Sucrose 6-α-D-Glucosyltransferase from *Streptococcus sobrinus*: Characterization of a Glucosyl–Enzyme Complex†

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ABSTRACT: A covalent glucosyl–enzyme was isolated from a quenched reaction of *Streptococcus sobrinus* sucrose 6-α-D-glucosyltransferase and radiolabeled sucrose. No complex was observed with heat-inactivated enzyme or when sucrose was replaced with radiolabeled maltose or glucose. The complex was stable at pH 2 in 1% sodium dodecyl sulfate, 6.0 M urea, and 4.0 M guanidine hydrochloride, but became increasingly labile with increased pH (32-min half-life at pH 7.0). D-Glucose was the exclusive radiolabeled compound identified when all radioactivity was released under mild alkaline conditions. Glucosyl–enzyme hydrolysis rates were linearly dependent on hydroxide ion concentration, giving a second-order rate constant of 2.15×10^5 M^{-1} min^{-1}. When compared to the base lability of known glycosyl amino acid derivatives, the pH dependency of the glucosyl–enzyme most closely paralleled a glucosyl linkage to a carboxyl group. A novel application of a carbohydrate high-performance liquid chromatography column in aqueous solution was used to identify the anomeric form of D-glucose released on (i) alkaline hydrolysis of denatured glucosyl–enzyme and (ii) native enzyme hydrolysis of sucrose. The β-anomer was identified in the former case and the α-anomer in the latter. The results with the denatured glucosyl–enzyme are consistent with a β-glucosyl ester linkage to an aspartic or glutamic acid that hydrolyzes at the ester carbon with retention of anomeric configuration; for native glucosyltransferase catalysis, the data are consistent with a β-glucosyl covalent intermediate as well, where deglucosylation occurs by attack at the acetal carbon with anomeric inversion. However, the glucosyl–enzyme complex could not be renatured to demonstrate catalytic competence, leaving open the possibility that the covalent bond formed during collapse of the enzyme active site. While the glucosyl bond was extremely base labile, it was found to be sufficiently stable at low pH to survive pepsin proteolytic cleavage. Active-site labels for this family of oral bacterial glycosyltransferases are virtually unknown, and the complex may be a useful probe to study the structure of the enzyme active site.

Cariogenic oral streptococci secrete several glycosyltransferases that use sucrose as a high-energy substrate to synthesize glucose and fructose polymers (Mukasa, 1985). The polysaccharides serve as structural support in dental plaque to aid bacterial colonization of smooth enamel surfaces (Gibbons & Banghart, 1967) and are largely responsible for the exceptional cariogenicity of the bacteria and of sucrose (Hamada & Slade, 1980).

Streptococcus sobrinus releases at least three glucosyltransferases: two synthesize α-1,6-linked water-soluble glucans (dextran), which differ in glucan affinity and degree of α-1,3 branching, and a third synthesizes an α-1,3-linked water-insoluble glucan (Shimamura et al., 1983). The *S. sobrinus* glucosyltransferase used in this study (sucrose:1,6-α-D-glucan 6-α-D-glucosyltransferase, EC 2.4.1.5) synthesizes a highly branched α-1,6-linked water-soluble glucan and is commonly referred to as GTF-S or simply GTF.[1]

The enzyme catalyzes glucosyl transfer from sucrose to dextran (dextran synthesis) and from sucrose to water (sucrose hydrolysis) and isotope exchange between fructose and sucrose (Mooser et al., 1985). Two lines of evidence suggest transfer involves a glucosyl–enzyme intermediate: The reaction shows retention of glucose anomeric configuration from substrate to product, implying a double-displacement mechanism (Koshland, 1954), and a partial reaction of isotope exchange between fructose and sucrose follows ping-pong kinetics. The data do not, however, distinguish between a covalent and a noncovalent form of the glucosyl–enzyme. Covalent enzyme–substrate complexes are best evaluated when isolation does not require irreversible enzyme denaturation, since demonstrating kinetic competence by returning the complex to reaction conditions is a strong criterion of authenticity (Purich, 1982). However, water is a very efficient glucosyl acceptor for GTF (Goodman et al., 1955; Luzio & Mayer, 1983; Mooser et al., 1985), making stabilization of a transient intermediate difficult in aqueous solution. As an alternative, we trapped a glucosyl–enzyme by acid denaturing a steady-state reaction of enzyme and radiolabeled sucrose. Data on the reactivity and stereochemistry of the complex provided a basis to draw conclusions about the active-site amino acid linked to glucose and the steric form of the glycosidic linkage.

Experimental Procedures

Materials. [U-^{14}C]Sucrose (673.0 mCi/mmol), [U-^{14}C]-glucose (329 mCi/mmol), [U-^{14}C]maltose (360 mCi/mmol), and [*glucosyl*-^{14}C]sucrose (200 mCi/mmol) were obtained from New England Nuclear. Guanidine hydrochloride (Gdn-HCl) was of ultrapure grade from Schwarz-Mann. α-D-Glucose and β-D-glucose were from Applied Science Laboratories, Inc. Twice crystallized porcine stomach pepsin was from Sigma Chemical Co. Water and acetonitrile used in high-performance liquid chromatography (HPLC) were HPLC-grade reagents from J. T. Baker Chemical Co., and

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[1] Abbreviations: GTF, sucrose:1,6-α-D-glucan 6-α-D-glucosyltransferase; Gdn-HCl, guanidine hydrochloride; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography.

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trifluoroacetic acid (TFA) was of ionate grade from Pierce Chemicals.

Preparation and Assay of Streptococcus sobrinus Glucosyltransferase. Glucosyltransferase was isolated from the culture broth of *S. sobrinus* (American Type Culture Collection *S. mutans* 6715-7) grown in the chemically defined media of Terleckyj et al. (1975). The enzyme was purified by affinity chromatography as previously described (Mooser et al., 1985). The maximum velocity at saturating concentrations of dextran T-10 (Pharmacia Fine Chemicals) and sucrose was 36.9 μmol min^{-1} mg^{-1} at pH 6.0, 37 °C. The enzyme synthesized water-soluble glucan only. Initial velocity enzyme assays were based on glucosyl transfer from [U-^{14}C]sucrose to dextran using a modified procedure of Chludzinski et al. (1974).

High-Performance Liquid Chromatography. HPLC equipment included Waters Associates Model 6000A and 510 pumps, Model 680 solvent programmer, and Model 441 absorbance monitor and Altex Model 156 refractive index monitor. Gel filtration chromatography was performed with a Bio-Sil TSK-250 column (300 mm × 7.8 mm) from Bio-Rad Laboratories. Fractions were eluted with 0.1% TFA, pH 2.0, at 1 mL/min, room temperature. Carbohydrate separations were performed on a Bio-Rad Aminex HPX-87C carbohydrate analysis column (300 mm × 7.5 mm) and monitored by refractive index. When injected samples contained protein or protein-bound radioactivity, it was retained on a guard column and did not interfere with carbohydrate detection. Glucose, fructose, and sucrose were resolved by elution with water at 0.6 mL/min, 85 °C; glucose anomers were resolved under the same conditions at 25 °C. Peptide separation was by reverse phase with a Vydac 5 μC$_4$ column (250 mm × 4.6 mm) eluted at 1 mL/min by using a linear gradient of solvent B at 1%/min. Solvent A was 0.1% trifluoroacetic acid, and solvent B was 0.1% trifluoroacetic acid in 90% acetonitrile.

Preparation of Glucosyl–Enzyme. Glucosyltransferase (0.1–1.2 mg) was lyophilized and then redissolved in 100 μL of 0.05 M phosphate–citrate buffer, pH 6.0. A 1-μL aliquot was removed, diluted 100-fold, and then assayed for dextran synthesis activity. Generally 10–20% of the activity was lost on lyophilization. The enzyme was added to 100 μL of [U-^{14}C]sucrose (300 μCi) in the enzyme buffer and mixed with a vortex pulse. The final sucrose concentration was 2.23 mM. After 6 s, 500 μL of 2% formic acid (pH 2.0) was added and rapidly mixed. The solution was loaded on a Bio-Sil TSK-250 column eluted with 0.1% TFA at 1.0 mL/min and monitored at 280 nm. Fractions were collected at 1-min intervals. Protein-bound radioactive fractions eluted before the large amount of free radioactivity. At times, two or three trailing protein fractions were chromatographed a second time to remove a small amount of contaminating free radioactivity. Protein-bound radioactivity was stable for at least 2 months at −20 °C in 0.1% TFA, 2% formic acid, or lyophilized.

Preparation of β-D-[^{14}C]Glucose. Approximately 1 μmol containing 1 μCi of mutarotated [^{14}C]glucose in 50 μL of water was loaded on an Aminex HPX-87C carbohydrate analysis column, eluted with water at 0.6 mL/min, 25 °C. Elution was monitored by refractive index, and fractions were collected in approximately 50-μL aliquots. The samples were immediately frozen in a dry ice/acetone bath, lyophilized, and stored desiccated at −20 °C. Reconstitution of a sample in water and rechromatography showed that approximately 96% was β-D-glucose.

Measurement of Mutarotation Rates. A 0.1 M solution of α-D-glucose was prepared in HPLC-grade water. At timed intervals, 20 μL was removed and loaded on the carbohydrate analysis column. The relative refractive index was recorded on a Shimadzu C-R3A integrating recorder to quantify α- and β-D-glucose peaks. The magnitude of the separate anomers was the same within the error of the chromatography system.

The degree of D-glucose mutarotation (M) was calculated by using eq 1, where the subscripts represent the relative

$$M = \frac{\alpha_t - \alpha_e}{\alpha_0 - \alpha_e} = \frac{\beta_t - \beta_e}{\beta_0 - \beta_e} \quad (1)$$

amounts of α- or β-anomer in the initial solution (0), at a given time (t), and at equilibrium (e). The mutarotation rate constant, k_m, was calculated according to the integrated reaction velocity equation for first-order reversible unimolecular equilibria (Isbell & Pigman, 1937)

$$k_m = k_f + k_r = 1/t \ln M \quad (2)$$

where k_f and k_r are the respective forward and reverse first-order rate constants for anomeric conversion.

General Procedures. Protein was assayed according to the method of Bradford (1976) using bovine γ-globulin as a standard. Radioactivity was measured with a Beckman Model LS 8000 liquid scintillation counter. Descending paper chromatography was performed by using Schleicher & Schuell 2043a chromatography paper developed with 1-butanol/glacial acetic acid/water (4:1:1 v/v) for 48 h. Thin-layer chromatography was performed with 10 × 10 cm HPTLC cellulose plates from EM Science. Radioactive samples were concentrated and spotted in 0.2-μL aliquots and then developed horizontally for 2–3 h with 1-butanol/acetic acid/water (4:1:1 v/v). Radiolabel on paper or thin-layer chromatograms was detected by autoradiography with Kodak DF-65 X-ray film. When radioactivity was quantified from paper chromatograms (analysis of the amount of sucrose utilized in preparation of glucosyl–enzyme), radioactive areas identified by autoradiography were cut from the chromatogram and counted.

Results

Preparation of Glucose–Enzyme Complex. A protein-bound radioactive fraction was among the products of a 200-μL reaction of 2.23 mM [U-^{14}C]sucrose and 1 mg of GTF that was quenched with acid 6 s after initiating the reaction. Free and protein-bound radioactivities were separated by size exclusion HPLC in 0.1% TFA (Figure 1). Cellulose paper chromatography of the free counts showed that approximately 20% of the sucrose had reacted. Both active enzyme and native substrate were necessary to produce protein-bound radioactivity. None was detected when GTF was heat inactivated (75 °C, 1 h), replaced with 1.0 mg of bovine serum albumin, or when [U-^{14}C]sucrose was replaced with 3 mM [U-^{14}C]maltose or [U-^{14}C]glucose.

Protein-bound radioactivity was labile under mild alkaline conditions. Exposing the protein to pH 10.0 for 10 min resulted in nearly total loss of protein-associated radiolabel. However, with the pH maintained below 2.5, the complex was stable in 6.0 M urea, 1.0% SDS, 4.0 M Gdn-HCl, and 1.0 M NaCl (Table I).

Alkaline-released radioactive fractions were collected from the size exclusion column, neutralized, concentrated, and rechromatographed on a Aminex HPX-87C carbohydrate analysis column at 85 °C. All radioactivity eluted coincident with D-glucose (Figure 2). To confirm identification, the radioactive fraction was allowed to mutarotate for 5 h and rechromatographed on the carbohydrate column at 25 °C. Two radioactive peaks eluted coincident with carrier α- and

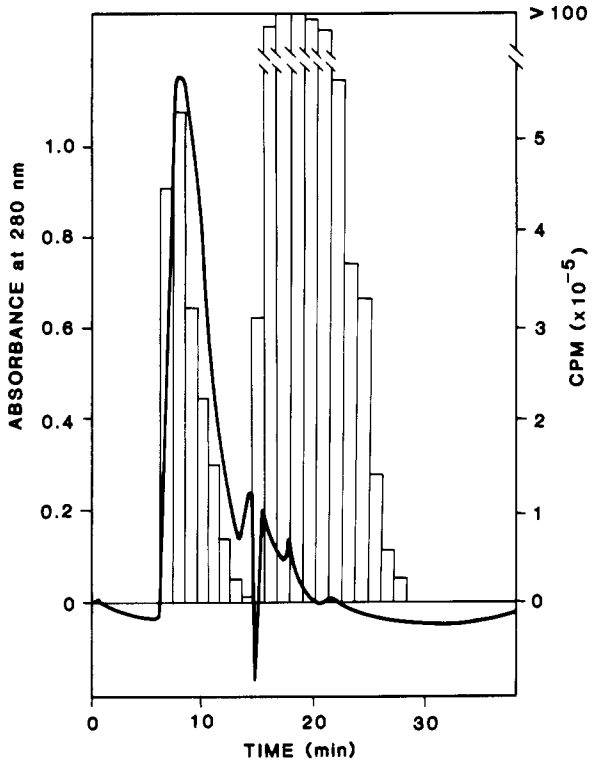

FIGURE 1: Size exclusion HPLC separation of free and protein-bound radioactivity from an acid-quenched steady-state reaction of 1 mg of GTF and 2.23 mM [U-^{14}C]sucrose (300 μCi). The reaction products were eluted from a Bio-Sil TSK-250 column with 0.1% TFA (pH 2.0) at 1 mL/min. Elution was monitored at 280 nm (solid line), and collected fractions were sampled to measure radioactivity (histogram). The large excess of unbound radiolabel eluted after the protein at approximately 15 min. Complete experimental details are given under Experimental Procedures.

Table I: Solvent Effects on GTF-Bound Radioactivity[a]

solvent	pH	% bound[b]
2% formic acid	2.0	96
1% sodium dodecyl sulfate/2% formic acid	2.0	96
6.0 M urea/2% formic acid	2.5	83
4.0 M Gdn-HCl/2% formic acid	1.7	92
1.0 M sodium chloride/2% formic acid	2.0	88
0.01 M sodium phosphate	10.0	3

[a] Approximately 3000 cpm of enzyme-bound radioactivity was incubated for 1 h in 200 μL of each solvent at 25 °C. Free and protein-bound radioactivities were quantified after separation on a TSK-250 column eluted with 0.1% TFA at 1 mL/min. [b] Percent bound radiolabel is the radioactivity in the protein-associated fractions relative to total eluted radioactivity, which was commonly about 95% of the injected material.

β-D-glucose at a ratio of 37.7:62.3 (α:β), consistent with previously reported values (38:62; Angyal, 1975). This and related experiments are discussed in greater detail in a later section.

The range of specific activities of five preparations of glucose–enzyme complex was 479 000–724 000 cpm/mg of protein, which represents approximately 0.07–0.10 mol of glucose/mol of enzyme. The results are consistent with selective substitution at a single site on the enzyme, but currently available kinetic data are not sufficient to determine if the value is consistent with the theoretical steady-state level of a transient covalent or noncovalent glucosyl–enzyme.

pH Dependency of the Glucose–Enzyme Linkage. The pH dependency of the glucose–enzyme bond was measured by incubating the complex in five solutions covering a pH range of 6.5–7.9. At periodic intervals aliquots were sampled and

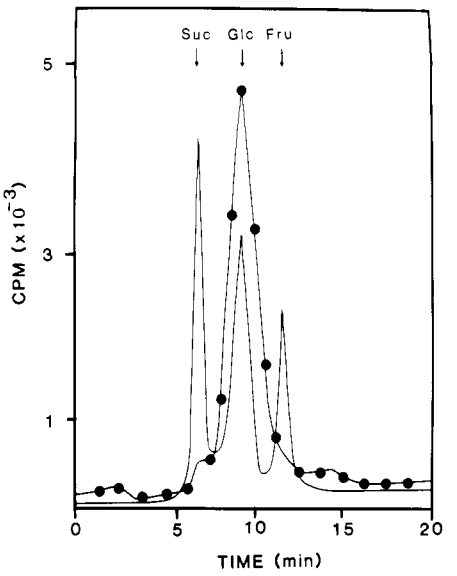

FIGURE 2: Chromatographic elution of sucrose, glucose, fructose, and the radioactive component released from the enzyme complex. Protein-bound radioactivity was hydrolyzed from the complex by incubation of approximately 15 000 cpm at pH 10 for 10 min. Unlabeled sucrose (Suc), glucose (Glc), and fructose (Fru) (1 μmol each) were added as carrier. The sample was chromatographed on an Aminex HPX-87C HPLC carbohydrate analysis column eluted with water at 0.6 mL/min, 85 °C. The elution profile shows radioactivity in each fraction (●) and relative refractive index of carbohydrate standards.

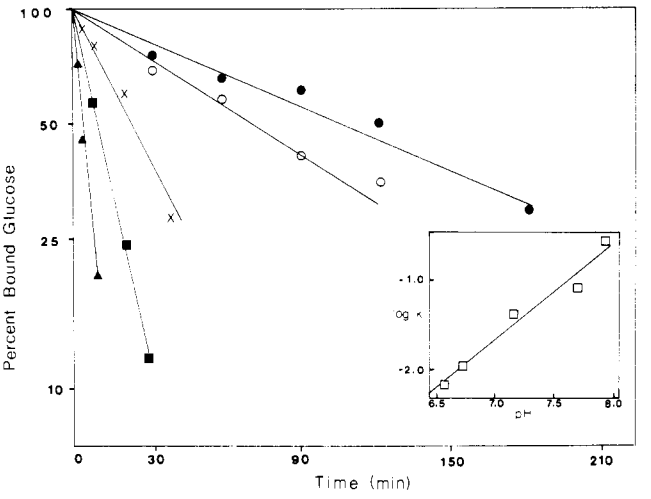

FIGURE 3: pH dependency of the glucose–enzyme bond. Approximately 12 000 cpm of protein-bound radiolabel was lyophilized and then reconstituted in 135 μL of 0.05 M phosphate–citrate buffer at 25 °C. The pH of five incubation mixtures was 6.55 (●), 6.72 (○), 7.15 (×), 7.68 (■), and 7.92 (▲). At selected time intervals, 20-μL aliquots were removed and added to 100 μL of 2% formic acid to halt hydrolysis of the glucose–enzyme bond. Free and protein bound radioactivities were separated and quantified. The pH of each mixture was determined from the remaining volume of each reaction. Zero-time and completely hydrolyzed controls were prepared by adding buffer at pH 2.0 in the former case and 1-h incubation at pH 10 in the latter. Percent bound radioactivity was calculated as $100(B_t - B_h)/(B_0 - B_h)$, where B_0 is bound radioactivity measured in the zero-time control, B_t is bound radioactivity at a given time, and B_h is bound radioactivity in the alkaline-hydrolyzed sample. The insert shows the log of the pseudo-first-order rate constants, k, plotted as a function of pH.

hydrolysis was halted by lowering the pH with 2% TFA. Free and protein-bound radioactivities were separated, counted, and plotted as the logarithm of the percentage of bound counts as a function of time (Figure 3); the linear slopes are consistent with a pseudo-first-order reaction. A replot of the logarithm

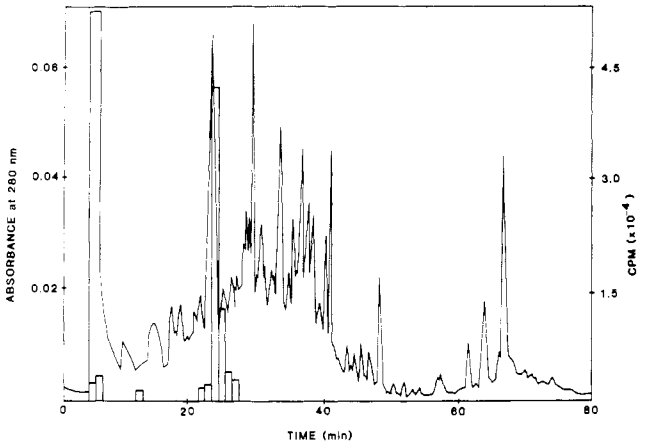

FIGURE 4: Elution profile of pepsin digest of glucosyl–enzyme. Approximately 0.15 mg of enzyme complex with 83 000 cpm bound glucose was digested with 15 μg of pepsin in 50 μL of 0.1% TFA for 18 h. The sample was chromatographed on a Vydac 5 μm C_4 reverse-phase column eluted at 1 mL/min with a 0.9%/min gradient of acetonitrile and a constant concentration of 0.1% TFA. Absorbance at 280 nm is shown by the solid line and radioactivity by the superimposed histogram.

of the rate constants as a function of pH was also linear with a slope of 1.07 ± 0.11 (insert, Figure 3). The calculated second-order rate constant was 2.15 ± 0.25 × 10^5 M^{-1} min^{-1}.

From these data it is possible to compare the relative glucosyl-transfer efficiency of native enzyme and glucose–enzyme complex. One micromole of glucose–enzyme complex at pH 6.0 releases glucose at a rate of 2.15 × 10^{-3} μmol min^{-1}. By comparison, 1 μmol of sucrose-saturated enzyme at the same pH catalyzes sucrose hydrolysis considerably more rapidly at 4.8 × 10^2 μmol min^{-1} (Mooser et al., 1985). The latter, which represents all rate-dependent steps in sucrose hydrolysis, is over 5 orders of magnitude more rapid than deglucosylation of the denatured glucose–enzyme complex. In a subsequent section where the anomeric form of glucose released from the complex and from sucrose are compared, it becomes clear that the large difference in rates does not simply represent a severely compromised active site but a fundamentally different mechanism of deglucosylation.

Pepsin Cleavage of Glucose–Enzyme. We hydrolyzed the glucose–enzyme complex with pepsin at pH 2.0 to determine if the glucose linkage would survive proteolysis. Peptide products of extensive cleavage (10% pepsin w/w, 24 h) were chromatographed by reverse-phase HPLC (Figure 4). A small amount of radioactivity eluting in the initial column volume was identified as free glucose. The majority of the radioactivity eluted later in the gradient as a peak associated with peptides. The peak was concentrated and exposed to pH 10 for 10 min to verify that the fractions contained glucose-bound peptides. Of 31 000 cpm treated in this manner, 28 321 cpm eluted coincident with D-glucose on the carbohydrate analysis column.

Anomeric Form of D-*Glucose following Alkaline Hydrolysis of Glucose–Enzyme Complex.* Glucose anomers were separated in aqueous solution on a carbohydrate analysis HPLC column. Preliminary experiments showed that authentic α- and β-D-glucose elute almost exclusively as their respective anomers, and mutarotation rates can be followed by sampling and chromatographing aliquots over time. The rate of change in anomer distribution, applied to the equation for a reversible unimolecular reaction, gave a mutarotation rate constant of 0.028 min^{-1} ($t_{1/2}$ = 24.8 min), consistent with previously reported values (0.024 min^{-1}; Macbeth & Traill, 1925; Lee et al., 1969). This result establishes the chromatographic method

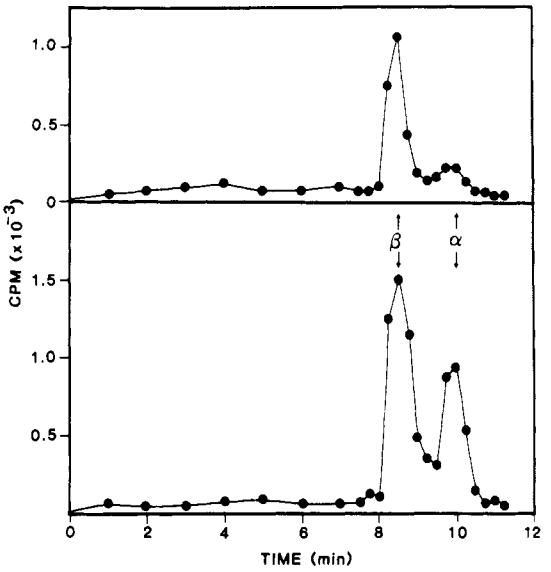

FIGURE 5: Distribution of D-glucose anomers following alkaline hydrolysis of glucosyl–enzyme linkage. (Top) A 210-μL sample containing 25 000 cpm bound glucose in 2% formic acid was cooled to −5 °C and then 40 μL of 0.4 M Na_3PO_4, pH 13.2, at the same temperature was added and rapidly mixed. A 50-μL aliquot was sampled after 6 s and loaded on an Aminex HPX-87C carbohydrate analysis column eluted with water at 25 °C. Fractions were collected and radioactivity measured (●). Approximately 75% of the glucose was released. (Bottom) Chromatographic elution profile of mutarotated [^{14}C]glucose after incubation under the same conditions as the glucosyl–enzyme preparation. The positions of α- and β-D-glucose elution are indicated on the graph.

as a valid measure of D-glucose anomer distribution.

D-Glucose mutarotation half-life was less than 5 min at pH 7.5 in 100 mM sodium phosphate, 25 °C. This is more rapid than the rate of glucose release from glucose–enzyme complex at the same pH and eliminates these conditions form consideration. However, the rate decreased substantially with decreased temperature; in distilled water D-glucose mutarotation half-life increased from 24.8 min at 25 °C to 292 min at 4 °C (Iwaoka, 1983). This presented the possibility of lowering the temperature to reduce mutarotation and increased pH to accelerate glucose release from the complex.

We cooled a glucose–enzyme sample in 2% formic acid to −5 °C and then rapidly added 0.4 M Na_3PO_4, pH 13.2, at the same temperature. After 6 s, an aliquot was removed and loaded onto the carbohydrate analysis column. Figure 5 shows the elution profile with the anomeric distribution significantly enriched in the β-form; the calculated percent β was 86.1 ± 2.7%, compared to 60.4 ± 1.4% for mutarotated D-glucose under the same conditions. The value agrees favorably with 88.6 ± 0.2% β-anomer found when glucose–enzyme was replaced with authentic β-D-glucose under identical reaction conditions (Table II).

The results establish covalent linkage of glucose to the enzyme through C-1; the glucose–enzyme complex can now more accurately be referred to as a glucosyl–enzyme complex.

Anomeric Form of D-*Glucose following Native GTF-Catalyzed Sucrose Hydrolysis.* The anomeric form of D-glucose released following GTF catalysis of sucrose hydrolysis has not been determined, although the α-form is predicted on the basis of retention of configuration observed with dextran synthesis. Nonetheless, it was important to firmly establish the form to compare with the results found with glucosyl–enzyme base hydrolysis.

A GTF reaction mixture was initiated with [*glucosyl*-^{14}C]sucrose. After 30 s, an aliquot was sampled and chro-

Table II: D-Glucose Anomer Distribution following Alkaline Hydrolysis of Glucosyl–Enzyme Complex and Sucrose Hydrolysis by Native Glucosyltransferase

treatment	temp (°C)	anomer %[a] α	anomer %[a] β
glucosyl–enzyme, alkaline treated[b]	−5	13.9 ± 2.7	86.1 ± 2.7
β-D-glucose, alkaline treated[c]	−5	11.4 ± 0.2	88.6 ± 0.2
native glucosyltransferase, sucrose hydrolysis[d]	25	92.4 ± 0.8	7.6 ± 0.8
D-glucose, mutarotated[e]	−5	39.6 ± 1.4	60.4 ± 1.4
D-glucose, mutarotated[e]	25	37.7 ± 0.6	62.3 ± 0.6

[a] D-Glucose anomers were separated on an Aminex HPX-87C column by elution with water, 0.6 mL/min, 25 °C. Glucose in all samples was ^{14}C-labeled. Carrier mutarotated D-glucose was in the syringe prior to loading each sample on the column. Elution was monitored by refractive index, and radioactivity was measured in collected fractions. Values are the average ± SE of three experiments. [b] Treated as described in Figure 5 (top). [c] Treated identically with alkaline-hydrolyzed glucosyl–enzyme except β-D-[^{14}C]glucose replaced the enzyme complex. [d] A 160-μL glucosyltransferase reaction in 0.05 M phosphate–citrate buffer, pH 6.0, was initiated with [*glucosyl*-^{14}C]sucrose. After 1 min, a 50-μL aliquot was sampled and loaded on the carbohydrate analysis column. [e] Mutarotated [^{14}C]glucose was incubated at ambient temperature for 18 h in the solution used in the alkaline-hydrolyzed samples. The solutions were then incubated for 5 h at the respective temperatures.

matographed on the carbohydrate analysis column. D-Glucose was significantly enriched in the α-anomer, consistent with the stereochemistry of dextran synthesis. These data, however, differ from those of the β-anomer observed on hydrolysis of denatured glucosyl–enzyme (Table II).

Attempts To Transfer Glucose from Glucosyl–Enzyme to Dextran or Fructose. Kinetic competence in transferring glucose in the reaction-completing step can be compelling evidence of authenticity of a covalent intermediate. Therefore, even though glucose transfer from glucosyl–enzyme is characteristically different from glucose transfer by native enzyme, we examined the potential of dextran and fructose to serve as glucosyl–enzyme acceptors. A solution of 10% dextran T-10 or 0.1 M fructose was incubated with 20000 cpm of lyophilized glucosyl–enzyme in 0.05 M phosphate–citrate buffer, pH 6.0 at 25 °C. After 2 h, the pH of the mixture was raised to 10.0 to release remaining protein-bound radioactivity that might comigrate and interfere with detection of radiolabeled dextran. Product analysis by cellulose thin-layer chromatography and autoradiography showed radioactivity migrating coincident with D-glucose, but neither [^{14}C]dextran in one reaction nor [*glucosyl*-^{14}C]sucrose in the other was detected.

Discussion

A glucosyl–enzyme intermediate is supported by GTF glucosyl transfer with retention of anomeric configuration and by isotope-exchange kinetics. The complete kinetic pattern is a hybrid rapid equilibrium random/ping-pong mechanism where glucosyl transfer from sucrose to dextran is rapid equilibrium random, but a partial reaction of exchange between sucrose and fructose follows classical ping-pong kinetics (Mooser et al., 1985). The isotope-exchange rate is more rapid than glucosyl transfer from sucrose to dextran, as expected for a partial exchange reaction (Purich, 1982), and, when the exchange and glucosyl transfer to water are monitored concurrently, sucrose utilization partitions between the reactions as a function of fructose concentration (Mooser et al., 1985). This demonstrates that fructose exchange occurs at the same site as GTF glucosyl transfer and does not result from enzyme or substrate contamination. Ping-pong kinetics is commonly associated with a double-displacement mechanism, but direct displacement cannot be discounted since a very small kinetic term can make a transient enzyme form undetectable. However, for this exchange reaction, where fructose and sucrose bind to the same site (on the basis of relatively high affinity of both reactants and structure similarities), direct displacement is improbable since the fructosyl moiety of sucrose must leave the active site before acceptor fructose binds (Jencks, 1969). The data support a glucosyl–enzyme along the reaction pathway, but the current information does not distinguish between glucosyl–enzyme forms or between S_N1 and S_N2 mechanisms since both a covalent complex and a tightly bound oxocarbonium can account for the results.

Isolation of a denatured covalent glucosyl–enzyme brings additional information to the GTF catalytic process. Formation of the complex is dependent on enzyme catalysis since both active enzyme and sucrose are mandatory for complex formation. When uniformly labeled [^{14}C]sucrose is used as the substrate, radiolabeled glucose, but not fructose or sucrose, is bound. Linear pseudo-first-order and second-order kinetics of base-catalyzed hydrolysis of glycosyl–enzyme indicate that glucose is linked to one type of functional group on the enzyme, and release of a single glucose anomer on alkaline hydrolysis establishes that the glucose linkage to the enzyme is through C-1.

The pH dependency of glucosyl–enzyme implicates a carboxyl group of aspartate or glutamate as the amino acid functional group bonded to glucose. N- and O-glycosides of serine, threonine, and asparagine are relatively stable in base. A β-D-glucopyranoside of *N*-glycyl-L-serine methylamide, for example, is stable for 24 h at pH 11, 37 °C (Derevitskaya et al., 1967), and the stability of the threonine derivative would be similar (Vercellotti et al., 1970). *N*-Acylglucosylamines are also quite stable; *N*-(L-aspartyl)-β-D-glucosylamine shows no degradation after 7 h in 0.5 N NaOH (Marks & Neuberger, 1961).

In contrast, fructosyl and particularly glucosyl esters of aspartate or glutamate carboxyls closely parallel the alkaline lability of GTF glucosyl–enzyme. Supporting evidence comes from pH dependency studies of a denatured glucosyl–enzyme of sucrose phosphorylase reported by Voet and Abeles (1970) and an analogous denatured fructosyl–enzyme of levansucrase described by Chambert and Gonzy-Treboul (1976). In both complexes the active site is destroyed to the extent that enzyme-catalyzed glycosyl transfer from the complex to water does not occur but base-catalyzed transfer does. The levansucrase fructosyl–enzyme is linked to the enzyme through an aspartate and hydrolyzes with a rate constant of 65 M^{-1} min^{-1}; this is about 3.5 pH units more stable than GTF glucosyl–enzyme at 2.15×10^5 M^{-1} min^{-1}. The denatured sucrose phosphorylase glucosyl–enzyme is bonded as an ester to either glutamate or aspartate (DeToma & Abeles, 1970). The half-life at pH 7.0 calculated from the first component of a biphasic pseudo-first-order decay is approximately 60 min (Voet & Abeles, 1970); the GTF glucosyl–enzyme under similar conditions has a half-life just slightly lower at 32 min, a difference that can result from different amino acids adjacent to the carboxylate.

GTF glucosyl–enzyme did not transfer glucose to either dextran or fructose. The complex did transfer glucose to water, but as the β-anomer not the α-anomer observed with native GTF hydrolysis of sucrose. The possibility that glucosyl–enzyme is an ester offers an explanation for the difference, since a glucosyl ester (acylal) can hydrolyze with either retention or inversion of configuration at C-1: Nucleophilic attack at the ester carbon leaves the anomeric form intact, and reaction

at the acetal carbon results in anomeric inversion.

Base hydrolysis of acylals occurs at the ester carbon (Brown & Bruce 1973; Fife & De, 1974), so β-D-glucose released from denatured GTF glucosyl–enzyme translates to a retained β-glucosyl linkage on the enzyme. On the other hand, native GTF glucosyl transfer occurs at the acetal carbon (Hestrin, 1961). If the reaction proceeds by an S_N2 displacement, the enzyme would form the same β-glucosyl–enzyme complex observed with the denatured complex, but in this case a second S_N2 attack would release the α-anomer.

The glucosyl–enzyme has characteristics distinct from the glucose and glucan–enzyme preparations of *Leuconostoc* and oral streptococcal glucosyltransferases described by Robyt and by Mayer and colleagues (Robyt et al., 1974; Robyt & Martin, 1983; Parnaik et al., 1983) since the latter are in a more native state. The complexes are prepared by reacting sucrose with enzyme bound to a chromatography resin and then washing the complex free of unbound carbohydrate. The preparations, however, cannot be truly native since a native intermediate would very rapidly deglucosylate along the sucrose hydrolysis pathway. Parnaik et al. (1983) stabilized a resin-bound *S. sobrinus* GTF complex by lowering the temperature to 4 °C. The temperature is higher than commonly required to stabilize enzyme intermediates (Fink, 1977), and the glucose/enzyme stoichiometry is over 100-fold greater than expected. Nonetheless, the complex transferred glucose to common glucosyltransferase acceptors (Luzio et al., 1983), which requires that some form of high-energy glucose remained associated with the enzyme.

It is difficult to put the *Leuoconostoc*, *S. sanguis*, and *S. sobrinus* GTF glucosyl–enzyme complexes into context since the data are incomplete. Purich (1982) has clearly outlined the problems and limitations of authenticating covalent catalysis, and while there is a large amount of accumulated information on glucosyltransferases, it is insufficient to distinguish a covalent from a noncovalent mechanism. For example, the GTF covalent glucosyl–enzyme reported here may result from collapse of an oxocarbonium ion during denaturation of the enzyme active site, and experiments on the glucose–enzyme complexes bound to chromatography resins have not rigorously excluded the possibility of a tightly bound noncovalent form and/or small amounts of a trapped substrate as were discovered with the phosphoryl–enzyme complex of phosphoglycerate kinase (Johnson et al., 1976).

Thus, even though a glucosyl–enzyme (stabilized by a carboxylate of glutamic or aspartic acid) is clearly part of the GTF reaction pathway, the reaction mechanism is not obvious. One mechanism that deserves serious consideration is an equilibrium between a covalent glucosyl ester and noncovalent oxocarbonium ion. Oxocarbonium ions have an extremely short life (Young & Jencks, 1977), and the small distance between the cation and a carboxylate could reversibly collapse to a glycosyl ester. Such an equilibrium has been proposed for several glycosyltransferases and glycosidases (Meiyal & Abeles, 1972; Dunn & Bruce, 1972; Sinnott, 1978; Rosenberg & Kirsch, 1981) and may be relevant to *S. sobrinus* GTF as well.

There are no active-site markers for GTF in part because modest structural modification of sucrose, glucose, or fructose commonly eliminates active-site affinity. Alternative substrates (Genghof & Hehre, 1972) and active-site reversible inhibitors (Thaniyavarn et al., 1981; Bhattacharjee & Mayer, 1985; Binder & Robyt, 1985) are rare, and active-site irreversible ligands are unknown. Because of this, there is no direct information on the structure of the active site, even though the complete amino acid sequence of one *S. sobrinus* glucosyltransferase (Ferretti et al., 1987) and other closely related glycosyltransferases [*S. mutans* GTF (Shiroza et al., 1987), *S. mutans* fructosyltransferase (Shiroza & Kuramitsu, 1988), and *Bacillus subtilis* levansucrase (Steinmetz et al., 1985)] have been deduced from the gene sequences. The covalent glucosyl–enzyme survives proteolysis under acid conditions and has the potential to serve as a probe of active-site structure of these enzymes.

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Fragmentation of an Endogenous Inhibitor upon Complex Formation with High- and Low-Ca^{2+}-Requiring Forms of Calcium-Activated Neutral Proteases

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ABSTRACT: The interaction of an endogenous inhibitor for the calcium-activated neutral protease (CANP or calpain EC 3.4.22.17) with CANP was examined by SDS–polyacrylamide gel electrophoresis, immunoblot analysis, and gel filtration. Fragmentation of the inhibitor (M_r 110K) by mCANP, a high-Ca^{2+}-requiring form, was shown only in the presence of Ca^{2+} ions of millimolar order, with decreased inhibitor activity recovered from gel extracts in the 110-kDa area. This fragmentation took place even when the inhibitor could completely inhibit the caseinolytic activity of mCANP. The fragmented inhibitor retained considerable inhibitor activity after the CANP–inhibitor complex was dissociated by the addition of EDTA, and 69% of the initial activity was recovered from the mixture reacted with excess mCANP lacking the 110-kDa band. A C-terminal fragment of CANP inhibitor produced in *Escherichia coli* (M_r 40K) was also hydrolyzed by mCANP in the presence of Ca^{2+}. The interaction of both forms of the inhibitor with μCANP, a low-Ca^{2+}-requiring form, led to the same phenomena in the presence of micromolar levels of Ca^{2+}. CANP inhibitor could not completely inhibit the autolysis of mCANP and μCANP, indicating that these were intramolecular events. Gel filtration analysis revealed that the mass of the smallest fragment with inhibitor activity was about 15 000 daltons. These results suggest that CANP inhibitor may act in the manner of a suicide substrate.

The calcium-activated neutral protease (CANP[1] or calpain, EC 3.4.22.17) is an intracellular cysteine protease distributed ubiquitously in various tissues and cells of vertebrates and is presumed to participate in various cellular functions mediated by Ca^{2+} (Suzuki et al., 1984; Pontremoli & Melloni, 1986). CANP has at least two isozymes with different calcium sensitivities: μCANP and mCANP, active at micro- and millimolar Ca^{2+}, respectively. Each CANP is composed of two different subunits, a large catalytic subunit with M_r 80K and

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[1] Abbreviations: CANP, calcium-activated neutral protease; mCANP and μCANP, CANPs which are active in the presence of millimolar and micromolar Ca^{2+}, respectively; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; TCA, trichloroacetic acid; PMSF, phenylmethanesulfonyl fluoride; kDa, kilodalton(s); BPB, bromophenol blue; sample diluter, 125 mM Tris-HCl, pH 6.8/4% SDS/10% 2-mercaptoethanol/20% glycerol/0.005% BPB.

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