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Photolabeling of dextransucrase from *Streptococcus sanguis* with *p*-azidophenyl α-D-glucopyranoside*

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ABSTRACT

Dextransucrase from *Streptococcus sanguis* ATCC 10558 was photolabeled using *p*-azidophenyl α-D-glucopyranoside with an apparent rate constant of inactivation of 1.40 min^{-1}. The dissociation constant for this compound, which acts as an acceptor molecule in the enzymatic reaction, is 90 μM. Apparently two acceptor binding sites exist on dextransucrase as shown by (*i.*) photolabeling the enzyme with *p*-azidophenyl-α-D-[5,6-^{3}H]glucopyranoside and (*ii.*) fluorescence titration experiments.

INTRODUCTION

Dextransucrase [EC 2.4.1.5] is an exocellular glucosyltransferase produced by various *Lactobacillus* and *Streptococcus* species. The enzyme from oral streptococci (*e.g.*, *S. sanguis* and *S. mutans*) appears to play a role in dental cariogenicity[1].

Dextransucrase (DS) produces a D-glucan (*i.e.*, dextran) by polymerization of D-glucosyl units from the substrate sucrose. Although the catalytic mechanism is unknown, the reactions (glucosyl transfer to acceptors, autopolymerization, isotope exchange, and sucrose hydrolysis) catalyzed by dextransucrase probably proceed through a glycosyl–enzyme intermediate[2,3]. The autopolymerization reaction proceeds with reducing end chain growth[4,5] while the chain appears to be covalently attached to the enzyme[5,6]. Glucosyl-residue transfer to acceptors competes with the autopolymerization reaction[7] for enzyme-bound glucosyl residues and results in the addition of monomeric units to the non-reducing terminus of the acceptor. A general catalytic scheme which could describe the glucosyl transfer reaction is shown in Scheme 1.

Information regarding the nature of the functional groups on the enzyme that participate in catalysis is limited and equivocal. Measurement of the rate of the reaction as a function of pH indicates the presence of catalytically important groups with pK_a values of 4.5 and 7.5 (ref. 8). Chemical modification of the enzyme with carbodiimides and glycine methyl ester results in non-specific modification of carboxylates and loss of

* Abbreviations used: APG, *p*-azidophenyl α-D-glucopyranoside; BCA, bicinchoninic acid (4,4′-dicarboxy-2,2′-biquinoline); DS, dextransucrase [EC 2.4.1.5]; [^{3}H]APG, *p*-azidophenyl-α-D-[5,6-^{3}H]glucopyranoside.

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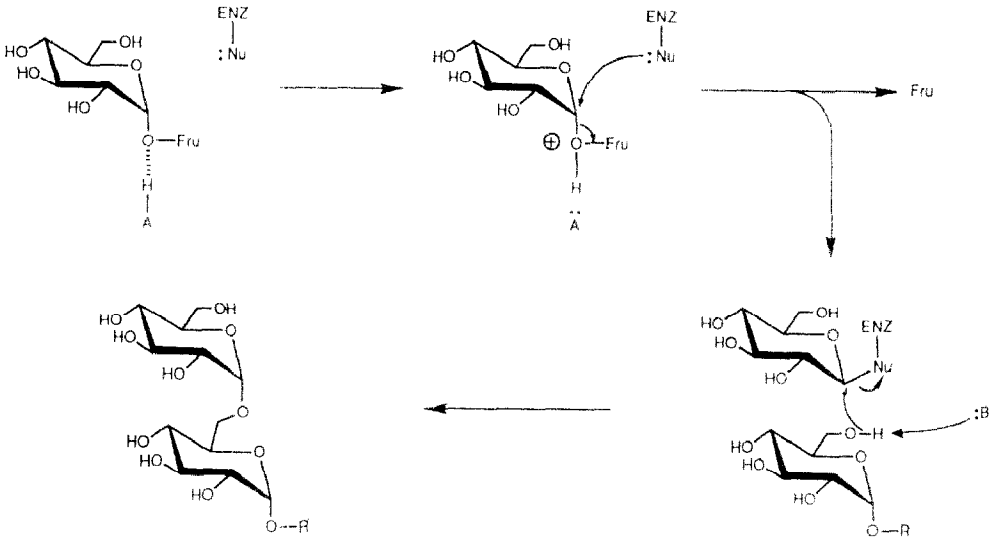

Scheme 1. Proposed reaction mechanism of dextransucrase. Nu, nucleophilic side chain on the enzyme; A/B, proton donor/acceptor side chain; R, linkage to another sugar residue (glucosyl transfer reaction).

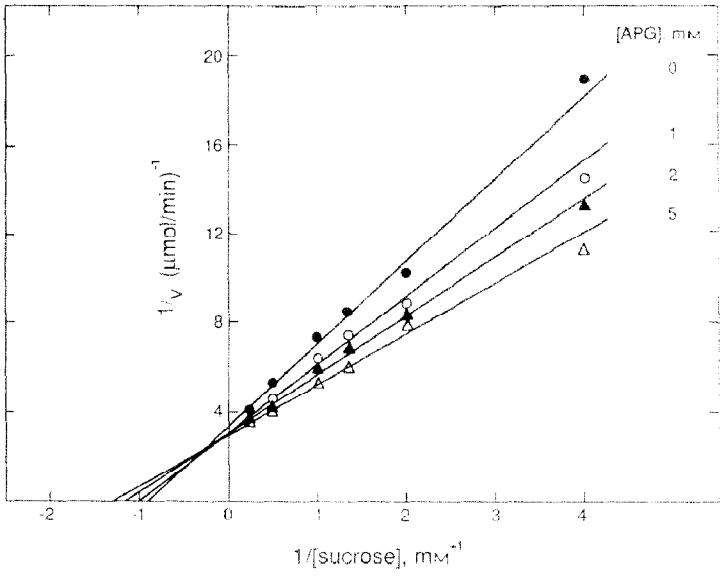

Fig. 1. Kinetics of dextransucrase in the presence of APG. Dextransucrease (0.05 unit) was reacted with varying concentrations of sucrose in the presence of the indicated concentrations of APG. Reactions, buffered with sodium phosphate (10mM, pH 6.3) in a final volume of 500 μL, were maintained at 37°. At intervals aliquots (50 μL) were withdrawn for activity analysis as described in the Experimental.

activity[9]. Dye-sensitized photooxidation of dextransucrase (from various sources) produced inactive enzyme, but the modification was not selective[10–12]. In the present study the use of probes that can be photoactivated was investigated to determine their suitability as active-site labels.

The enzyme from both *S. mutans* and *Streptococcus sobrinus* has been reported to have glucan-binding sites[13–15] that may be distinct from catalytic sites. It has been postulated that there are two such sites based on indirect evidence[14]. Using photoaffinity labelling we provide evidence that the enzyme from *S. sanguis* has two sites that bind acceptor sugars.

RESULTS AND DISCUSSION

Kinetics of reaction of p-*azidophenyl* α-D-*glucopyranoside with dextransucrase.* — In order to determine whether APG is a substrate for dextransucrase, reactions were carried out at several APG concentrations in the presence of varying of sucrose. The results (Fig. 1) indicate that the rate is stimulated as a function of APG concentration. This behavior is similar to that observed for compounds that serve as acceptor sub-

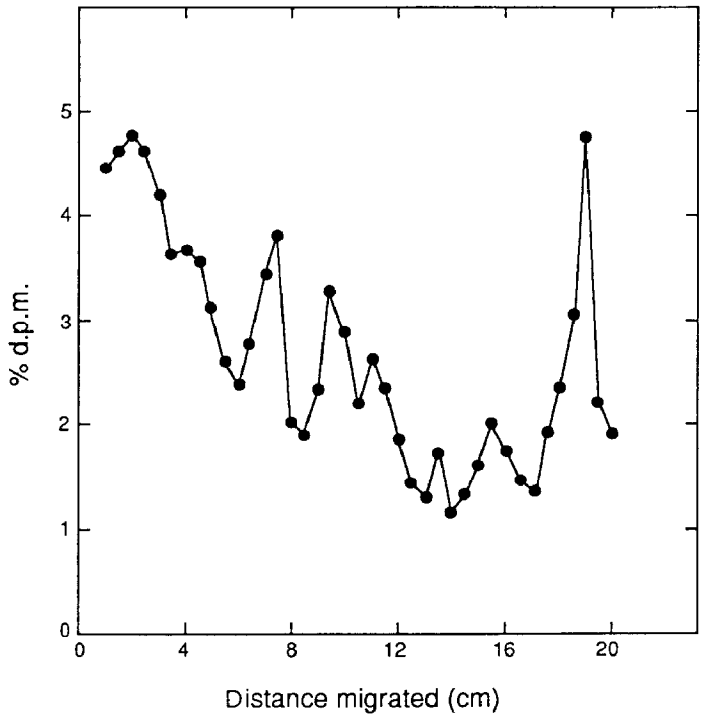

Fig. 2. Product analysis of reactions with APG. Dextransucrase (0.05 unit, D-[U-^{14}C-glucose]sucrose (100mM, 0.05 μCi) and APG (200mM) were reacted for 120 min at 37° in sodium phosphate buffer (10mM, pH 6.3) in a final volume of 200 μL. An aliquot (50 μL) was spotted on Whatman 3MM paper and developed in 3:1:6 2-propanol–ethyl acetate–water. Radioactivity was determined by liquid scintillation counting after the chromatogram was cut into strips (0.5 × 2 cm).

strates[16]. A more direct evaluation of this point was accomplished by using D-[U-^{14}C-glucose]sucrose and APG in the reaction mixtures. The products of this reaction were separated by paper chromatography. The results (Fig. 2) show that a homologous series of low molecular weight products were formed. This is similar to the pattern of product formation observed with acceptors such as maltose and methyl α-D-glucopyranoside[16]. These data indicate that APG most likely serves as an acceptor substrate in the reaction with dextransucrase.

Thin-layer chromatographic analysis of reactions of dextransucrase with APG indicated no hydrolysis of APG for reaction times up to 48 h (data not shown). Thus we conclude that APG, unlike *p*-nitrophenyl-α-D-glucopyranoside[17], is not a donor substrate for this enzyme.

Photoinactivation of dextransucrase. — At either 25° or 0°, in the absence of APG, dextransucrase was inactivated very rapidly (within 30 s) by exposure to radiation at 254 nm or 300 nm, and more slowly (within 2 min) at 350 nm. However, in a frozen matrix at 77 K, the rate of photoinactivation in the absence of APG was sufficiently slow so that the effect of increasing quantities of APG could be measured accurately. Samples were irradiated at 300 nm, which activated the azido group of APG and produced the

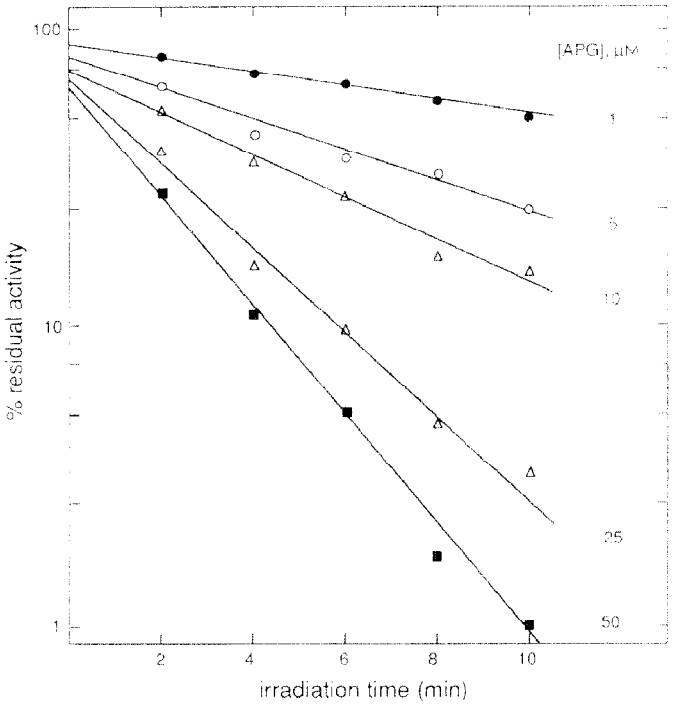

Fig. 3. Photoinactivation of dextransucrase with APG. Solutions containing dextransucrase (2 U/mL), APG (0–50 μM) and sodium phosphate buffer (50mM, pH 6.3) were dripped into liquid nitrogen from a syringe fitted with a 26-gauge needle. Photolysis of the samples was performed using 300 nm light for 0 to 15 min. Following photolysis, samples were thawed at 25°, and enzyme activity was determined. Pseudo-first order rate constants were calculated from the slopes of semi-log plots for each concentration of APG.

triplet-state nitrene derivative[18]. Inactivation of dextransucrase is enhanced by the matrix photolysis technique because the triplet nitrene does not decay to the singlet state at 77 K, and diffusion of the reactive species from the binding site is limited[19].

The photoinactivation of dextransucrase in the presence of APG follows pseudo-first order kinetics (enzyme concentration limiting) as shown in Fig. 3. The pseudo-first order rate constants of inactivation show a linear relationship to APG concentration (Fig. 4, inset). The binding constant of APG with DS was determined from a double-reciprocal plot of the photoinactivation data (Fig. 4); K_d = 1/x-intercept = 90μM. The apparent rate constant of photoinactivation (k_{app} is 1.40 ($\pm$0.32) min^{-1}, as given by equation 1:

$$k_{app} = k\psi K_d\,[\text{APG}] \qquad (1)$$

The specificity for binding of acceptor substrates to dextransucrase is dependent upon the structural charateristics of the sugar moiety at the non-reducing end. A D-glucosyl group provides the most effective substrate. Thus, obvious choices of active-site protecting reagents are molecules which contain D-glucose units. Sucrose is unattractive because of its reactivity in the autopolymerization reaction, and dextran is unsuitable due to its size and difficulty of removal prior to activity determinations. D-Glucose was selected since it is non-reactive and meets the structural requirements for binding to the active site of the enzyme. Photoinactivation with APG was carried out in the presence of varying amounts of D-glucose. Approximately 80% of the enzymic

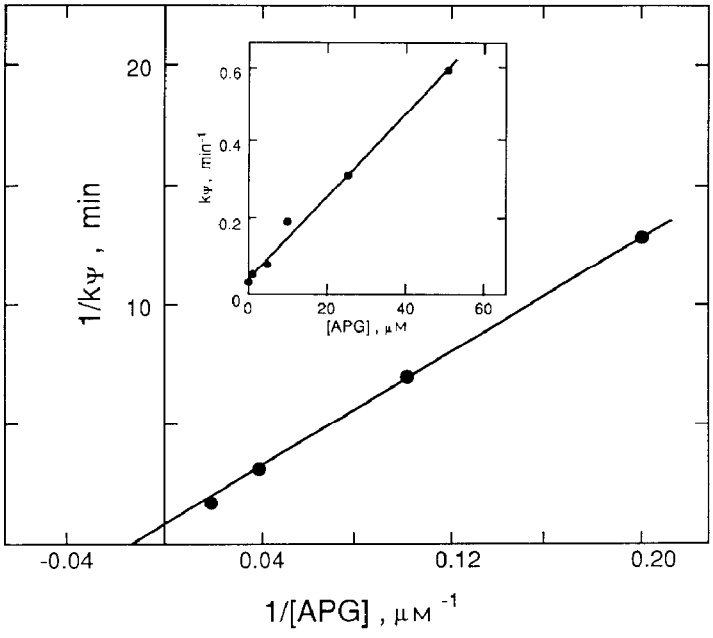

Fig. 4. Determination of the dissociation constant of APG with dextransucrase. General conditions were the same as those cited for Fig. 3. The double-reciprocal plot of $k\psi$ *vs.* [APG] is linear, with the 1/x-intercept = K_d (90μM). Inset: Plot of $k\psi$ as a function of [APG] to determine the rate constant of photoinactivation.

activity is retained at 20μM D-glucose (which is an [APG]:[D-glucose] ratio of 0.5) as illustrated in Fig. 5. These data indicate that the specifity of photoinactivation for the APG-binding site of DS resides principally with the D-glucosyl moiety.

D-Glucosyl-binding sites of dextransucrase. — Approximately 2.5 mol. equiv. of [^{3}H]APG were incorporated into DS, with about 40% residual activity (Fig. 6), following matrix photolysis at 300 nm. The results from several experiments indicated that approximately two molar equiv. of [^{3}H]APG per mol. equiv. of enzyme were incorporated following photoinactivation, although the inactivation was not greater than 80%. All attempts to determine the specifity of labeling and the extent of non-specific labeling, by peptide mapping, were unsuccessful. This was due to the relatively small amounts of protein that could be photolyzed using the matrix technique.

An alternative method to determine the number of binding sites is utilization of spectrofluorometry. The intrinsic fluorescence of DS decreases when dextran binds to the enzyme. Two dextran-binding sites were observed for dextransucrase when fluorescence measurements of the enzyme were determined as a function of dextran concentration (Fig. 7). The titration curve is biphasic with linear segments between 0–75μM and 75–130μM dextran, which suggests the presence of two binding sites with different affinity[20] for dextran. These data substantiate an hypothesis of differential affinity binding of dextran to DS from *S. mutans*[14].

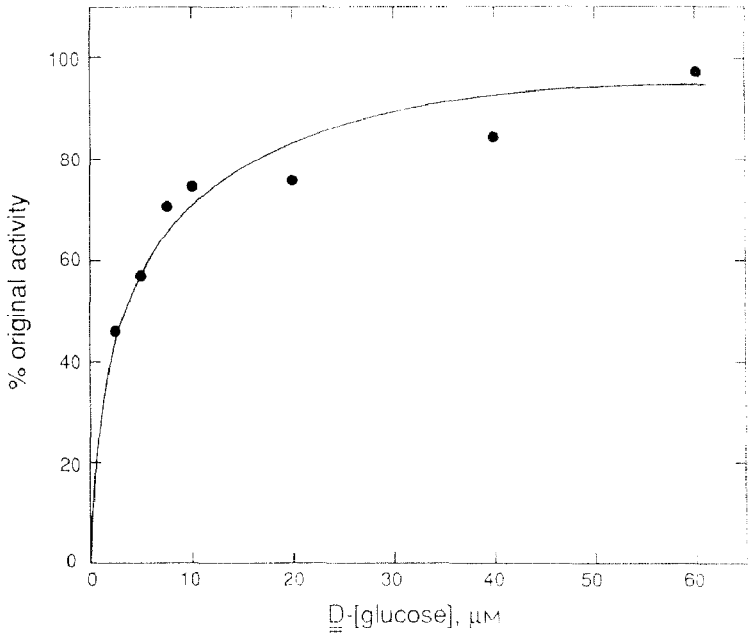

Fig. 5. Protection against photoinactivation. Dextransucrase (2 U/mL), APG (10μM) and D-glucose (0–60μM) in sodium phosphate buffer (10mM, pH 6.3), in a final volume of 1.0 mL, was dripped into liquid nitrogen to form frozen pellets. Approximately 100 μL of these enzyme pellets were photolyzed for 5 min at 300 nm, as described in the Experimental section. Enzyme activity was determined on aliquots of each solution after the pellets were thawed at 25 .

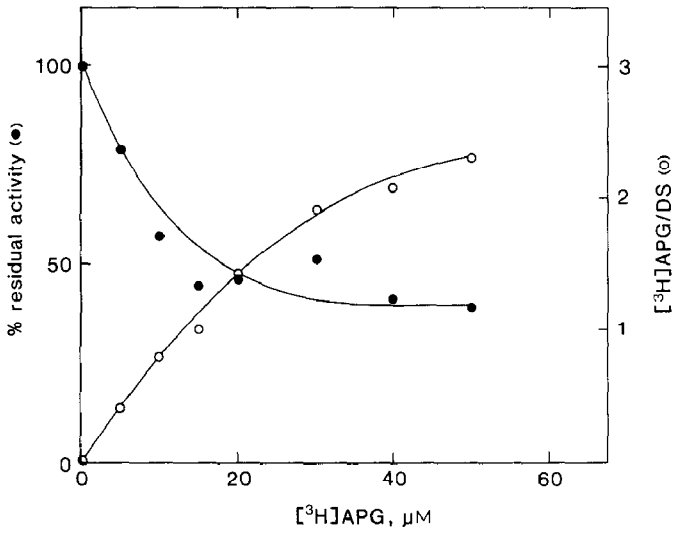

Fig. 6. Photoinactivation with [^{3}H]APG. Photoinactivation was performed as described for Fig. 3. Dextransucrase (2 U/mL), [^{3}H]APG (0–50μM; 1.51 μCi μmol^{-1}) in sodium phosphate buffer (10 mM, pH 6.3), in a final volume of 1.0 mL, was frozen as described in the Experimental. Approximately 200 μL of enzyme pellets were irradiated (300 nm) for 5 min. After thawing at 25°, aliquots were withdrawn for activity determinations and the measurement of radioactivity. The remainder was chromatographed on Bio-Beads SM-2 to adsorb materials not bound to DS. Protein and radioactivity measurements were made on aliquots of each column eluate.

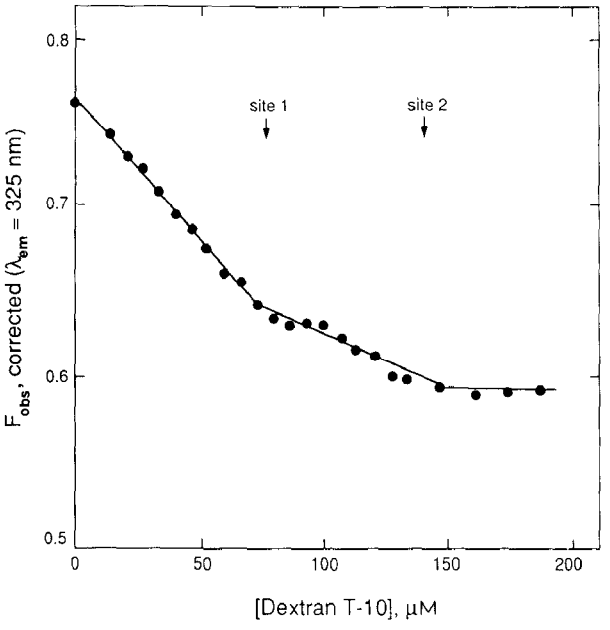

Fig. 7. Fluorescence of dextransucrase as a function of dextran binding. Fluorescence measurements were made on solutions containing dextransucrase (7.2μM) and dextran (0–180μM) in 5mM sodium phosphate, pH 6.3, in a final volume of 1.15 mL. Excitation light was at 295 nm and emission intensity was measured at 325 nm. Observed fluorescence (F_{obs}) was corrected for dilution from successive addition of stock dextran solution.

EXPERIMENTAL

Materials. — *p*-Nitrophenyl α-D-glucopyranoside was obtained from Aldrich Chemical Co. Bio-Beads SM-2 resin, AG 50W-X8 [H^+] resin, AG 501-X8 mixed-bed resin, and hydroxyapatite (HTP) were from Bio-Rad Laboratories. D-Glucose was purchased from Sigma Chemical Co. Dextran T-10 (Pharmacia) was dialyzed extensively against water (dialysis tubing molecular weight cut-off = 6000–8000) and lyophilized to dryness. New England Nuclear Corp. was the source of D-[5,6-^{3}H]glucose and D-[U-^{14}C-glucose]sucrose. Bicinchoninic acid (BCA) protein assay was obtained from Pierce Chemical Co.

Methods. (a) Synthesis of p-azidophenyl α-D-*glucopyranoside.* — *p*-Azidophenyl α-D-glucopyranoside (APG) was synthesized according to a general procedure reported for the β anomer[21] except that reduction of the nitro group was achieved using $Na_2S_2O_4$ (ref. 22). The final purification step included adsorption chromatography on Bio-Beads SM-2. Yield: 77%; m.p. 142–143°; R_f = 0.82 (Silica Gel G, 3:1:1 ethyl acetate–acetic acid–water); λ max 252 nm (ε 15 500)[23]; ^{1}H-n.m.r. (200 MHz, D_2O), δ 3.49 (2 H, s), 3.65 (1 H, dd), 3.87 (2 H, m), 4.03 (1 H, dd), 5.71 (1 H, d), 7.25 (4 H, m); i.r. (Nujol mull) 3110, 2950, 2120, 1460, 1375, 1225, 1100, 1020, 725 cm^{-1}; m.s. (*m/z*, %) 297.1 (0.26) 269.1 (1.74), 135.0 (24.3), 109.0 (100), 107.0 (48.6), 85.0 (21.8), 73.0 (27.4); exact mass calculated for $C_{12}H_{15}O_6N_3$: 297.27, found 297.11.

Anal. Calc. for $C_{12}H_{15}O_6N_3$: C, 48.49; H, 5.09; N, 14.13; O, 32.29; Found: C, 48.47; H, 5.48; N, 13.90; O, 32.13 (by difference).

(b) Synthesis of p-azidophenyl α-D-[5,6-^{3}H]*glucopyranoside. p*-Azidophenyl α-D-[5,6-^{3}H]glucopyranoside ([^{3}H]APG) was synthesized from D-[5,6-^{3}H]glucose. The starting material, consisting of 4.1 mCi of [5,6-^{3}H]glucose (82.9 Ci mmol^{-1}) plus 70 mg of D-glucose, was peracetylated[24]. The peracetylated glucose (a mixture of α and β anomers) was separated from starting material and partially acetylated forms by preparative thin-layer chromatography (Silica Gel G, 500 μm, ethyl acetate). Radiolabeled products were located using a Berthold counter, and 80% of the sugar was determined to be fully acetylated. The peracetylated D-glucose derivatives was *p*-nitrophenylated at the anomeric position using $ZnCl_4$ as a catalyst[25]. *p*-Nitrophenyl α-D-[5,6-^{3}H]glucopyranoside was isolated by preparative thin layer chromatography (Silica Gel G, 500 μm; 7:3 benzene–ethyl acetate). Isotope scanning indicated that 50% of the starting material was converted to the α derivative. The *p*-azidophenyl derivative was synthesized as described for the non-radioactive compound. The overall yield was 30%, and the specific activity of [^{3}H]APG = 15.09 μCi μmol^{-1}. The purity of this compound was assessed by thin-layer chromatography, liquid scintillation counting, and by u.v.–vis. spectroscopy before and after photolysis.

(c) Assay procedures. Dextransucrase activity was determined using a coupled-enzyme assay system[26]. [^{3}H]APG and photolysis by-products were removed from labeled protein by adsorption onto a column (0.7 × 8 cm) of Bio-Beads SM-2 equilibrated in water. Bicinchoninic acid microassay was used to determine protein concentration. The radioactivity of the labeled protein was measured by liquid scintillation

counting (Packard Tri-Carb 460CD instrument) of an aliquot of the mixture solubilized in ScintiVerse E cocktail (10 mL). Fluorescence spectra were obtained using an SLM Aminco SPF-500C spectrofluorometer. A Kontron Uvikon 810 spectrophotometer was utilized for u.v.–vis. spectroscopic measurements.

(*d*) *Enzyme preparation.* Dextransucrase was prepared as previously described[27]. Samples for photolysis were prepared with appropriate amounts of enzyme and APG and frozen by dripping from plastic tuberculin syringes, equipped with 26-gauge needles, into liquid nitrogen.

(*e*) *Photolysis.* Photolysis was performed on samples immersed in liquid nitrogen, using low-pressure mercury vapor lamps (Rayonet RPR-3000 Å). Frozen pellets of enzyme were placed into Pyrex tubes (0.4 × 45 cm) that were suspended in a Dewar flask containing liquid nitrogen, which was placed between two lamps in the photolysis apparatus. The sample was centered 2.5 cm from the lamp surface, and the Pyrex tubing was rotated 90° during photolysis to ensure equivalent irradiation of the entire sample. Photolysis was continued for a designated time interval, at which time the tube was removed, excess liquid nitrogen was allowed to evaporate, and the sample was stored at −15° until it was ready for analysis. Prior to analysis, the frozen enzyme pellets were allowed to thaw at 25°.

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