Sucrase-isomaltase (SI) is an intestinal membrane-associated α-glucosidase that breaks down di- and oligosaccharides to absorbable monosaccharides. SI has two homologous functional subunits (sucrase and isomaltase) that both belong to the glycoside hydrolase family 31 (GH31) and differ in substrate specificity. All GH31 enzymes share a consensus sequence harboring an aspartic acid residue as a catalytic nucleophile. Moreover, crystallographic structural analysis of isomaltase predicts that another aspartic acid residue functions as a proton donor in hydrolysis. Here, we mutagenized the predicted proton donor residues and the nucleophilic catalyst residues in each SI subunit. We expressed these SI variants in COS-1 cells and analyzed their structural, transport, and functional characteristics. All of the mutants revealed expression levels and maturation rates comparable with those of the wild-type species and the corresponding nonmutated subunits were functionally active. Thereby we determined rate and substrate specificity for each single subunit without influence from the other subunit. This approach provides a model for functional analysis of the single subunits within a multidomain protein, achieved without the necessity to express the individual subunits separately. Of note, we also found that glucose product inhibition regulates the activities of both SI subunits. We experimentally confirmed the catalytic function of the predicted proton donor residues, and sequence analysis suggested that these residues are located in a consensus region in many GH31 family members. In summary, these findings reveal the kinetic features specific for each human SI subunit and demonstrate that the activities of these subunits are regulated via product inhibition.

Sucrase-isomaltase (SI; EC 3.2.1.48 and 3.2.1.10) is an integral intestinal membrane α-glucosidase that catalyzes the final step of carbohydrate digestion by breaking disaccharides and oligosaccharides to absorbable monosaccharides (1–3). Reduced or absent enzymatic levels of SI can lead to carbohydrate malabsorption with gastrointestinal symptoms, such as osmotic diarrhea, bloating, flatulence, and vomiting (4). SI deficiencies can occur primarily as a consequence of mutations in the coding region of the SI gene, referred to as congenital sucrase-isomaltase deficiency (CSID) (5). Deleterious mutations are associated with alterations in the intracellular trafficking, functional deficits, and missorting of SI (5–7). Secondary SI deficiencies, on the other hand, arise collaboratively to other organ pathologies in the intestine in which the integrity and/or the normal physiology of the intestinal epithelium is severely affected, for example in intestinal ulcers or infections (8, 9) and inflammatory bowel disease (10).

SI belongs to the glycoside hydrolases of family 31 (GH31); according to the Carbohydrate Active Enzymes (CAZy) database, http://www.cazy.org (11) and is composed of two luminal catalytic subunits including sucrase (SUC) at the C terminus of SI and isomaltase (IM) at the N terminus (1). The GH31 family encompasses various eukaryotic α-glucosidases like SI, which cleave terminal carbohydrate moieties from substrates of different length and of different glycosidic binding types (12). Other examples of this group are the endoplasmic reticulum glucosidases I and II (13), lysosomal α-glucosidase (14), and intestinal maltase-glucoamylase (15). Different members of this group possess one or a combination of α-1,2-α-1,3-α-1,4-, and α-1,6-glucosidase activities (16, 17). Except α-glucanases, GH31 enzymes function via an acid/base catalyzed double replacement mechanism, also known as classical Koshland retaining mechanism involving a glycosyl-enzyme intermediate (18–20). Retaining enzymes typically have two carboxylic amino acid residues surrounded by consensus motifs. One residue acts as a catalytic nucleophile, and the second acts as a proton donor or acid/base catalyst (21, 22). From active site-directed inhibition and labeling studies, an aspartic acid within the (G/F/Y)(L/I/V/M/F)WXDM(N/S/A)E consensus motif of GH31 (PROSITE PS00129) is demonstrated to function as the catalytic nucleophile (23, 24).

Within SI, SUC is the major α-1,2-glucosidase of the intestinal lumen (25), and IM is the main enzyme that hydrolyzes oligosaccharides including the α-limit dextrin at the α-1,6 linkages (17, 25). Both subunits together contribute to 60–80% of maltose hydrolysis in the intestine (17, 26); however, their individual specificity toward maltose digestion and their contribution to maltase-glucoamylase function are not defined yet. Based on sequence similarities, Asp⁵⁰⁵ in IM and Asp¹³⁹⁴ in SUC within the conserved motif of DGL-

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3 The abbreviations used are: SI, sucrase-isomaltase; CSID, congenital sucrase-isomaltase deficiency; GH31, glycoside hydrolases of family 31; SUC, sucrase; IM, isomaltase; endo H, endo-β-N-acetyl-glucosaminidase; MGAM, maltase-glucoamylase.

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WIDMNE (underlined) constitute the catalytic nucleophile residues in the active site (27, 28). By resolving the crystal structure of the N-terminal domain of SI, Asp604 in the HWLGDN motif has been predicted to function as the proton donor in the IM active site cleft for the acid/base catalysis (27). Crystal structure of the SUC domain is not resolved yet; however, based on its strong sequence homologies to the IM subunit, a potential proton donor residue in SUC can be predicted by \textit{in silico} analysis.

Characterization of the function of a single active site in either SUC or IM requires specific inactivation of the other subunit. Given the striking structural homology between SUC and IM and their conserved active sites, the inhibition of the catalytic activity of one subunit by a particular substance may also inhibit the other subunit (29). Elucidation of the functional capacities of SUC and IM by expression of the individual subunits is potentially possible. Nevertheless, such an approach ignores the complexity of the domain-domain interactions of SUC and IM during folding and acquisition of functional maturity and therefore does not reflect the native structure of the complete and intact SI (30).

In this study we have investigated the implication of the motif HWLGDN in the functional capacities of IM and SUC with particular emphasis on the two aspartic acid residues predicted to participate in the \(\alpha\)-glucosidase activity as proton donors. The study utilized site-directed mutagenesis of the individual aspartate residues. The generated mutants have provided a model to study enzymatic characteristics of IM and SUC without the functional overlapping of the other subunit.

Results and discussion

\textit{The aspartate-targeted mutants within the HWLGDN motif in SUC and IM are trafficked normally}

The nucleophilic catalytic residue of human SI is located in the DGLWIDMNE sequence, which is conserved among all members of the GH31 family of enzymes (12, 28, 31). By resolving the crystal structure of the N-terminal domain of human SI, Asp604 in the HWLGDN motif is predicted to function as the proton donor for the acid-base catalysis in IM (27) (Fig. 1). To experimentally validate the contribution of this residue to the sugar hydrolysis, Asp604 was exchanged by mutagenesis PCR to amino acid residues that have similar or different physicochemical characteristics as presented in Fig. 2. SUC reveals a striking sequence homology to IM; however, its crystal structure is not defined yet. Therefore, based on amino acid sequence alignment, we predicted Asp1500 in the consensus sequence of HWLGDN in SUC to function as the proton donor residue for hydrolysis (Fig. 2). This site was subjected to mutagenesis similar to that of IM. As a control, Asp505 in IM or Asp1394 in SUC within the conserved DGLWIDMNE catalytic site was mutated to glutamic acid.

The SI mutants were transiently expressed in COS-1 cells, and their biosynthetic forms were analyzed by endoglycosidase H (endo H) treatment and immunoblotting. As shown in Fig. 3, properly processed wild-type SI appears mainly as a complex \(N\)-and \(O\)-glycosylated 245-kDa endo H-resistant form (SIc) (32). This form represents the mature and functionally active SI that is properly processed in the Golgi apparatus (1). The minor endo H-sensitive band at \(\sim\)200 kDa corresponds to the immature mannose-rich ER form of SI (SIh) (1, 34). Analysis of the SI
mutants revealed essentially a similar electrophoretic pattern as that of the wild-type protein with a comparable ratio of SIc to SIh (Fig. 3). These results indicate that the biosynthesis, processing, and trafficking of the SI mutants were not altered because of the introduced mutations in the HWLGDN or DGLWIDMNE motifs. Similar relative protein expression levels of the mutants compared with SI wild type (Table 1) provide further evidence that the SI mutants are not subjected to any trafficking hindrances or proteolysis. Additionally, similar biosynthetic patterns strongly suggest that causal alterations on the protein folding of the mutants may have not occurred. Nevertheless, the possibility of partial misfolding cannot be completely excluded, because the exchanged amino acids in the SI mutants differ from aspartate in their physiochemical characteristics. We first addressed the folding of the SI-D604E and SI-D1500E mutants by comparing their tryptic digestion profiles to that of the wild-type SI, a procedure that has been frequently utilized to investigate the folding of the SI protein (30, 35). It is based on unique structural properties of SI that harbors 149 potential trypsin cleavage sites in its primary structure, and yet only one of these sites becomes exposed and cleaved by trypsin in the intestinal lumen when SI is properly folded (36). The cleavage of properly folded SI generates the two subunits, SUC and IM, which maintain their association through noncovalent interactions (32). The susceptibility of the SI mutants to trypsin can be assessed in immunoblots by antibodies specific to IM or SUC (Fig. 4). Given the large number of potential trypsin sites in the linear SI sequence, a different tryptic pattern would be anticipated in the case of an altered folding of SI. As shown in Fig. 4, mutations in the predicted proton donor residue Asp1500 of SUC did not affect the tryptic pattern of the SI mutants, neither in the IM nor in the SUC domains compatible with proper folding of these mutants. Likewise the D505E and the D1394E mutations in the nucleophilic residue did not induce alterations in the tryptic pattern of the SI mutants. However, SI mutants in Asp604, the predicted protein donor in the IM subunit, revealed different sensitivities toward trypsin. Although the folding of the SUC domain in all the Asp604 mutations did not change, the IM domain was affected except for the D604E mutation. These folding alterations in IM are likely not substantial, because the trafficking and maturation of the corresponding SI mutants were comparable with that of wild-type SI (Fig. 3). Nevertheless, we took into consideration these folding variations when analyzing the functional capacities of the SI mutants in which Asp604 has been mutagenized.

Inactivation of one subunit of SI by mutagenesis is not paralleled by loss or reduction in the functional capacity of the other

Having analyzed the SI mutants and determined that their maturation, post-translational processing, and trafficking were comparable with those of wild-type SI, we assayed the functional capacities of the mutants toward sucrose, maltose, and isomaltulose to determine the α-1,2-, α-1,4-, and α-1,6-hydro-
lytic activities, respectively. For this purpose, the mutants or wild-type SI were expressed in COS-1 cells, immunoprecipitated, and subjected to enzyme activity measurements. Fig. 5 shows that all mutant variants of SUC and IM at the potential proton donor residue revealed drastic reductions or even complete loss of enzyme activities of the targeted subunit. Thus the mutations targeting the Asp1500 residue of SUC including D1500E, D1500N, D1500S, and D1500Y became inactive toward /H9251-1,2-glucosidic linkages, the SUC preferred substrate configuration. The IM activity on the other hand was completely retained, and in comparison with wild-type SI, a slight to moderate reduction in the /H9251-1,4-hydrolytic activity of the SUC-based mutants was observed. This latter reduction strongly suggests that SUC contributes to the overall digestive capacity of maltase-glucoamylase in vivo.

Likewise, mutations of the Asp604 residue in IM entirely abolished the IM activity, reduced maltose hydrolysis, but did not lead to a loss or reduction in SUC (Fig. 5). The SI mutants that targeted Asp605 in IM and Asp1394 in SUC, both in the conserved well established DGLWDMDNE catalytic site, also revealed similar activity profiles as the SI mutants of HWLGDN. Taken together, these results support the view that predicted proton donor residues in HWLGDN are implicated in the enzyme function of SUC and IM.

We have aligned the amino acid sequence of 59 different members of the GH31 family and compared them for the stretch containing the proton donor residue in the human SI subunits. The data illustrate a conserved region surrounding the target aspartic acid residue (Fig. 6), suggesting a consensus motif in this family, most likely with a conserved function for the aspartic acid residue in its center as a proton donor for carbohydrate hydrolysis.

**Figure 4.** Tryptic structural analysis to determine the folding of SI catalytic site variants. Immunoprecipitants of wild-type SI or its variants prepared from transiently expressing COS-1 cells were subjected to 500 BAEE units of trypsin and detected by Western blotting in comparison with the untreated controls. Immunoblotting (IB) was either performed with the HBB2/614/88 antibody to detect the SUC subunit or the HBB3/705/60 antibody to detect the IM subunit.

**Figure 5.** Sucrase, maltase, and palatinase activities of SI active site mutants compared with the wild type. The SI variants were immunoprecipitated from cell lysates of transiently expressing COS-1 cells and assayed for their enzymatic function in hydrolyzing sucrose, maltose, or isomaltulose. The relative specific activities were calculated based on the SI protein content in each sample detected by Western blotting. The relative specific activities of SI normalized to the wild type are presented as means ± S.E. of at least four independent experiments.

**Contribution of SUC and IM to the activity of maltase-glucoamylase (MGAM)**

As mentioned earlier, SI shares the α-1,4-glucosidic activity in the intestine with its homologue disaccharidase MGAM. Because of its higher abundance in the intestine than MGAM, SI is assumed to be responsible for 60–80% of the maltase activity in the intestine (26, 37, 38); however, the contribution of each SI subunit to this activity is not defined yet. Based on the data presented in Fig. 5, single active SUC and IM respectively express ~80 and ~50% of the total maltase activity of the wild-type SI. Surprisingly, the sum of the SUC and IM maltase activities exceeds that recorded for the wild-type nonmutagenized SI species. Likewise, the hydrolysis of the sucrose or isomaltose substrates by the individual subunits SUC or IM respectively increases in the absence of an active form of the other subunit. We have therefore addressed the elevated catalytic capacity of the mutagenized SI species by analyzing their kinetic parameters in comparison with wild-type SI in hydrolyzing maltose. SI-D1500Y variant representing active IM and SI-D604Y variant representing active SUC species of SI were used for this purpose. Based on the Michaelis-Menten equation, the theore-
ical maximum velocity ($V_{\text{max}}$) calculated for SUC is comparable with that of wild type, whereas IM presents almost 60% of the wild-type $V_{\text{max}}$ in hydrolyzing maltose (Fig. 7). The affinity of the active sites to maltose is higher in intact SI as implied by a lower $K_m$ value, which is expected because of the presence of two active subunits in the wild-type protein. However, upon increasing substrate concentration, the rate of maltase activities is reduced, which indicates a gradual inhibitory function in correlation with maltose concentration. Different species show unique thresholds in responding to the inhibitory effect, among which the wild type is the most sensitive one. Analyzing the kinetics of maltose digestion of wild-type SI, SUC, and IM constructs with the Hill equation does not reveal any negative cooperativity between the functional subunits of SI (data not shown).

Potential product inhibitory mechanism may account for the increased activities of IM and SUC toward maltose

The observed inhibition cannot be exclusively considered as substrate inhibition, because the sensitivity of the tested constructs for the inhibitory effect appears at different concentrations of the substrate; i.e. $>4$ mM for wild type, $>10$ mM for IM (SI-D1500Y), and $>20$ mM for SUC (SI-D604Y). Therefore we evaluated a potential role for product inhibition in regulating SI catalytic function. For this purpose human intestinal brush border membrane preparations containing the fully active in vivo forms of SI and sucrose or isomaltose substrates were used to determine Michaelis-Menten kinetics in the presence or absence of excess glucose. As presented in Fig. 8, both SUC and IM activities are subjected to a functional inhibition in the presence of 0.7 mM glucose. Reduction in the $V_{\text{max}}$ value combined with an increased $K_m$ value indicates a mixed type of inhibition of SI activities in the presence of glucose, i.e. a combination of competitive and uncompetitive inhibitions. Competitive inhibition is compatible with the affinity of glucose to the active sites of SUC and IM. This type of inhibition is likely to occur at high glucose concentrations. The uncompetitive inhibition, on the other hand, is an allosteric inhibition of the catalysis by affecting the conversion of enzyme-substrate complex to the enzyme and the product. In total, the combination of these inhibitions reduces the catalytic efficiency of sucrase and isomaltase by almost 20% in comparison with the control sample (Fig. 8). In view of these data, the increased maltase capacity of IM or SUC variants in comparison with wild-type SI as presented in Fig. 5 can be explained by the microenvironmental concentrations of glucose. Appearance of functional inhibition is linked to a threshold concentration of glucose, which is achieved at similar activity levels for all constructs (Fig. 7). In the wild-type enzyme, both subunits are active, thus increasing the concentration of liberated glucose in the microenvironment with a higher rate in comparison with the variants with a single active subunit. IM or SUC constructs, on the other hand, reach this level of activity at higher substrate concentrations. Therefore collection of maltase activities from IM and SUC in separate reactions results in a higher value than that of wild-type SI.

The inhibitory characteristics of glucose on SI mimic the function of $N$-butyl deoxynojirimycin, an iminosugar analogue of $D$-glucose on intestinal SI (29, 39). In fact, we have previously reported that this iminosugar can inhibit intestinal $\alpha$-glucosidases including SI in a mixed inhibitory manner (29, 39). It can be postulated that $N$-butyl deoxynojirimycin shares the same allosteric binding site on SI with glucose. In a physiological context, product inhibition or negative feedback is a known
The inhibitory function of glucose on the SI activity is likely a regulatory mechanism in the body to control excess release of glucose in the intestine and so modulate the glycemic level in the blood. The average luminal concentration of glucose in the small intestine is estimated to range between 0.4 and 24 mM (42). Because of experimental limitations, the amount of glucose applied in this study belongs to the lowest levels of this range. Therefore it is plausible to propose a more effective role for this inhibitory function when the luminal concentration of the glucose is elevated by consumption of carbohydrate-rich diet.

**Conclusions**

This study demonstrates the implication of the sequence motif HWLGDN in SUC and IM in the functional capacities of the two enzymes, emphasizing the role of the two aspartic acid residues predicted to participate in the α-glucosidase activity as potential proton donor residues. Three-dimensional resolution of the IM subunit suggested a role of aspartate within the HWLGDN motif in the catalytic activity of IM (27). Additionally, sequence alignments illustrate that the stretch HWLGDN containing the predicted proton donor residue is also found in SUC and conserved among members of the GH31 family as a consensus motif (43). The current study provides direct experimental evidence on the role of this residue in the context of the HWLGDN motif and the SI function. The exchange of the predicted proton donor residue in IM and its counterpart in SUC in the overall enzyme function of SI. The (G/F/Y)(L/I/V/M/F)WXXDM(N/S/A)E pattern, including the nucleophilic aspartic acid, is a well known characteristic of the GH31 family of enzymes (consensus motif PS00129, PROSITE). Our data unequivocally demonstrate that (T/L/G/S/I/A)(G/Y)DXXX(W/Y/F) is a conserved motif in the GH31 family of enzymes that likely contains the proton donor aspartic acid for the catalysis in all of the members of this family. However, the latter pattern cannot be assigned as a consensus pattern unique to the GH31 family because it is also present in some other classes of proteins (e.g. kinases, helicases, cytochromes, etc.) as analyzed by ScanProsite online tool (44).

Another observation that sheds light on the interactive digestive functions of intestinal disaccharidases is the contribution of SUC, and to a lesser extent also IM, to the digestive capacity of MGAM. It has been proposed that SUC, by virtue of its digestive capacity toward α-1,4-glucosidic linkages, and partly also IM both hydrolyze substrates that are primarily predestined for MGAM. In fact, a reduction in SUC or IM is paralleled by partial loss of maltase activity, as demonstrated in many cases of CSID (5, 45). CSID-associated mutations exclusively alter protein folding, post-translational processing, trafficking, and also function of SI, i.e. SUC and IM. The data presented here verify and strengthen this view and demonstrate that abolishment of SUC or IM is also paralleled by substantial reductions of maltase digestion capacity in the intestine and at the same time shed light on the primordial role of SI in the overall digestion of starch and other carbohydrates.

Finally, the inhibitory function of glucose on the activity of human SI corresponds to the higher digestive rates of α-1,4-glucosidic linkages (MGAM-like) in individual SUC or IM versus the intact SI protein. This so far undescribed product inhibition of SI presumably functions as a regulatory mechanism to control glucose release from carbohydrate digestion in the intestine and balance the glycemic levels in the blood.
**Carbohydrate hydrolysis by human SI**

### Materials and reagents

Tissue culture dishes were obtained from Sarstedt (Nümbrecht, Germany). DMEM, penicillin, streptomycin, FCS, trypsin-EDTA, protease inhibitors, DEAE-dextran, and protein A-Sepharose were purchased from Sigma-Aldrich. Acrylamide, tetramethylmethylenediamin, SDS, Tris, DTT, PVDF membranes, sucrose, isomaltose, and maltose were purchased from Carl Roth GmbH (Karlsruhe, Germany). Glucose oxidase-peroxidase monoreagent was obtained from Axiom GmbH (Bürstadt, Germany). DMEM, penicillin, streptomycin, FCS, trypsin were obtained from Fisher Scientific. Isis proofreading DNA polymerase was purchased from MP Biomedicals. Endo H was acquired from Roche Diagnostics. All other reagents were of superior analytic grade.

### Immunochemical reagents

The anti-SI mAbs produced from the hybridoma cell lines HBB 1/691/79, HBB 2/614/88, HBB 2/219/20, HBB 3/705/60, and HSI 2 were provided by Dr. Hans-Peter Hauri (Biocenter, Basel, Switzerland) and Dr. Erwin Sterchi (University of Bern, Bern, Switzerland). A combination of these antibodies was used to detect different conformations and glycoforms of sucrase-isomaltase wild-type and mutant forms. Horseradish peroxidase-conjugated anti-mouse IgG was acquired from Thermo Fisher Scientific.

### Site-directed mutagenesis

Expression plasmids encoding sucrase-isomaltase (pSG8-SI) with amino acid substitutions of the target aspartic acid (Asp<sup>604</sup>, Asp<sup>505</sup>, Asp<sup>604</sup>, Asp<sup>1500</sup>, or Asp<sup>1394</sup>) in its catalytic site motifs were generated by site-directed mutagenesis. PCR was performed using Isis proofreading polymerase and different complementary mutagenic primers (Table 2) (7). PCR products were treated with DpnI restriction enzyme to remove the methylated template, then transformed into DH5α bacteria, and screened for the correct amino acid exchanges by DNA sequencing after plasmid preparation.

### experimental procedures

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**Carbohydrate hydrolysis by human SI**

### Table 2

Oligonucleotide primers used in site-directed mutagenesis for generation of aspartic acid SI variants

| Primer     | Sequence of mutagenesis primer (5′ → 3′) |
|------------|------------------------------------------|
| D605E forward | GACCTGGAGAAGTGGACATGGAAATGTCGTAATGGTCGCT |
| D605E reverse | TCTAGAAGTGGACATGGAAATGTCGTAATGGTCGCT |
| D604E forward | GATTTAGGAGAAGTGGACATGGAAATGTCGTAATGGTCGCT |
| D604E reverse | TCTAGAAGTGGACATGGAAATGTCGTAATGGTCGCT |
| D604N forward | TCTAGAAGTGGACATGGAAATGTCGTAATGGTCGCT |
| D604N reverse | TCTAGAAGTGGACATGGAAATGTCGTAATGGTCGCT |
| D604S forward | TCTAGAAGTGGACATGGAAATGTCGTAATGGTCGCT |
| D604S reverse | TCTAGAAGTGGACATGGAAATGTCGTAATGGTCGCT |
| D604Y forward | TCTAGAAGTGGACATGGAAATGTCGTAATGGTCGCT |
| D604Y reverse | TCTAGAAGTGGACATGGAAATGTCGTAATGGTCGCT |
| D604N forward | TCTAGAAGTGGACATGGAAATGTCGTAATGGTCGCT |
| D604N reverse | TCTAGAAGTGGACATGGAAATGTCGTAATGGTCGCT |
| D604Y forward | TCTAGAAGTGGACATGGAAATGTCGTAATGGTCGCT |
| D604Y reverse | TCTAGAAGTGGACATGGAAATGTCGTAATGGTCGCT |

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- Glucose oxidase-peroxidase monoreagent was obtained from Axiom GmbH (Bürstadt, Germany).
- Restriction enzymes, molecular weight standards for SDS-PAGE, and SuperSignal<sup>™</sup> West Femtometric maximum sensitivity Western blot substrate were obtained from Thermo Fisher Scientific. Isis proofreading DNA polymerase was purchased from MP Biomedicals. Endo H was acquired from Roche Diagnostics. All other reagents were of superior analytic grade.

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- PCR products were treated with DpnI restriction enzyme to remove the methylated template, then transformed into DH5α bacteria, and screened for the correct amino acid exchanges by DNA sequencing after plasmid preparation.

**Cell culture and transient transfection**

- COS-1 cells were cultured in DMEM containing 1000 mg/liter glucose supplemented with 10% (v/v) FCS, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. The cells were maintained at 37°C in humidified incubators with 5% CO₂. Transfection with SI constructs was performed by the DEAE-dextran method as described before (46). 2 days after transfection, the cells were lysed and used for experiment.

**Cell lysis, immunoprecipitation, deglycosylation, and tryptic structural analysis**

- Transfected COS-1 cells were solubilized in 25 mM Tris-HCl, pH 8.0, containing 50 mM NaCl, 0.5% Triton X-100 (v/v), 0.5% sodium deoxycholate, and a protease inhibitor mixture for 1 h at 4°C. After removing the cell debris with cold centrifugation, lysates were subjected to protein A-Sepharose beads preconjugated with a combination of monoclonal anti-SI antibodies and rocked for 1 h at 4°C. The immunoprecipitants were washed successively with wash buffer 1 (0.5% Triton X-100 and 0.05% deoxycholate in PBS) and wash buffer II (125 mM Tris, 10 mM EDTA, 500 mM NaCl, 0.5% Triton X-100, pH 8.0) each two times. Immunoprecipitants were treated with endo H for deglycosylation experiments as described previously (47) or subjected to partial proteolysis with 500 BAEE units of trypsin for 1 h at 37°C to analyze the SI structure.

**SDS-polyacrylamide gel electrophoresis and Western blotting**

- Immunoprecipitated SI was boiled in Laemmli buffer containing 100 μM DTT for 5 min at 95°C, and proteins were resolved on 6% polyacrylamide gels and wet-transferred to a PVDF membrane at 240 mA for 100 min. After blocking with 5% milk in PBS, the membranes were used for immunoblotting with primary antibodies either HBB2/705/60 detecting the isomaltase subunit or HBB3/705/60 detecting the aspartic acid subunit of SI or HBB614/88 recognizing the sucrose subunit followed by incubation with goat anti-mouse IgG antibody conjugated with horseradish peroxidase. Protein bands were visualized via enhanced chemiluminescent peroxidase substrate and documented with a ChemiDoc XRS System (Bio-
Measurement of enzymatic activities

Immunoprecipitated SI was washed three times with PBS, pH 6.2, and incubated with maltose (100 mM), sucrose (75 mM), or isomaltulose (100 mM) substrates for 1 h at 37 °C. Activities were calculated based on liberated glucose determined by glucose oxidase-peroxidase monoreagent method with calorimetric measurement at 492 nm. Activities were correlated to the relative amount of SI protein in each sample that was detected by Western blotting. Relative specific activities are reported in comparison with the wild type.

To determine the kinetics of maltose digestion by different SI variants, COS-1 cells transiently expressing SI mutants or wild-type protein were collected in phosphate-citrate buffer, pH 6.2, and homogenized by multiple passages through a 0.4-mm syringe. Cell debris were removed by centrifuging at 2000 relative centrifugal force for 5 min at 4 °C, and the homogenate was used to determine Michaelis-Menten kinetics as described before (29). Catalytic efficiency of SI in hydrolyzing sucrose or isomaltose was calculated by dividing V_{max} by K_{m} for each condition and is presented as a ratio of the control sample as a percentage.

To analyze the effect of glucose on the function of SI, brush-border preparation of the human intestine was used as enzyme source, and the kinetics of sucrose and isomaltose hydrolysis by SI in the presence or absence of 0.7 mM glucose was determined based on Michaelis-Menten method as described before (29). Each unit is equal to the amount of enzyme activity that liberates 1 μmol glucose/h.

Statistical analysis and bioinformatics

Statistical tests were performed using Microsoft Excel software. Michaelis-Menten plots were generated by Microsoft Excel software or GraphPad Prism. The data were analyzed by paired Student’s t test and are presented as means ± S.E. of values from at least three independent experiments. Statistical significance is depicted as * for p < 0.05, ** for p < 0.01, and *** for p < 0.001. Polypeptide sequence alignments were performed by PRALINE multiple sequence alignment (48), and the amino acid sequence logo was generated by the WebLogo online tool (49). Protein three-dimensional structures were visualized and aligned by UCSF Chimera software (50). The crystal structure of isomaltase subunit (PDB code 3LPO) (27) was used for alignment. Comparative modeling of the three-dimensional structure of sucrase subunit was performed by MODELLER (33) based on its homology to glucoamylase subunit of intestinal maltase-glucoamylase protein (PDB code 3TOP) (51).

Author contributions—B. G. and N. S. performed the experiments, analyzed the results, and drafted the manuscript. M. A. performed the experiments and analyzed the results. M. A. and H. Y. N. designed the study, interpreted the results, and drafted the manuscript.

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