De Novo Glycosidic Linkage Synthesis by Glycosylases: α-D-Glucosyl Fluoride Polymerization by Dextransucrase (36662)

DOROTHY S. GENGHOF AND EDWARD J. HEHRE

Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York 10461

Recent experimental findings (1–3) provide proof for the first time that the biochemical process of glycosyl transfer, contrary to the accepted view of this process, is able to create glycosidic linkages *de novo* and not solely from preexisting glycosidic linkages. These findings support a concept that recognizes glycosyl–proton interchange as the basis of all carbohydrase actions and as one of the truly major adaptable type-reactions of living matter. Maltosaccharide syntheses from β-maltose by β-amylase (1), from β-D-glucopyranose by glucoamylase (1), and from α-D-glucosyl fluoride and α-maltosyl fluoride (in excess of hydrolysis of these substrates) by α-amylases (2, 3) prove that glycosylation extends to *de novo* glycosidic linkage formation; the results also place these classic enzymes, traditionally considered "glycoside hydrolases" (EC 3.2.1), in a new light.

The present report describes a further aspect of evidence for the generality of the glycosylation concept (2, 3), namely, the utilization of α-D-glucosyl fluoride by a polysaccharide-synthesizing enzyme, dextransucrase. Hitherto, only sucrose and 4^F β-D-galactosylsucrose ("lactulosucrose") were known to support dextran synthesis by this enzyme (4); indeed, every donor substrate known to support polysaccharide synthesis by any "glycosyl transferase" (EC 2.4) has possessed, and has been assumed to owe its functionality to, a glycosidic linkage.

Yet, as shown below, α-D-glycosyl fluoride which is a stereoanalog of α-D-glucopyranose and not a glycoside, parallels sucrose in being utilized by dextransucrase both for glucosylating D-fructose and (depending on the enzyme) for synthesizing water-soluble or water-insoluble high polymer dextrans. The conversion to insoluble dextrans holds special interest since the formation of such dextrans from sucrose by certain streptococci of human source (5, 6) appears related to the genesis of dental plaque and dental caries (6–10) as well as to subacute bacterial endocarditis (5).

Material and Methods. The α-D-glucopyranosyl fluoride was a recrystallized sample, $[\alpha]_D^{24}$ + 88° (c, 2), synthesized as previously described (2). Sucrose was a sample of beet sugar free from the traces of dextran and other polysaccharides that accompany most lots of commercial and reagent sucrose (11); ^{14}C-D-fructose, from New England Nuclear Corp., was purified by paper chromatography before use; isomaltose was the gift of Dr. Allene Jeanes.

Dextransucrase from *Leuconostoc mesenteroides* B-512F was a lyophilized sample kindly supplied by Dr. H. M. Tsuchiya and kept at 4° for some years; it assayed 0.58 unit/mg (*i.e.*, each mg converted 0.58 μmole of sucrose to dextran and fructose per min at pH 5.1 and 30°). Dextranase from *Penicillium funiculosum* NRRL 1768 (12), also furnished by Dr. Tsuchiya, assayed 350 units/mg at 30°. Analytical grade yeast invertase (β-D-fructofuranosidase) was purchased from Difco Corp.; honey invertase (α-D-glucosidase) was prepared from a sample of unheated honey according to directions kindly supplied by Dr. J. W. White, Jr.

Chromatography was carried out by the descending method, using Whatman no. 1 paper and *n*-butanol:pyridine:water (6:4:3). Carbohydrate was measured by the phenol–H_2SO_4 technique (13); free fluoride (in the

TABLE I. Sucrose Synthesis from α-D-Glucosyl Fluoride by Dextransucrase.[a]

Donor substrate	Incubation (min)	Radioactivity under well-defined peaks: Sucrose[b] 20–25 cm	Leucrose[c] 14–17 cm	D-Fructose 30–38 cm
α-D-Glucosyl fluoride	30	2750	300	58,000
	120	2850	450	54,800
Sucrose	30	3900	300	48,000
	120	3650	850	48,000

[a] Mixtures (0.20 ml) in 0.05 *M* acetate buffer (pH 5.1) contained donor substrate (10 μmoles), ^{14}C-D-fructose (10 μmoles, 5 μCi), and *L. mesenteroides* dextransucrase (0.1 mg, 0.06 unit). After incubation at 30°, and inactivation (80°, 10 min), 10 μl was chromatographed. Radiochromatograms were examined with a Baird Atomic scanner equipped with integrator, then stained with $AgNO_3$.

[b] Identical in chromatographic mobility and slow $AgNO_3$ staining with authentic sucrose. All counts disappeared after treatment of the mixtures either with yeast invertase (β-D-fructofuranosidase) or with a honey invertase (α-D-glucosidase) preparation free from β-D-fructosidase activity.

[c] Tentatively identified on the basis of chromatographic mobility and by the fact that the counts were unaffected by treatment of the mixtures with yeast or honey invertase.

presence of glycosyl fluoride) by a micromethod previously described (2).

Results and Discussion. The first evidence that α-D-glucosyl fluoride is an effective substrate for dextransucrase was given by experiments showing the formation of sucrose and a second disaccharide [presumably leucrose, *O*-α-D-glucopyranosyl-(1 → 5)-D-fructopyranose (14)] in mixtures of enzyme, glucosyl fluoride, and ^{14}C-D-fructose (Table I). Dextransucrase is known to synthesize these disaccharides from sucrose plus fructose (4, 14, 15), and a direct comparison confirms that glucosyl fluoride supports the same transfer reactions as sucrose (Table I). At the incubation times tested (30 and 120 min), somewhat lower concentrations of the transfer products were found in the mixtures with glucosyl fluoride than with sucrose, *e.g.*, approximately two-thirds as much labeled sucrose was present. However, the levels of labeled sucrose (which changed little between 30 and 120 min) cannot be used to measure the rates of synthesis since sucrose is further polymerized to dextran. Indeed, all reaction mixtures became opalescent in the course of incubation, indicating the occurrence of polysaccharide synthesis and raising the question of whether glucosyl fluoride is polymerized by dextransucrase or is converted only to sucrose (perhaps by traces of an accompanying α-glucosidase or sucrose phosphorylase (16)).

Further study showed that, in mixtures without D-fructose (sucrose synthesis excluded) α-D-glucosyl fluoride is directly converted to dextran and that the product resembles the dextran formed from sucrose by the particular dextransucrase use. Thus, when *L. mesenteroides* B-512F dextransucrose [1.6 units in 3.0 ml of 0.1 *M* acetate buffer (pH 5.1)] was incubated with α-D-glucosyl fluoride or sucrose (300 μmoles) opalescence developed rapidly and, after 90 min at 30°, was intense in both mixtures.[1] A highly polymerized, water soluble dextran was recovered from each by repeated precipitation with ethanol (60% by volume) and drying *in vacuo*. The

[1] In other experiments, under similar conditions, the enzymic polymerization of 100 m*M* glucosyl fluoride was found to follow zero-order kinetics to at least 30% substrate utilization, and to yield equimolar amounts of dextran and free fluoride. The enzyme was essentially without action on 200 m*M* glucosyl fluoride though highly active upon 10–100 m*M* substrate. Departure from the Michaelis-Menten equation, long known for the dextransucrase–sucrose system (18), proved far more pronounced in the case of α-D-glucosyl fluoride.

yield from glucosyl fluoride (17.2 mg, 35% of theory) was approximately three-fourths that obtained from the sucrose (22.6 mg, 46% of theory).

Solutions of each product gave visible precipitation at 0.25 to 4 μg/ml with a standardized type 2 pneumococcus rabbit antiserum, characteristic of highly polymerized dextrans with very high proportions of α-1,6-linked anhydroglucose units (17). Solutions (1 mg in 1 ml) treated with *P. funiculosum* dextranase (20 μg, 7 units) immediately lost their opalescence; by 30 min at 30°, each product appeared extensively hydrolyzed since addition of ethanol (to 60%) gave no turbidity, and chromatograms showed the abundant presence of a reducing sugar identical in migration with an authentic sample of isomaltose.

The polymerization of α-D-glucosyl fluoride, as well as sucrose, to water-insoluble dextrans was demonstrated using enzymes from two strains of *Streptococcus* variety DS (5, 19) isolated from the blood of patients with subacute endocarditis. In 5% sucrose broth these strains produced voluminous zoogleal gels of dextran, a phenomenon first described for streptococci by Hehre and Neill in 1946 [cf. Fig. 1 in Ref. (5)]. For enzyme preparation, each strain was grown in dialyzed medium (20) with D-glucose (but no sucrose) under conditions described by Carlsson, Newbrun and Krasse (21); the neutralized culture fluids, freed of cells by centrifugation at 13,000*g* (30 min), were used.

As shown in Table II and Fig. 1, both α-D-glucosyl fluoride and sucrose were converted by each of these enzymes into water-insoluble dextran gels of high serological activity. Under the conditions used, gel formation was evident with both donors after 3.5 hr incubation at 30°. Analysis at 20 hr showed that the total dextran formed from glucosyl fluoride was 75–80% of that from sucrose; also, that 15–20% of the polymer from the glucosyl fluoride was water soluble whereas almost all of the sucrose-derived product was insoluble.

A noteworthy finding was that these and similar streptococcal dextran gels are solubilized by dimethyl sulfoxide (22). That is, when centrifuged and washed gels (8–10 mg dextran/ml) were treated with 10 vol of dimethyl sulfoxide, clear viscous solutions resulted. Dialysis of these provided clear aqueous solutions of dextran (ca. 0.5 mg/ml) from which gels were obtained when concentration was attempted, *e.g.*, by vacuum evaporation at 35°. These observations suggest that gel formation involves a final, nonenzymic, molecular association process.

Studies by Hestrin (23) and Eisenberg and

TABLE II. Insoluble Dextran Synthesis from α-D-Glucosyl Fluoride by Enzymes from Endocarditis Streptococci.[a]

Substrate	*Streptococcus* DS var. (strain 53) enzyme		*Streptococcus* DS var. (strain 135) enzyme	
	Insoluble dextran gel (μmoles)[b]	Soluble dextran (μmoles)[b]	Insoluble dextran gel (μmoles)[b]	Soluble dextran (μmoles)[b]
α-D-Glucosyl fluoride	16.7[c]	4.4	14.4	2.2
Sucrose	26.0[c]	0.6	21.3	0.6
D-Glucose	0.0	0.0	0.0	0.0

[a] Mixtures of substrate (100 μmoles), streptococcal enzyme (0.50 ml), benzyl penicillin K salt (50 units), and 0.1 *M* pH 6.8 phosphate buffer (0.50 ml) were incubated at 30° (20 hr). After heat inactivation and centrifugation, gel and fluid components were separated. Gels were washed three times by suspension in 1% sodium acetate and centrifugation (1500*g*, 30 min); finally dissolved in 1 *N* NaOH and analyzed for carbohydrate (13). Dextran in fluids was separated by repeated precipitation with ethanol and determined similarly (13).

[b] Equivalent to percentage of the theoretical yield from 100 μmoles of substrate.

[c] Identified as dextran on the basis of precipitation at 0.3–0.5 μg/ml with type 2 pneumococcus rabbit antiserum (17), as well as hydrolysis by *P. funiculosum* dextranase.

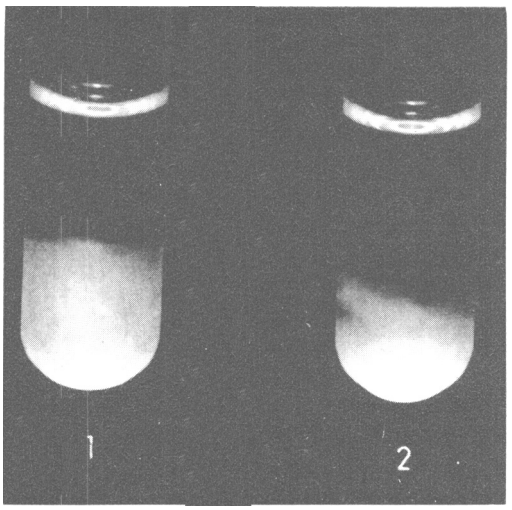

FIG. 1. Insoluble dextran gels produced by *Streptococcus* DS 53 dextransucrase incubated with (1) 0.1 *M* sucrose; (2) 0.1 *M* α-D-glucosyl fluoride. The enzyme–substrate mixtures, lightly centrifuged (700 *g*, 15 min), show the near absence of opalescence of the fluids. 2×. See Table II for analyses.

Hestrin (24) have established that dextransucrase is a glucosylase that does not transfer the glycosidic oxygen bridge atom of sucrose to the dextran product. The present finding that α-D-glucosyl fluoride is a substrate shows further that a preexisting glycosidic linkage in the donor substrate is unnecessary for the activity of this enzyme. The *de novo* synthesis of glycosidic linkages by dextransucrase supports the view (2, 3) that the process of glycosyl transfer is not, as commonly believed, restricted to the redistribution of already existing glycosidic linkages.

Summary. α-D-Glucosyl fluoride, a stereoanalog of α-D-glucopyranose, parallels sucrose in serving as an effective substrate for dextransucrase. Its polymerization to soluble dextran by *L. mesenteroides* enzyme, and to water-insoluble dextran gels by enzymes from certain endocarditis streptococci (*Streptococcus* var. DS), are the first known examples of enzymic polysaccharide synthesis from a donor substrate lacking a glycosidic linkage.

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