined electrophoresis and blotting is considered in Table I.

trinase catalytic sites. Alternatively, sucrase could be separated from its α-dextrinase partner, thereby producing a conformational change to unmask additional dextrinase catalytic sites. Further experiments were designed to explore the mechanism of these catalytic changes in S–D in response to differential denaturation of brush border membrane vesicles.

Because exposure to SDS was carried out at individual temperatures rather than sequentially, endogenous membrane protease not inhibited by the DTT–EDTA–PMSF mixture may have destroyed some α-dextrinase activity in the 4 °C SDS-treated experiment; at higher temperatures (45 and 55 °C), the proteases may have been rendered inactive, and α-dextrinase would then not be attacked, leading to an apparent increase in α-dextrinase activity. Therefore, we carried out sequential denaturation experiments of individual samples at increasing temperatures to examine the potential protease artifact. As shown in Table II, sequential prior exposure of intact BB membrane vesicles to 1% SDS–30% sorbitol at 4 → 37 → 55 °C followed by electrophoresis and immunoblotting produced appreciable loss of sucrase activity and a 5.4-fold increase in α-dextrinase activity. This is reflected in the 10-fold decrease in the S/D activity ratio during the sequential exposure to SDS at increasing temperatures. Allowing for the slightly greater recovery of protein (+14%) and S–D immunoactivity (+17%) after electrophoresis and blotting of the SDS-treated membrane at the higher temperatures (cf. Table I), the increase in α-dextrinase catalytic activity was still nearly 500%, more than double that originally applied to the gels (Table II). Hence, the incremental increase in α-dextrinase activity observed in these experiments appears to be real. Neither proteolytic destruction in the single 4 °C experiments nor enhanced recovery at the higher temperature can account for the major recruitment of dextrinase activity.

Interdependent Changes in Catalytic Kinetics for S–D during Denaturation. On the basis of the dramatic gain in α-dextrinase activity and reciprocal loss of sucrase activity by exposure to SDS–sorbitol at 45 °C (Figure 1), 4 and 45 °C (30 min) were chosen as pretreatment temperatures for enzyme kinetic studies. Intact brush border membrane vesicles (40 milliunits of sucrase) were subjected to acrylamide electrophoresis, electroblotting, and assay on the NC strips, as described under Experimental Procedures. The kinetic study was performed at 11 different substrate concentrations encompassing more than 2 times the K_m (sucrose, 3.7–44 mM; isomaltose, 0.7–8.8 mM). Table III shows that the K_m for neither sucrase nor α-dextrinase changed significantly when intact brush border membranes were exposed to 1% SDS–30% sorbitol at 4 or 45 °C for 30 min prior to SDS electrophoresis. In marked contrast, there was a dramatic tripling of the V_{max} for α-dextrinase in response to the SDS exposure at 45 °C, while the V_{max} for sucrase declined only slightly (–27%) (Table III). These reciprocal changes of S and D catalytic rates were

Table III: Kinetic Study of Sucrase–α-Dextrinase on Nitrocellulose Blots[a]

		pretreatment temp (°C)		% change
		4	45	4 → 45 °C
sucrase	V_{max}	15	11	–27
	K_m	20	22	+10
	r^b	0.90	0.96	
α-dextrinase	V_{max}	17	47	+176
	K_m	4.2	3.8	–9
	r	0.98	0.95	

[a] Intact brush border membrane vesicles (40 milliunits) were pretreated in 1% SDS–30% sorbitol at 4 or 45 °C for 30 min, subjected to SDS gel electrophoresis, and transferred to an NC membrane sheet. Individual lanes were then cut out. Kinetic analyses were performed on the NC membrane strips and computed to Eadie–Hofstee plot. V_{max} is expressed as nanomoles per minute per strip and K_m as millimolar. [b] Correlation coefficient for linearity of Eadie–Hofstee plot.

not stoichiometric, the net gain of α-dextrinase activity being 8 times greater than the loss of sucrase activity (Table III). Because the changes in V_{max} occurred without an apparent change in the affinity of either active site for its substrate (as estimated from the constant K_m value), the conformation of the catalytic sites did not appear to change significantly. The correlation of S and D structure with its function is considered further below.

Molecular Species, Immunoactivity, and Catalytic Activity of S–D during Differential Denaturation. The changes in activity strongly suggested an intricate structural interaction of the S–D active sites in the native enzyme complex. When intact BB membranes were used as the starting material, analysis of the S and D species for both immunoactivity and catalytic activity on the same NC sheet allowed further insight into the functional and structural relationship of the subunits. Appreciable renaturation of S–D species occurred after transferring from the electrophoretic gel to the nitrocellulose replica, allowing precise localization of catalytic activity and immunoactivity on the blots. Figure 2 shows a typical immunoblot (Figure 2A) and catalytic blot replicas (Figure 2B,C) after equal quantities (15 milliunits of sucrase) of intact brush border membrane vesicles had been differentially denatured for 30 or 60 min each at 4, 37, 45, 55, or 100 °C for 5 min. The expected immunoreactive S and D species were noted on the NC blot after full denaturation at 100 °C for 5 min (Figure 2A, far right lane). These were designated as the α (140 kDa), β (125 kDa), and γ (110 kDa) subunits according to their migration on the gel. Even when intestinal membranes were homogenized in the presence of a protease inhibitor mixture (PMSF, antipain, chymostatin, leupeptin, pepstatin A, and ovomucoid) and the crude pellet was exposed immediately to 1% SDS at 100 °C for 5 min, the γ species was identified along with the α and β by electrophoresis–im-

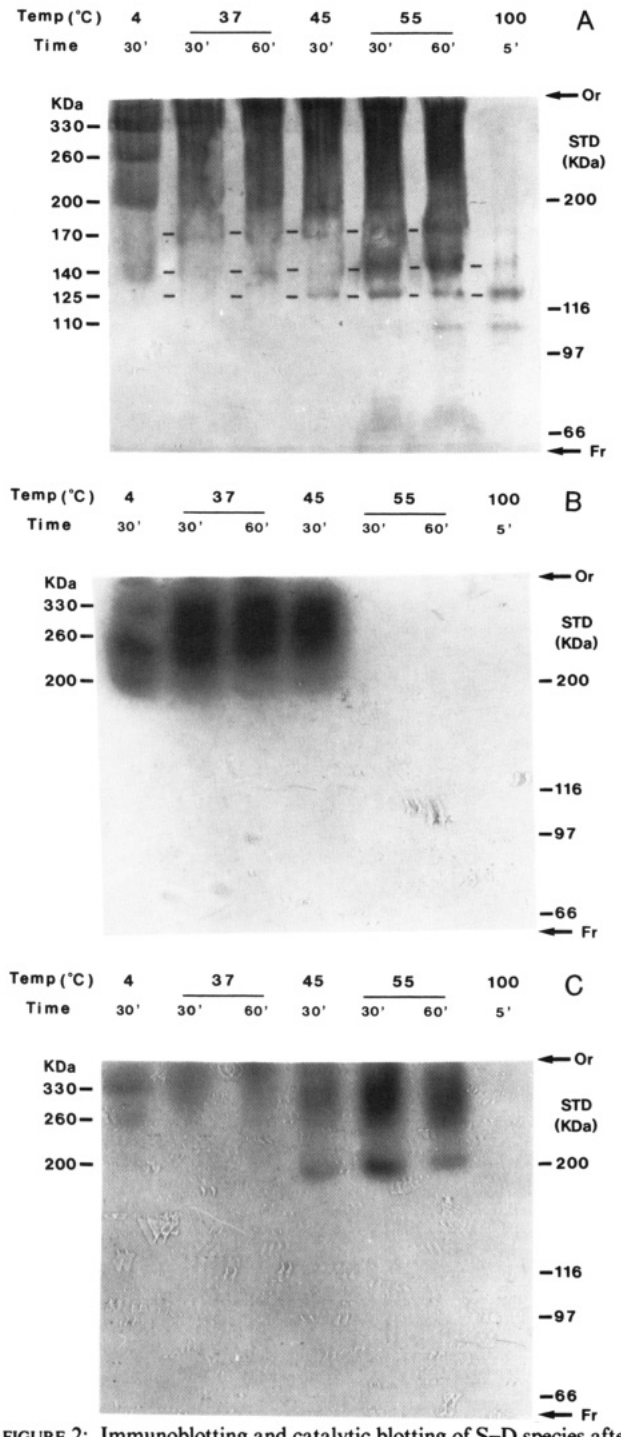

FIGURE 2: Immunoblotting and catalytic blotting of S–D species after differential denaturation. Equal quantities of intact brush border membrane vesicles were exposed to 1% SDS–30% sorbitol at 4, 37, 45, and 55 °C (30 or 60 min) or at 100 °C (5 min), subjected to SDS electrophoresis, and transferred to an NC sheet. Immunoreactive S–D (panel A), detected by specific anti-S–D serum, was compared with in situ activites of sucrase (panel B) and α-dextrinase (panel C) by catalytic blotting. Molecular masses of standard proteins are on the right and those for each S–D species on the left.

munoblotting (data not shown). As typically found in our laboratory, the subunits were noted to be of unequal density with the β unit predominating, both in purified brush border membranes and rapidly prepared total intestinal membrane samples when analyzed either by electrophoresis–immunoblot or by SDS electrophoresis of immunoprecipitates. Despite removal of the denaturants by the blotting technique, no hydrolytic activity was recovered for any of these three subunits after 100 °C, 5-min treatment (Figure 2B,C). In contrast,

Table IV: Sucrase–α-Dextrinase Species after Differential Treatment in SDS[a]

species (kDa)	pretreatment temp (°C)				
	4	37	45	55	100
330	+ (S, D)	+ (S, D)	+ (S, D)	+ (D)	–
260	+ (S, D)	+ (S, D)	+ (S, D)	+ (D)	–
200	+ (S)	+ (S)	+ (S, D)	+ (D)	–
170	–	+	+	+	–
140	+	+	+	+	+
125	–	–	+	+	+
110	–	–	–	+	+
~70	–	–	–	+	–

[a] Abbreviations: (+) presence or (–) absence of immunoreactive S–D on the immunoblot. S or D in parentheses indicates the in situ sucrase or dextrinase hydrolytic activity present in the particular molecular species on the catalytic blots.

after exposure to SDS at low temperature (4 °C, 30 min), immunoreactive species were identified at 330, 260, 200, and 140 kDa (Figure 2A). Because release of the 140-kDa α preceded that of the other subunits (Figure 2A, 4 °C lane) and, indeed, was associated with a loss of sucrase activity even in the 4 °C experiment (the normal S/D ratio in untreated brush border of 1.0–1.2 was decreased to 0.8 by SDS exposure at 4 °C), the α unit was a candidate for the sucrase-carrying subunit. Catalytic assay of the same blot revealed sucrase activity localized to the 330-, 260-, and 200-kDa units (Figure 2B), with the 260-kDa species predominating. α-Dextrinase activity was associated with only the 330- and 260-kDa species and predominated in the 330-kDa moiety (Figure 2C). Exposure to SDS at 37 °C (30 or 60 min) was associated with the loss of the discrete definition of the 330-, 260-, and 200-kDa macromolecular species on the immunoblot and the appearance of a smaller immunoreactive species (170 kDa) (Figure 2A). Catalytic activity for sucrase persisted at 330, 260, and 200 kDa (Figure 2B), and α-dextrinase activity continued to be associated predominantly with the 330-kDa unit (Figure 2C). Exposure to 45 °C resulted in the appearance of a 125-kDa unit (β) on the immunoblot (Figure 2A). The catalytic blot showed reduction of sucrase activity with no qualitative change in the localization (Figure 2B), but α-dextrinase activity increased and shifted (Figure 2C) dramatically to lower molecular units (260 and 200 kDa). Notably, this qualitative and quantitative shift of the active α-dextrinase species was associated with a recruiting of D active sites manifested by a 3-fold increase in the V_{max} of α-dextrinase (cf. Figure 1, Table III); this was associated with the release of the inactive 125-kDa β unit from the active oligomers (Figure 2A, 45 °C lane). Heating at even higher temperature (55 °C; 30 or 60 min) resulted in the complete loss of sucrase activity (Figure 2B) and the concomitant persistence of α-dextrinase activity associated with the 330-, 260-, and 200-kDa species; indeed, the dextrinase activity appeared to increase further after the 55 °C exposure (Figures 1 and 2C). Although there was some loss of α-dextrinase activity associated with the longer 60-min exposure at 55 °C, the overall pattern of the catalytic blot was similar to that seen at 30 min. The complete loss of the sucrase activity by exposure to 55 °C was associated with the presence of the 125-kDa species, the emergence of a predominant 140-kDa moiety, and the appearance of additional inactive immune species at 110 kDa (the γ species) and a diffuse band at 70 kDa (Figure 2A). A summary of the effect of differential denaturation at varying temperatures on the apparent masses of the immunoreactive and catalytically active species is given in Table IV.

Composition of the S–D Molecular Species and Assignment of the Active Sites. Because the active 260- and 330-kDa

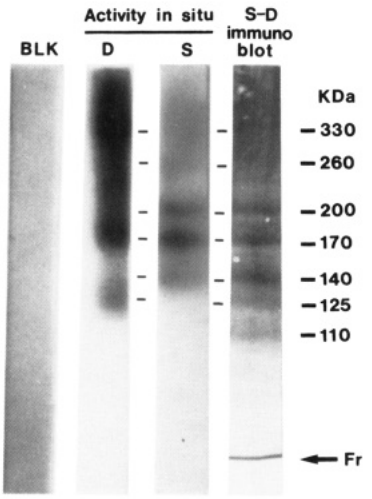

FIGURE 3: Species of purified S–D after 4/42 °C treatment. The two equal aliquots (250 milliunits) of purified S–D were exposed to SDS–sorbitol for 30 min at 4 and 42 °C, respectively, and then recombined, applied to SDS electrophoresis in a trough across a 6% acrylamide gel, and electrophoresed. The expected protein species (330, 260, 200, 170, 140, 125, and 110 kDa) were identified by immunoblotting and catalytic blotting on vertical test strips after transferring to NC. The minimal mass of the species associated with sucrase activity (S) was 140 kDa and with dextrinase activity (D) 125 kDa. BLK, catalytic blot blank without saccharide substrate.

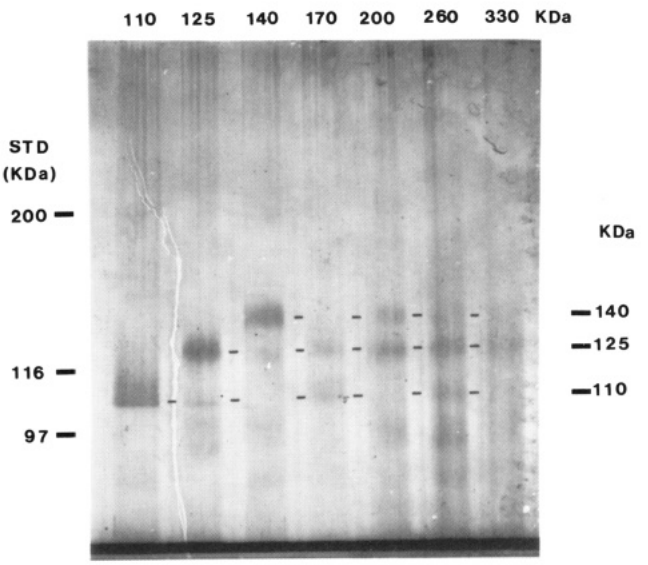

FIGURE 4: Subunit composition of individual S–D species. Individual species were isolated by cutting horizontal strips across the gel from the experiment in Figure 3, with the immunoblot strip used as the template. Each species was totally denatured by exposure to 1% SDS, 4 M urea, 10 mM DTT, and the protease inhibitor mixture at 100 °C for 5 min followed by electrophoresis on a 6% SDS–urea gel. The monomeric subunits comprising each species were identified by silver staining. The molecular masses at the top of the figure are the masses (in kDa) of the particular species applied. Note that only the 140- (α), 125- (β), and 110-kDa (γ) species appear to be true monomers.

species carrying both sucrase and dextrinase catalytic sites found at the lower denaturing temperatures were as large as or larger than the putative 230–260-kDa S–D hybrid (Figure 2), it was important to determine the subunit composition of each moiety. S–D, purified as detailed under Experimental Procedures to a specific activity of 20 units/mg of protein, was homogeneous by denaturing acrylamide electrophoresis, having the typical α, β, γ pattern (not shown). The purified S–D was divided into two equal aliquots (250 milliunits each), one incubated in 1% SDS at 4 °C for 30 min and the other at 42 °C for 30 min. They were then recombined and applied across the top of a 7.5 × 5.5 cm SDS gel via a trough. After electrophoresis, the protein species were electrophoretically transferred to a NC sheet which was then cut into vertical strips. As expected, all S–D protein species (330, 260, 200, 170, 140, 125, and 110 kDa) were identified by both Coomassie Blue staining and immunoblotting, and the several active species were observed by catalytic blotting of the test NC blots (Figure 3). In these combined 4 and 42 °C pretreated samples, catalytically active dextrinase was readily identified in the several large species, was most prominent in the 330-kDa species, and was also present in the 170- and 125-kDa (β) units. In contrast, sucrase activity was most prominent in the 200- and 170-kDa species and was readily detected in the 140-kDa (α) unit. This pattern was found for both detergent-solubilized and autolyzed tissue. Notably, the association of sucrase with the larger α subunit and dextrinase with the smaller β subunit is the reverse of the assignments generally accepted for mammalian S–D, based on the work of Brunner et al. (1979) on rabbit S–D.

Each molecular species of S–D was isolated by cutting the appropriate horizontal gel strips from the 4–42 °C experiment (see above and Figure 3). This allowed analysis of the subunit composition of each individual S–D immunospecies under fully denaturing conditions (1% SDS, 4 M urea, 10 mM DTT, and 1.0 mM PMSF at 100 °C for 5 min). As shown in Figure 4, the 140- (α), 125- (β), and 110-kDa (γ) moieties appeared to be legitimate monomers since they were maintained as the predominant species after maximal denaturation. The larger oligomers consisted of various combinations of the 140-, 125-, and 110-kDa units, and those known to be associated with appreciable catalytic activity (the 330-, 260-, and 200-kDa units) also included smaller molecular units of 90 and 70 kDa as components. This was particularly notable for the 260-kDa species (260 lane, Figure 4). Overall, these experiments revealed that the S–D complex is predominantly a 330- and 260-kDa oligomer composed of α, β, and γ subunits (and possibly other subunits) in varous combinations. Interestingly, the 170-kDa species contained β and γ monomers and some other fragments. Although both S and D activities were demonstrated in the 170-kDa unit by catalytic blotting (Figure 3), no α band could be detected with the sensitive silver staining. At first analysis, this appeared to be an enigma since only the β subunit possessed activity for dextrinase and the γ unit was not associated with activity. However, when associated with smaller fragments of 70 or 90 kDa, the γ may possess activity or, alternatively, the β may change conformationally and express sucrase activity. As shown below, the N-terminal amino acid sequence of the γ subunit shows high homology with a region of the α subunit, suggesting that it is a product of the α.

N-Terminal Amino Acid Sequencing of the α, β, and γ Subunits. Since the 140-, 125-, and 110-kDa species were not fragmented further by stringent denaturation at 100 °C for 2–10 min, they appeared to be monomeric forms of the oligomer. To verify this, we separated these species in the pure S–D protein on 6% acrylamide gels, blotted them to PVDF membranes, localized them by Coomassie Blue staining, and sequenced each species directly from the blots. As shown in Figure 5, the sequence of α shows high homology with that previously published for the N-terminus of the P precursor and for that of the α, in the pig, human, and rabbit. Similarly, the N-terminal sequence of β begins with the Ile residue near the center of the P precursor (residue 1008 of a total of 1827, based on the full-length rabbit cDNA) and agrees completely with the analogous region of a recently cloned partial cDNA

α Subunit:

	1								10									19
Rat*:	A	K	K	K	F	S	A	L	E	I	S	L	I	V	L	F(V)	(I)	(V)
Human**:	-	R	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-
Hog§:	-	R	-	-	-	-	G	-	-	-	X	-	-	-	-	-	A	-	-
Rabbit¶:	-	-	R	-	-	-	G	-	-	-	T	-	-	-	-	-	-	-	-

β Subunit:

	1008	1010										1020					1025
Rat*:	I	K	L	P	S	N	P	I	S(E)	L	R	V	E	V	K	Y(H)	
Rat†:	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	-	-
Hog§:	-	-	-	-	-	D	-	-	P	T	-	-	-	-	-	-	-
Rabbit¶:	-	T	-	-	-	E	-	-	T	N	-	-	-	-	-	-	-

γ Subunit:

	186				190											200			204
Rat*:	A	D	T	L	Y	D	V	Q	V	S	E	N	P	F	X	I	K(V)	(I)	
Human**:	S	-	-	-	-	-	-	K	-	A	Q	-	-	-	S	-	Q	-	-
Rabbit¶:	T	E	-	-	-	-	-	-	-	T	-	-	-	-	S	-	-	-	-

* Data for rat in this paper.
** Deduced from human cDNA sequence (Green et al, 1987).
§ From Sjöström et al, 1982.
¶ Deduced from rabbit cDNA sequence (Hunziker et al, 1986).
† Deduced from rat cDNA sequence (Broyart et al, 1990).

FIGURE 5: Comparison of N-terminal amino acid sequences of the S–D subunits. S–D subunits (α, β, and γ), determined as detailed in the text, are compared with those previously published for rat and other mammalian species. The γ sequence is aligned with the deduced sequence from the rat, rabbit, and human cDNAs. Parentheses, tentative assignments; X, unknown residue; –, identical residue to that for rat.

for rat S–D (Broyart et al., 1990). The N-terminal sequence of γ subunits is clearly derived from additional processing near the N-terminus of the P precursor or most probably its α subunit, since it has major homology beginning at residue 186 of the P on the basis of the rabbit cDNA.

Discussion

Structural and Functional Implications of Differential Denaturation of S–D Oligomers. Whereas the primary structure of the P precursor is known from cloning of the cDNA (Hunziger et al., 1986), the complex postinsertional processing to the S–D oligomer can only be established by analysis of its glycoprotein structure and correlation with its catalytic function. The use of SDS–sorbitol at varying temperatures to partially denature the intact brush border membrane glycoproteins followed by maximal renaturation by blotting to remove the denaturant revealed a dramatic change in the relative and absolute hydrolytic activities of the sucrase and dextrinase components of S–D (cf. Figure 1). Notably, the complete loss of sucrase activity at 55 °C was associated with a nearly 3-fold increase in dextrinase activity (Figure 1). The possibility of destruction of dextrinase at 4 °C by contaminating proteases but not at 45–55 °C was eliminated by similar findings when brush border membranes were initially exposed to SDS at 4 °C and then in sequence to higher temperatures (cf. Table II). Since this was associated with a dramatic increase in V_{max} without a significant change in K_m (cf. Table III), there was clearly a major recruitment of functional dextrinase catalytic sites. Although the exact mechanism of the changes in these α-glucosidase active sites remains to be established, the marked gain in dextrinase activity and loss of all sucrase activity was associated with the release of the monomeric subunits (α, β, and γ) from the active 300-, 260-, and 200-kDa oligomers. Because the release of the 140-kDa α unit which harbors the sucrase active site (cf. Figure 3) was particularly prominent when the dextrinase was maximally activated (cf. Figure 2, 55 °C lanes), we suspect that the association of sucrase with its dextrinase partner promotes steric hindrance of the normal conformation of one or more auxiliary dextrinase active sites. Of course, conversion of the sucrase to dextrinase site is possible, but these two active sites have no substrate cross-specificity for their primary substrates in the native enzyme, even though they are both α-glucosidases (Conklin et al., 1975).

These insights were possible because we have capitalized on the use of blotting of partially denatured membrane glycoproteins from SDS–acrylamide gels which allows removal of denaturants and recovery of appreciable amounts of functional catalytic activity. Thus, detailed analysis of the structural and functional correlation of S–D was possible while it was still a component of the brush border membrane. When brush border membranes were exposed to SDS at 4 °C, immunoblots and catalytic blots revealed clearly that there are two major species of active oligomers (260 and 330 kDa) possessing both sucrase and dextrinase activity but with different relative activities of the two α-glucosidases (cf. Figure 2). It is important to point out that these active oligomeric species may not differ appreciably in their molecular mass, and our reference to the active species according to their apparent mass is for operational purposes. Their precise molecular masses can only be approximated because they are not completely denatured and hence may not migrate as a strict function of mass on SDS gels. Yet, in other experiments not detailed here, these active α-glucosidase oligomers differed consistently in their migration on acrylamide gels at several effective pore sizes (5.3, 5.6, 5.9, and 6.4% total acrylamide), in each instance the calculated molecular masses corresponding to 330–350 and 250–260 kDa for the respective active oligomers. Therefore, a subtle difference in charge alone is unlikely to account for the differences in migratory behavior. Being separable on the basis of their relative resistance to movement through the gel interstices, these active oligomers are, at a minimum, conformationally distinct. Notably, unlike our experiments on intact brush border membranes, all previous studies depended upon more severe treatments, including chemical attack and extraction of the S–D from the membrane, perturbations that might be expected to be much more likely to evoke artifactual structural rearrangement of the S–D moiety.

These findings indicate that S–D, rather than being a single hybrid dimer as commonly found after its solubilization with proteases or detergents, exists in its native state within the intact brush border membrane in at least two major oligomeric forms. Although each contains both sucrase and dextrinase activities, the 330-kDa unit possesses most of the dextrinase activity and the 260-kDa unit harbors mainly sucrase activity (Figure 2, compare panels B and C). The prominence of the 330- and 260-kDa species in intact brush border membranes exposed to SDS at low temperature and their relative differences in sucrase and dextrinase specificity suggest they are distinct native α-glucosidase units in the brush border. Also notable are previous electron microscopic analyses of solubilized, purified S–D which revealed a variable morphology compatible with the presence of several (up to six) subunits by direct negative staining (Nishi et al., 1968) or after reinsertion into artificial lipid membranes (Cowell et al., 1986). Our findings of at least two active oligomeric S–D in intact membranes is compatible with these microscopic structure studies.

Exposure of the 330- and 260-kDa species to SDS at higher temperatures produced the release of true monomeric subunits (cf. Figure 2A) and the associated enhancement of dextrinase and loss of sucrase activity (cf. Figure 1). This suggests that the oligomeric association in intact brush border membrane

involves steric adaptations favoring the proper conformation of the sucrase catalytic site while blocking dextrinase active sites. That dextrinase sites are completely inhibited rather than having their substrate affinity altered is supported by the kinetic studies where the K_m for dextrinase was unchanged, but its V_{max} increased dramatically (cf. Figure 1 and Tables II and III) when the oligomer was reduced in mass from 330 to 200 kDa by virtue of release of the 140-kDa α subunit and other subunits (cf. Figure 2A).

α, β, and γ Subunits and Assignment of Active Sites. When the native S–D 330- and 260-kDa oligomers were dissociated into their subunit components by exposure to SDS at increasing temperatures, sucrase and dextrinase activities could be readily monitored by the in situ catalytic blotting technique (cf. Figure 2). Somewhat to our surprise, the largest true monomeric component, the 140-kDa α subunit, having the appropriate N-terminal sequence of the membrane-anchoring subunit (cf. Figure 5) carried the sucrase active site (cf. Figure 3). The 125-kDa β subunit, having the appropriate sequence to position its N-terminus near the center of the single-chain precursor (cf. Figure 5), displayed dextrinase activity (cf. Figure 3). Neither of these minimal active units possessed any detectable activity against each other's specific substrate. The specific localization of a single type of α-glucosidase active site was most pronounced when differential denaturation was performed on purified detergent-solubilized S–D (Figure 3). However, in other experiments not shown here, direct exposure of intact brush border vesicles to SDS–sorbitol in the 4/42 °C protocol (as detailed for the experiments in Figure 3 with solubilized S–D) also yielded detectable sucrase and α-dextrinase associated with the 140- and 125-kDa subunits, respectively.

While our assignment of α-glucosidase activities to the α and β subunit is the reverse of that generally accepted on the basis of stringent treatment and separation of the S–D units with citriconic anhydride (Brunner et al., 1979), three earlier studies (where citriconic anhydride or high alkaline pH was used to separate solubilized sucrase and α-dextrinase activity) had found sucrase associated with the largest monomeric unit (Cogoli et al., 1973; Braun et al., 1975; Takesue et al., 1977). In all previous analyses, there were major losses in catalytic activity of both subunits, and the assignment was inferred from migration of residual material on denaturing acrylamide gels. In contrast, we were able to recover the majority of the sucrase and dextrinase catalytic activities after differential denaturation under mild conditions of SDS exposure followed by maximal renaturation by electroblotting. Furthermore, this is the first study of S–D to directly demonstrate catalytic activity associated with the monomeric α and β subspecies in situ after their electrophoretic separation (Figures 2 and 3). Thus, we are confident that the 140-kDa α unit carries the sucrase active site and the 125-kDa β the dextrinase active site. The close correlation of the subunit masses and the amino acid sequences (see below and Figure 5) with previous work eliminates the possibility of subunit reversal by proteolytic artifact as an explanation for the catalytic assignments. For instance, while the larger subunit (α) could be made smaller by cleavage from its C-terminal end, its N-terminal sequence would have identified it as a cleaved α rather than as the β. If the N-terminus of the α had been attacked, it would have produced a unique N-terminal sequence. Instead, our β subunit showed the previously recognized sequence. Although there may be species differences, notably in nonmammals such as the chicken, where the smaller β subunit possesses the membrane anchor and is the dextrinase-containing segment (Hu et al., 1987), no significant structural differences among mammalian S–D's have been observed in rat, rabbit, pig, and human intestine. Their similarity is confirmed by the very high homology of the N-terminal sequences of the α, β, and γ species, comparing the rat with other mammalian species (cf. Figure 5).

N-Terminal Amino Acid Sequences of the Subunits. Previous amino acid sequence analyses of S–D had revealed appreciable interspecies homology (Semenza, 1986). As compared with those from human, hog, and rabbit intestine, our N-terminal sequences of the rat S–D subunits, performed on at least two separately purified samples from both Triton-solubilized and autolyzed (presumably protease-solubilized) S–D proteins, have verified the high homology (Figure 5). For the α subunit from detergent-solubilized samples, there was considerable discrepancy from the original rat sequence (Frank et al., 1978) but excellent agreement with a subsequently proposed sequence from residues 1 through 10 (Hauri et al., 1982). Probably because of the action of the numerous adherent pancreatic proteases present in crude intestinal preparations, no reproducible N-terminal sequence was found for the α subunit from autolyzed intestine (data not shown). In contrast, the N-terminal sequences of the β and γ subunits were the same for both the autolyzed and detergent-solubilized S–D.

Although the γ subunit has not been previously isolated and sequenced, it was found to have extremely high homology with the segment of the N-terminal α subunit beginning at residue 186, on the basis of alignment with the human and rabbit sequences deduced from their known cDNAs (Green et al., 1987; Hunziker et al., 1986). There were only four differences as compared to the rabbit (79% identity) and five with respect to the human sequences (74% identity) (cf. Figure 5). Hence the 110-kDa γ subunits is clearly derived from the original P precursor protein, or its α product, either by direct cleavage from the parental precursor or by removal of an N-terminal segment from the α subunit. Notably, the 170-kDa unit released during differential denaturation/renaturation possesses both sucrase and dextrinase activities (cf. Figure 3), and it is likely to consist of two peptides: (1) either the β or the γ, as the major unit; (2) one or more minor units (cf. Figure 4). The β carries the dextrinase active site, and the γ, comprising all of the sucrase-containing α except the N-terminal 185 residues, is likely to be responsible for the sucrase activity in the 170-kDa moiety. Constitutive endogenous endopeptidases are known to reside in the brush border membrane of the kidney and intestine (Bond et al., 1986; Butler et al., 1987; Barnes et al., 1989; Song et al., 1986; Toll et al., 1991) and may play a physiologic role in processing proteins within the same membrane (Shapiro et al., 1991). However, for cogent reasons, we doubt that these endogenous peptidases acted on S–D during the differential denaturation–renaturation studies. All are metalloenzymes that are inhibited by metal ion chelators such as EDTA. Endopeptidase 24.11, having specificity mainly for relatively small peptides, may serve to process hormone peptides in the intestinal lumen, but it has low specificity for large proteins (Bond et al., 1986). Endopeptidase 2 (Barnes et al., 1989) and meprin (Butler et al., 1987), although having the capacity to cleave larger proteins, are comprised of disulfide-linked subunits and are readily inhibited by reagents such as DTT. Notably, the γ unit, a product of the P precursor or its large (140 kDa) α product, was readily identified in both rapidly prepared and highly purified intestinal membranes in the presence of EDTA and DTT. Hence, we interpret it to be an endogenously modified species rather than a proteolytic

product released during membrane isolation or exposure to SDS-sorbitol.

Whether all the active species identified during differential denaturation of intact membranes are actually present under native conditions is of course uncertain. Any putative monomeric subunit smaller than the γ species was relatively faint on the gels and is likely to be a minor artifact of the denaturation-renaturation technique. Certainly this approach has allowed new insights into the structural and functional relationships of the S-D, and several features of the prominent oligomers indicate that the subunit interactions within the surface membrane are appreciably more complex than generally assumed: (1) there are two rather than a single oligomeric major species comprised of the α (carrying the sucrase activity), β (harboring the dextrinase activity), and γ subunits formed from postinsertional processing of the S-D precursor; (2) the 260-kDa oligomer is relatively enriched in sucrase activity and the 330-kDa unit in α-dextrinase activity; and (3) subunit association is essential for maintenance of optimal catalytic activity, ensuring a balance between the two α-glucosidase activities.

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Registry No. Sucrose, 57-50-1; α-dextrin, 10016-20-3.

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Molecular Basis for Vancomycin Resistance in *Enterococcus faecium* BM4147: Biosynthesis of a Depsipeptide Peptidoglycan Precursor by Vancomycin Resistance Proteins VanH and VanA†

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ABSTRACT: Vancomycin resistance in *Enterococcus faecium* BM4147 is mediated by vancomycin resistance proteins VanA and VanH. VanA is a D-alanine:D-alanine ligase of altered substrate specificity [Bugg, T. D. H., Dutka-Malen, S., Arthur, M., Courvalin, P., & Walsh, C. T. (1991) *Biochemistry 30*, 2017–2021], while the sequence of VanH is related to those of α-keto acid dehydrogenases [Arthur, M., Molinas, C., Dutka-Malen, S., & Courvalin, P. (1991) *Gene* (submitted)]. We report purification of VanH to homogeneity, characterization as a D-specific α-keto acid dehydrogenase, and comparison with D-lactate dehydrogenases from *Leuconostoc mesenteroides* and *Lactobacillus leichmanii*. VanA was found to catalyze ester bond formation between D-alanine and the D-hydroxy acid products of VanH, the best substrate being D-2-hydroxybutyrate (K_m = 0.60 mM). The VanA product D-alanyl-D-2-hydroxybutyrate could then be incorporated into the UDPMurNAc–pentapeptide peptidoglycan precursor by D-Ala-D-Ala adding enzyme from *Escherichia coli* or by crude extract from *E. faecium* BM4147. The vancomycin binding constant of a synthetic modified peptidoglycan analogue *N*-acetyl-D-alanyl-D-2-hydroxybutyrate (K_d > 73 mM) was >1000-fold higher than the binding constant for *N*-acetyl-D-alanyl-D-alanine (K_d = 54 μM), partly due to the disruption of a hydrogen bond in the vancomycin–target complex, thus providing a molecular rationale for high-level vancomycin resistance.

The recent emergence of bacterial resistance to the glycopeptide family of antibiotics in strains of *Enterococcus faecium* and *Enterococcus faecalis* (Courvalin, 1990) has prompted considerable interest in the mechanism of resistance: both because of the increasing use of the glycopeptide vancomycin in treatment of life-threatening Gram-positive bacterial infections and because of the unusual mode of action of the glycopeptide antibiotics. Vancomycin does not appear to penetrate the cell membrane or interact with cellular proteins but functions by complexation of peptidyl-D-Ala-D-Ala termini on the bacterial cell surface (Barna & Williams, 1984), thereby preventing transglycosylation and cross-linking of the peptidoglycan layer (Reynolds, 1989; Nagarajan, 1991). In view of the apparent ubiquity of the D-Ala-D-Ala terminus of bacterial peptidoglycan, it is difficult to envisage a simple mechanism for vancomycin resistance, which may explain the lack of bacterial resistance for over 30 years of clinical use. How then is this recently emergent vancomycin resistance achieved?

High-level vancomycin resistance in *E. faecium* BM4147, a class A vancomycin-resistant strain (Schlaes et al., 1991), is associated with production of a 38-kDa membrane-associated protein VanA, whose amino acid sequence has been determined (Dutka-Malen et al., 1990). Sequence similarity was found with Gram-negative D-Ala-D-Ala ligases, which are cytoplasmic enzymes responsible for synthesis of the D-Ala-D-Ala dipeptide for peptidoglycan assembly (Walsh, 1989). VanA has been purified, and shows D-Ala-D-Ala ligase activity, but has substantially modified substrate specificity, compared with Gram-negative D-Ala-D-Ala ligases (Bugg et al., 1991). VanA is able to synthesize a number of mixed dipeptides including D-Ala-D-Met and D-Ala-D-Phe in preference to D-Ala-D-Ala, suggesting that its cellular role may be synthesis of a D-Ala-X dipeptide, which is incorporated into peptidoglycan and is able to be cross-linked but is not recognized by vancomycin.

Sequencing of an open reading frame adjacent to the *vanA* gene on plasmid pIP816 that is also required for resistance has identified a further vancomycin resistance protein named VanH (Arthur et al., 1991). The amino acid sequence of VanH shows 19–30% sequence identity with sequences of three D-specific α-keto acid reductases: D-hydroxyisocaproate dehydrogenase, D-3-phosphoglycerate dehydrogenase, and D-erythronate phosphate dehydrogenase. This raises the pos-

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