*Carbohydrate Research*, 93 (1981) 125–133
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

# INACTIVATION OF D-GLUCOSYLTRANSFERASES FROM ORAL *Streptococcus mutans* AND *Streptococcus sanguis* BY PHOTOCHEMICAL OXIDATION

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(Received August 11th, 1980; accepted for publication, November 4th, 1980)

## ABSTRACT

Cell-free D-glucosyltransferase of D-glucose-grown *Streptococcus mutans* AHT was completely inactivated in the presence of 0.002% of Methylene Blue at 25° and pH 7.0 after illumination with a 150-W incandescent lamp. The rate of inactivation was decreased at pH values <7.0. Histidine was the only amino acid residue modified to a significant extent, and the rates of oxidation of histidine residues and loss of enzyme activity closely agreed. Production of both water-insoluble and -soluble D-glucan fractions from sucrose by the oxidized D-glucosyltransferase preparations was significantly inhibited. Photooxidation with 0.002% of Rose Bengal at pH 7.0 or higher also induced complete inactivation of the D-glucosyltransferase. These results strongly suggest that the imidazole portion of histidine may function as part of the active sites of both D-glucosyltransferase isozymes of *S. mutans* AHT, which are responsible for the synthesis of (1→3)- and (1→6)-α-D-glucosidic linkages. The D-glucosyltransferases from *S. mutans* 6715 and AHT-mutant M1, and *Streptococcus sanguis* ATCC 10558 were also almost completely inactivated by Methylene Blue-sensitized photooxidation.

## INTRODUCTION

Water-insoluble and -soluble α-D-glucans produced from sucrose by oral *Streptococcus mutans* and *Streptococcus sanguis* have been shown to play an active role in the accumulation of the bacteria on tooth surfaces[1,2]. Firm attachment of cariogenic *S. mutans* cells to smooth surfaces (cell-to-surface adherence) requires *de novo* synthesis of water-insoluble α-D-glucan[3–8]. Cell agglutination of *S. mutans*[9,10], *S. sanguis*[11], and *Actinomyces viscosus*[12], and aggregation between these streptococcal cells and *A. viscosus* cells[11,13] (cell-to-cell adherence) are mediated by insoluble and

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soluble α-D-glucans. These α-D-glucans are synthesized from sucrose by constitutive cell-free and cell-bound D-glucosyltransferases (EC 2.4.1.5). Several investigators have attempted to purify and characterize D-glucosyltransferases from these oral streptococci, but have not succeeded in complete resolution of the isozymes because they occur in high-molecular-weight aggregated forms[2,7,14-19]. This has impaired progress in the elucidation of the mechanism of synthesis of these α-D-glucans by the streptococcal D-glucosyltransferases.

Studies using photosensitized oxidation, which is a mild and moderately selective method for modifying amino acids in proteins and peptides, have demonstrated that histidine residues are involved in the active sites of a large variety of enzymes[20]. The D-glucosyltransferases from the strains of *Leuconostoc mesenteroides* and *S. sanguis* are inhibited by photooxidation reaction using Methylene Blue or Rose Bengal[21,22]. Imidazole catalyzes the hydrolysis of sucrose to D-glucose and D-fructose[23]. The present studies were initiated to support the hypothesis that the imidazole portion of histidine functions as part of the active site of D-glucosyltransferase isozymes from oral *S. mutans* and *S. sanguis*.

## EXPERIMENTAL

*Streptococcal strains.* — *S. mutans* AHT (a subculture AHT-k, serotype *g*)[24,25] and 6715 (serotype *d*) were obtained as described previously[26]. A *S. mutans* AHT-k mutant strain, M1 was obtained by chemical mutation[5,27]. *S. sanguis* ATCC 10558 (type I/II) was provided by S. Hamada (Osaka University, Osaka).

*Preparation of cell-free* D-*glucosyltransferase.* — The *S. mutans* strains were grown in Trypticase (BBL Microbiology Systems, Cockeysville, MD 21030)–0.5% D-glucose broth[1] to the early stationary phase at 37° under an atmosphere of 90% of nitrogen, 5% of carbon dioxide, and 5% of hydrogen. The *S. sanguis* strain was cultured in the same manner, except in air. The spent culture-liquor was obtained by centrifugation at 6000*g* for 20 min at 4°, and ammonium sulfate was added to a 50% saturation as described previously[5]. The precipitates were dissolved in 5mM

TABLE I

CELL-FREE D-GLUCOSYLTRANSFERASE PREPARATIONS FROM THE *S. mutans* AND *S. sanguis* STRAINS

| *Strain* | D-*Glucosyltransferase activity (U/L)* | *Protein (g/L)* | *Specific activity (U/g protein)* |
|---|---|---|---|
| *S. mutans* | | | |
| AHT | 43.1 | 6.3 | 6.8 |
| AHT-mutant M1 | 452.6 | 5.9 | 76.7 |
| 6715 | 112.0 | 5.4 | 20.7 |
| *S. sanguis* | | | |
| ATCC 10558 | 14.9 | 4.1 | 3.6 |

phosphate buffer (pH 6.0), and are designated as cell-free, D-glucosyltransferase preparations. Enzyme activities and protein contents of the D-glucosyltransferase preparations were determined as described in the next paragraph, and by the Folin–Ciocalteu method[28], respectively, and the results are summarized in Table I. No D-fructosyltransferase (EC 2.4.1.10) activity was detected in any enzyme preparation.

*Assay for D-glucosyltransferase activity.* — The reaction mixture contained an appropriately diluted, D-glucosyltransferase solution (5 μL) and 0.1 μCi of [U-$^{14}$C]-sucrose (5.1 Ci/mol, New England Nuclear, Boston, MA 02118) in 0.1M sodium citrate buffer (25 μL, pH 5.5). After incubation for 1 h at 37° in the dark, an aliquot (10 μL) of the assay digest was withdrawn and deposited on a filter-paper square (15 × 15 mm, No. 514, Toyo-roshi, Tokyo) in triplicate. The filter-paper squares were soaked three times in methanol, as described by McCabe and Smith[29], and dried. Radioactivity retained on paper squares was measured by a scintillation spectrophotometry with toluene scintillant. One unit of enzyme activity was defined as the amount of enzyme that transformed 1 μmol of sucrose to D-glucan per min, under the conditions described.

*Photochemical oxidation of D-glucosyltransferase.* — Methylene Blue and Rose Bengal were purchased from Ishizu Pharmaceutical Co., Ltd. (Osaka) and Wako Pure Chemicals (Osaka), respectively. They were used without further purification.

The typical mixture consisted of D-glucosyltransferase (~10 mU) and dye (20 μg) in 50mM sodium phosphate buffer (1.0 mL, pH 7.0). In some experiments, 50mM sodium citrate buffer (pH 3.0–6.0), sodium phosphate buffer (pH 6.0–8.0), and tris(hydroxymethyl)aminomethane · hydrochloride buffer (pH 7.5–9.0) were used. Aliquots (2 or 10 mL) of the mixture were distributed in 13 × 100-mm, glass test tubes or in 18 × 180-mm tubes, respectively, and placed 2 cm below the surface of water maintained at 25° in a glass water-bath. They were irradiated for 0–120 min with a 150-W, incandescent lamp placed 20 cm from the solution. During the illumination, the mixtures were vibrated for 10 s every 5 min with a Vortex mixer. The test tubes were removed from the light source at appropriate time intervals and stored in the dark. For controls, mixtures without dye, and/or mixtures with dye placed in the test tube wrapped with aluminum foil, were treated in the same way.

*Amino acid analyses of D-glucosyltransferase.* — The D-glucosyltransferase solution (40 mL, collected from a quadruplicate set of the 10-mL test mixture), with or without Methylene Blue, was treated with activated charcoal (4 g, granule type, Wako Pure Chemicals) for 24 h at 4° in the dark, and the mixture was filtered through a filter paper (No. 101, Toyo-roshi). To reduce salt concentration, the filtrate was transfered into an Amicon ultrafiltration-cell, type 202, equipped with a Diaflow membrane PM10 (exclusion limit, 10000 Daltons, Amicon Corp., Lexington, MA 02173). The membrane was washed twice with 200 mL each of distilled water, and the filtrate was freeze-dried. A portion (5 mg) of the powdered D-glucosyltransferase was hydrolyzed for 24 h at 110° in glass-distilled 6M hydrochloric acid (1.0 mL), in a vacuum-sealed hydrolysis tube (10 × 100 mm, Pierce Chemical Co., Rockford, IL 61105). After complete removal of hydrochloric acid by repeated evaporation of

water under diminished pressure at 40°, the dried, acid hydrolyzates were dissolved in 0.1M phosphate buffer (1.0 mL, pH 6.5). A portion (50 μL) of the solution was analyzed for amino acid composition by the procedures of Spackman *et al.*[30] with a high-speed amino acid analyzer (Type 835, Hitachi Ltd., Tokyo). The content of tryptophane was monitored on an unhydrolyzed sample of D-glucosyltransferase by the ninhydrin method of Gaitonde and Dovey[31].

*Production and fractionation of* D-*glucans.* — A portion (5 mL) of D-glucosyltransferase solution, oxidized in a 10-mL mixture, was allowed to react with sucrose (1.5 g) in 0.1M phosphate buffer (30 mL, pH 6.0) containing 0.02% of Thimerosal, for 16 h at 37° in the dark. The assay was run in triplicate. The D-glucans produced were fractionated into one water-insoluble and three water-soluble fractions by the procedures described earlier[26]. Briefly, the insoluble fraction ISG and soluble fraction SG were isolated by centrifugation (20000*g*, 15 min). Fraction SG was subdivided, in a Bio-Gel P-100 column, into two fractions with high (SG-A) and low (SG-B) molecular weight, and Fraction SG-A was further resolved into two subfractions (SG-A-I and SG-A-II) by 20% and 50% ethanol precipitation, respectively. The amount of D-glucans was determined by the anthrone method[32].

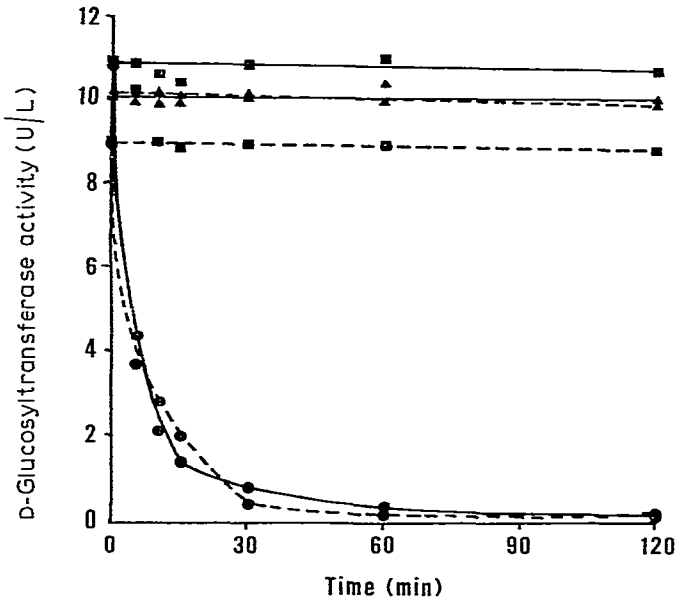


Fig. 1. Time course of the photochemical oxidation of *S. mutans* AHT D-glucosyltransferase. The mixture consisted of cell-free D-glucosyltransferase from *S. mutans* AHT (~10 mU) and 20 μg of Methylene Blue (——) or Rose Bengal (– – – –) in 50mM phosphate buffer (1.0 mL, pH 7.0). The mixture (2 mL) was placed in a 13 × 100-mm test tube and irradiated at 25° with a 150-W, incandescent lamp, placed 20 cm from the solution. Mixtures were shaken for 10 s every 5 min. Aliquots (2 mL) were removed from the light at different times and assayed for the remaining D-glucosyltransferase activity in triplicate in the dark, as described in the Experimental section: ●, illuminated in the presence of dye; ▲, illuminated in the absence of dye; and ■, kept in the dark in the presence of dye.

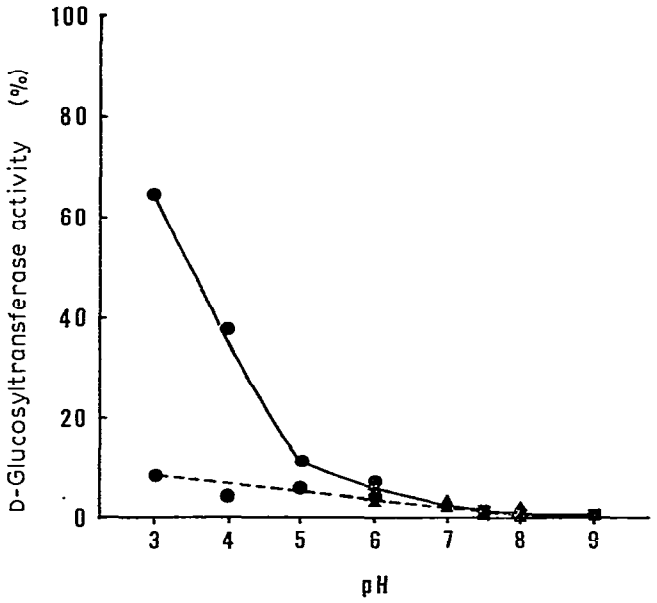


Fig. 2. pH Dependency of the photochemical oxidation of *S. mutans* AHT D-glucosyltransferase. The experimental conditions were the same as those given in the legend to Fig. 1, except that D-glucosyltransferase was illuminated for 30 min in 50mM citrate buffer, pH 3.0–6.0 (●); phosphate buffer, pH 6.0–8.0 (▲); or Tris · HCl buffer, pH 7.5–9.0 (■). The activity of the oxidized D-glucosyltransferases was expressed as percentage of the activity of the respective controls which were similarly treated in the absence of dye: (——) Methylene Blue and (– – – –) Rose Bengal.

## RESULTS

*Inactivation of streptococcal* D-*glucosyltransferase by photochemical oxidation.* — Cell-free D-glucosyltransferase of *S. mutans* AHT was completely inactivated after illumination for 30–60 min at pH 7.0 in the presence of 0.002% of Methylene Blue or Rose Bengal (Fig. 1). No apparent loss of enzyme activity was observed with the controls, which were either illuminated in the absence of dye or not exposed to light in the presence of dye.

As shown in Fig. 2, the extent of inactivation increased with the decrease of hydrogen-ion concentration in the assay medium; almost complete inactivation of D-glucosyltransferase was obtained at pH values higher than 7.0, after oxidation for 30 min with 0.002% of Methylene Blue; oxidation catalyzed by 0.002% of Rose Bengal also induced strong inactivation of D-glucosyltransferase at the higher pH values, but this did not show a pH dependency as clear as the Methylene Blue-catalyzed inactivation.

Inactivation of the D-glucosyltransferases from *S. mutans* 6715, AHT-mutant M1, and *S. sanguis* ATCC 10558 also was induced by illumination at pH 7.0 in the presence of 0.002% of Methylene Blue (Fig. 3). The D-glucosyltransferase from strain 6715 was inactivated at the same rate as the AHT enzyme (Fig. 1), but inactivation

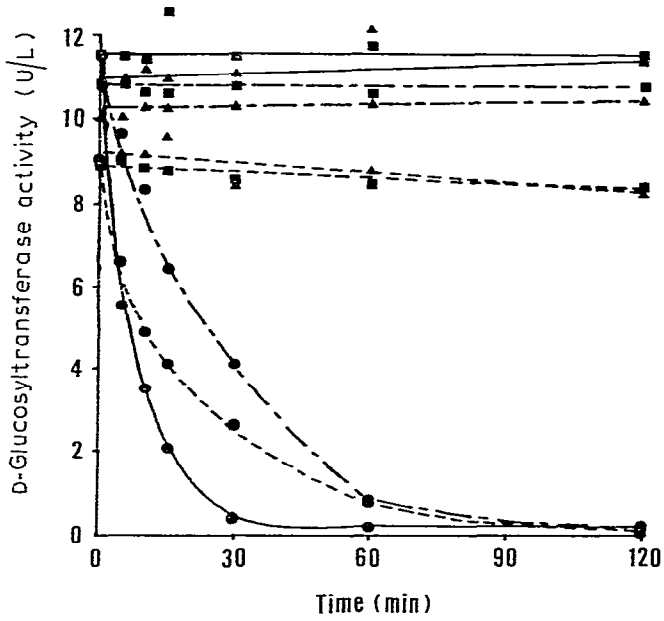


Fig. 3. Inactivation of cell-free D-glucosyltransferases from *S. mutans* 6715 and AHT-mutant M1, and *S. sanguis* ATCC 10558 by the Methylene Blue-sensitized photooxidation. The D-glucosyltransferases from strains 6715 (——), M1 (–·–), and ATCC 10558 (– – –) were exposed to light in the presence (●) or absence (▲) of 0.002% of Methylene Blue, or kept in the dark in the presence of the dye (■) under the same conditions as those described in the legend to Fig. 1.

of the D-glucosyltransferases from strains M1 and ATCC 10558 proceeded a little slower (Fig. 3).

*Amino acid analyses of photooxidized D-glucosyltransferase.* — Inactivation of D-glucosyltransferase was a little slower when a larger reaction-volume was used, probably because of restricted access of oxygen (Fig. 4). Amino acid analysis of the oxidized AHT D-glucosyltransferase demonstrated that histidine was the only amino acid residue modified to a significant extent (Fig. 4). The rates of oxidation of histidine residues and the loss of enzyme activity closely agreed. Methionine, tyrosine, cysteine (half-cystine), and tryptophan (Fig. 4), and aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, isoleucine, leucine, phenylalanine, lysine, arginine, and proline (not shown) were not oxidized significantly.

*Synthesis of D-glucans by the oxidized D-glucosyltransferase.* — Production of the water-insoluble α-D-glucan fraction from sucrose by the oxidized AHT D-glucosyltransferase gradually decreased as the time of photochemical oxidation increased (Fig. 5). The rate of the suppression of synthesis of ISG closely paralleled the loss of enzyme activity (Fig. 4), but inhibition of D-glucosyltransferase activity responsible for the production of the water-soluble fraction SG proceeded rapidly at the very early oxidation stage, and thereafter almost ceased. Synthesis of the SG subfractions (SG-A-I, SG-A-II, and SG-B) was inhibited to various extents.

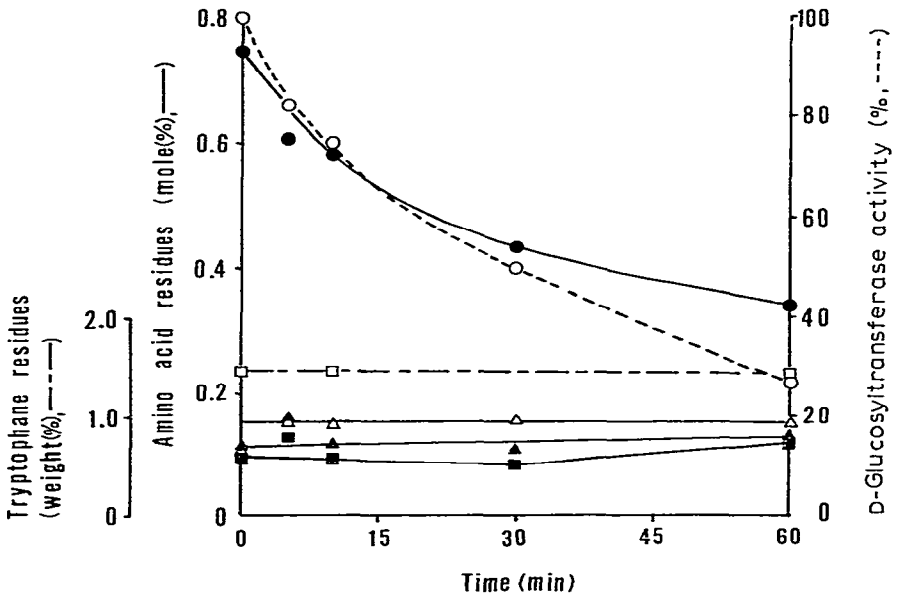


Fig. 4. Amino acid analyses during the Methylene Blue-sensitized photooxidation of *S. mutans* AHT glucosyltransferase. The mixture (described in the legend to Fig. 1) (10 mL) containing AHT D-glucosyltransferase (~ 100 mU) and 0.002% of Methylene Blue was illuminated in a 18 × 180-mm test tube. The D-glucosyltransferase activity (○) of the oxidized enzymes was determined as described in the Experimental section. The enzyme activity of the control that had not been exposed to light in the presence of the dye was arbitrarily taken as 100%. The oxidized D-glucosyltransferase solution (~40 mL, combined from a quadruplicate set of the 10-mL mixture) was treated with activated charcoal to remove the dye and filtered. The filtrate was washed and concentrated by ultrafiltration, and freeze-dried. A portion (5 mg) was analyzed for amino acid composition by the procedure of Spackman *et al.*[30]: ●, histidine; ▲, methionine; ■, tyrosine; and △, cysteine (half-cystine). The content of each amino acid was expressed as per cent mol of those of the amino acids and ammonia. Tryptophan (□) was monitored on an unhydrolyzed sample by the method of Gaitonde and Dovey[31], and its content was expressed as per cent (weight) of enzyme protein.

## DISCUSSION

Of the 18 naturally occurring amino acids, only histidine, tryptophan, tyrosine, methionine, and cysteine are readily susceptible to the dye-sensitized photooxidation[33]. In the presence of Methylene Blue, methionine and tryptophan are more easily oxidized under acidic, tyrosine more easily under alkaline, and histidine more easily under neutral to weakly alkaline conditions[34]. Rose Bengal induces a specific oxidation of histidine[35]. Cell-free D-glucosyltransferase of *S. mutans* AHT was readily inactivated at neutral to weakly alkaline pH values by the photochemical oxidation (Fig. 2). Amino acid analyses demonstrated that histidine was the only amino acid residue modified by the Methylene Blue-sensitized photooxidation, and that the rate of destruction of the amino acid residue and the decrease of enzyme activity were similar (Fig. 4). These results suggest that histidine residues may function at the active sites of cell-free D-glucosyltransferase of *S. mutans* AHT. The presence of a

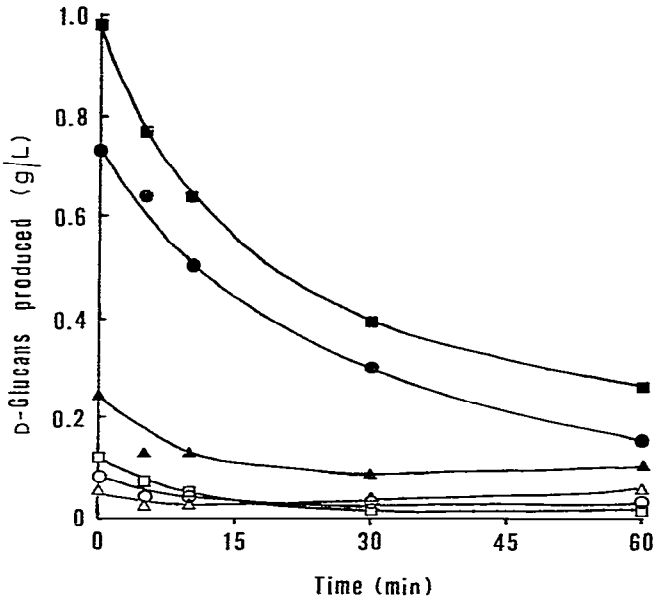


Fig. 5. Synthesis of D-glucans from sucrose by the photooxidized D-glucosyltransferase of *S. mutans* AHT. A portion (5 mL) of D-glucosyltransferase solution, oxidized in a 10-mL mixture, was allowed to react with sucrose (1.5 g) in 0.1M phosphate buffer (30 mL, pH 6.0) containing 0.02% of Merthiolate, for 16 h at 37° in the dark. The assay was performed in triplicate. The α-D-glucans produced were fractionated into ISG (●) and SG (▲) by differential centrifugation (20000*g*, 15 min), and SG was further separated into 3 sub-fractions, SG-A-I (○), SG-A-II (△) and SG-B (□) by a combination of Bio-Gel P-100 gel filtration and ethanol precipitation at 20 and 50% sarulations as described previously[26]. Amounts of total D-glucans (■) were obtained as the sum of those of ISG and SG.

similarly active histidine residue in the D-glucosyltransferases from other strains of *S. mutans* and *S. sanguis* also was indicated (Fig. 3). The involvement of histidine residues in the active sites of the D-glucosyltransferase from *L. mesenteroides* NRRL B-512F (ref. 22) and *S. sanguis* 804 (ref. 21) also has been suggested on the basis of photochemical oxidation studies.

The present results, and the recent observation that imidazole catalyzes the hydrolysis of sucrose into D-glucose and D-fructose[23], suggest that the imidazole portion of a histidine residue may be involved in the first step of the synthesis of α-D-glucans from sucrose by D-glucosyltransferase from different sources. This may be true for both D-glucosyltransferase isozymes responsible for the formation of (1→3)- and (1→6)-α-D-glucosidic linkages, as the enzyme from *S. mutans* AHT, which produces an ISG, containing a high proportion of (1→3)-linked-α-D-glucosyl residues, and an SG consisting almost exclusively of (1→6)-linked-α-D-glucosyl residues[5], was inactivated completely by the Methylene Blue-sensitized photooxidation (Fig. 1). Production of both ISG and SG by the oxidized enzyme was inhibited, but the extent of inhibition of SG production was lower than that of ISG production (Fig. 5). The D-glucosyltransferases from *S. mutans* AHT-mutant M1 and *S. sanguis* ATCC 10558, both of which have been shown[5,27] to produce only SG, were in-

activated more slowly by photochemical oxidation than were the enzymes from *S. mutans* AHT and 6715, which produced[26] preponderantly ISG (Figs. 1 and 3). This suggests that the structure of the enzyme molecule near the sucrose hydrolysis-catalytic site may differ between D-glucosyltransferase isozymes responsible for the synthesis of (1→3)- and (1→6)-α-D-glucosidic linkages. Possibly, a portion of the histidine residues at the catalytic site of the isozyme responsible for α-D-(1→6)-linkage formation may be buried in the interior of the enzyme protein, and hence may be resistant to oxidation.

ACKNOWLEDGMENTS

We thank Miss Hideko Dhomeki, Naka Works, Application Technical Center, Hitachi Ltd., for performing the amino acid analyses, and Professor Eric E. Smith, University of Miami School of Medicine, for reviewing the manuscript.

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