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Dextransucrase and the mechanism for dextran biosynthesis

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ABSTRACT

Remaud-Simeon and co-workers [Moulis, C.; Joucla, G.; Harrison, D.; Fabre, E.; Potocki-Veronese, G.; Monsan, P.; Remaud-Simeon, M. *J. Biol. Chem.*, **2006**, *281*, 31254–31267] have recently proposed that a truncated *Escherichia coli* recombinant B-512F dextransucrase uses sucrose and the hydrolysis product of sucrose, D-glucose, as initiator primers for the nonreducing-end synthesis of dextran. Using ^{14}C-labeled D-glucose in a dextransucrase–sucrose digest, it was found that <0.02% of the D-glucose appears in a dextran of M_n 84,420, showing that D-glucose is not an initiator primer, and when the dextran was treated with 0.01 M HCl at 80 °C for 90 min and a separate sample with invertase at 50 °C for 24 h, no D-fructose was formed, indicating that sucrose is not present at the reducing-end of dextran, showing that sucrose also was not an initiator primer. It is further shown that both D-glucose and dextran are covalently attached to B-512FMC dextransucrase at the active site during polymerization. A pulse reaction with [^{14}C]-sucrose and a chase reaction with nonlabeled sucrose, followed by dextran isolation, reduction, and acid hydrolysis, gave ^{14}C-glucitol in the pulsed dextran, which was significantly decreased in the chased dextran, showing that the D-glucose moieties of sucrose are added to the reducing-ends of the covalently linked growing dextran chains. The molecular size of dextran is shown to be inversely proportional to the concentration of the enzyme, indicating a highly processive mechanism in which D-glucose is rapidly added to the reducing-ends of the growing chains, which are extruded from the active site of dextransucrase. It is also shown how the three conserved amino acids (Asp551, Glu589, and Asp 622) at the active sites of glucansucrases participate in the polymerization of dextran and related glucans from a single active site by the addition of the D-glucose moiety of sucrose to the reducing-ends of the covalently linked glucan chains in a two catalytic-site, insertion mechanism.

1. Introduction

In 1941 Hehre[1] reported the first cell-free synthesis of dextran using sucrose as the substrate. Shortly thereafter, the enzyme dextransucrase [EC 2.4.1.5] was named as the enzyme responsible for the synthesis. It was found that both *Leuconostoc mesenteroides* strains and *Streptococcus* sp. elaborated dextransucrases. Dextransucrase actually is a generic name for a family of enzymes that synthesize dextrans with different structures from sucrose. Dextrans are glucans with contiguous α-(1→6) glucosidic linkages in the main chains that have various arrangements (random, continuous, and alternating single glucose residues and long dextran branched chains with three different kinds of branch linkages [α-(1→2), α-(1→3), or α-(1→4)], depending on the dextransucrase that is produced by the specific strain of *L. mesenteroides* or *Strep.* sp. In 1954, Jeanes et al.[2] reported the synthesis of many different kinds of dextrans by 96 strains of *L. mesenteroides* and *Streptococcus* sp. Both types of bacteria are Gram-positive cocci that are closely related. *L. mesenteroides* strains are facultative anaerobes, while *Streptococci* are strict anaerobes. Another notable difference between them is that *L. mesenteroides* strains require sucrose in their culture medium to induce the formation of the glucansucrases, whereas *Streptococcus* sp. are constitutive for glucansucrases and do not require sucrose in their medium for their elaboration. In 1994, Kim and Robyt,[3] using ethyl methanesulfonate as a mutagen, reported the mutation and selection of several *L. mesenteroides* strains (B-512FMC, B-742CA, B-742CB, B-1142C, B-1299C, B-1355CA, and B-1355CB) that were constitutive for their glucansucrases and did not require sucrose in their culture medium for

Abbreviations: DS, dextransucrase; LMW, low molecular weight; HMW, high molecular weight; M_n, number average molecular weight; DP, degree of polymerization; HA, hydroxyapatite; HA–DS, hydroxyapatite immobilized dextransucrase; CP, conversion period, which is the theoretical time necessary to convert the substrate into product for the amount of enzyme present; B-512FMC, dextransucrase constituent mutant of *Leuconostoc mesenteroides* B-512F; APTS, 8-amino-1,3,5-pyrenetrisulfonic acid, derivatizing fluorescent reagent; SDS, sodium dodecylsulfate; TFA, trifluoroacetic acid.

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induction and elaboration of their glucansucrases. The glucansucrases that were secreted into the culture medium were thus devoid of glucan and other products produced by glucansucrase. The mutants also were selected to elaborate only one of the two glucansucrases for those wild-type organisms that elaborated two kinds of glucansucrases. The mutant glucansucrases synthesized glucans that had the same structure and characteristics as the corresponding glucansucrases that were produced by the wild-type organisms. All of the mutants also produced 3–22 times the activity when grown on D-glucose than the amount produced by the wild-type parent strains when grown on sucrose.

Besides the synthesis of dextrans, dextransucrases also catalyze secondary transglycosylation reactions in which the D-glucose moiety of sucrose is transferred to mono- and oligosaccharides present or added to the digest to give oligosaccharide products.[4–7] This is called an 'acceptor reaction' and occurs at the expense of dextran synthesis.[7] The acceptor reactions also involve (a) the transfer of D-glucose to an acceptor monosaccharide or oligosaccharide,[6,7] (b) the transfer of D-glucose to a dextran chain to give D-glucosyl branch linkages, and the transfer of the dextranyl chain to a dextran chain to give dextranyl branched dextran chains,[8] (c) a very minor acceptor reaction in which the D-glucose moiety of sucrose is transferred to water to give the hydrolysis of sucrose,[9] (d) and the transfer of the dextranyl chain to water and/or to an acceptor saccharide (D-glucose, D-D-fructose, sucrose, or maltose) to release the dextran from the active site and terminate polymerization.[6,9]

In 1974 Robyt et al.,[10] using pulse and chase experiments with [^{14}C]-sucrose, showed that Bio-Gel P2-immobilized dextransucrase was transferring D-glucose from sucrose to the reducing-ends of the growing dextran chains. The enzyme formed covalent intermediates with the C1–OH of D-glucose and with the C1–OH at the reducing-end of the growing dextran chain. A two-site insertion mechanism was proposed for the synthesis of dextran in which D-glucose is inserted between the enzyme and the reducing-end of the growing dextran chain by means of the transfer of the dextran chain to the covalent D-glucosyl unit. In 1983, Robyt and Martin,[11] also using pulse and chase techniques with *Streptococcus mutans* 6715 dextransucrase (GTF-S) and mutansucrase (GTF-I), showed that the two *S. mutans* glucans, dextran and mutan, were also synthesized by the two enzymes from the reducing-end of the growing glucan chains. In 1984, Ditson and Mayer[12] also showed that the synthesis of dextran by *S. sangius* dextransucrase was from the reducing-end by the two-site insertion mechanism and not by a primer, nonreducing-end mechanism.

For over 20 years, 1956–1976, various investigators searched for an enzyme that branched dextran. A branching enzyme was never found. In 1976, Robyt and Taniguchi[8] showed that D-glucose and a dextran chain, covalently linked to B-512F dextransucrase, were released from the active site by exogenous dextran chains to form α-(1→3) branch linkages. They showed that the biosynthesis of the branch linkages did not require a separate branching enzyme and took place by an exogenous acceptor dextran chain, displacing the covalent glucosyl–dextransucrase complex or displacing the covalent dextranyl–dextransucrase complex to give α-(1→3) branched single glucose units and α-(1→3) branched dextran chains, respectively.

In 1978, Robyt and Walseth[6] showed that the mechanism of the acceptor reactions was the attack of the acceptor on the D-glucosyl- and dextranyl-covalent complexes, releasing them from the active sites of B-512F dextransucrase. In 1983, Robyt and Eklund[7] reported the relative quantitative effects of 17 saccharide acceptors. Maltose was the best acceptor, followed by isomaltose, D-glucose, and D-fructose in that order, and that the increase in the concentration of maltose gave an exponentially decreasing amount of dextran, indicating the inhibition of dextran synthesis.

Recently in 2006, there appeared a paper in the *Journal of Biological Chemistry* by Remaud-Simeon and co-workers,[13] using N- and C-terminal truncated *L. mesenteroides* B-512F dextransucrase that was cloned in *E. coli*. They state that this enzyme synthesizes B-512F dextran by a nonprocessive or semiprocessive reaction in which D-glucose and sucrose act as initiator primers and the D-glucose moiety of sucrose is added to the C-6–OH of D-glucose and to the C-6–OH of the D-glucose moiety of sucrose to give dextran polymerization from a single active site by the addition to the nonreducing-ends of isomaltodextrins and not by the two-site insertion mechanism, as had previously been established by several studies.

In the present study, we show that D-glucose produced by the hydrolysis of sucrose, and sucrose itself are not initiator primers, and that D-glucose is not added to the nonreducing-ends of isomaltodextrin primers for the synthesis of HMW dextran, as proposed by Remaud-Simeon and co-workers.[13] Using pulse and chase experiments, we reaffirm that D-glucose from sucrose is added to the reducing-end of a growing dextran chain. We also reaffirm that D-glucosyl- and dextranyl-covalent enzyme complexes are formed during the polymerization reaction and that the enzyme catalyzed reaction is highly processive and not nonprocessive or semi-processive, as was proposed by Remaud-Simeon and co-workers.[13] Further, we show how only one set of the three amino acids (Asp-551, Glu-589, and Asp-622), known to be conserved at the active sites of the so-called GH-family 70 glucansucrases, can participate in the two catalytic-site, insertion mechanism for the polymerization of glucans by glucansucrases, also contrary to the claim by Remaud-Simeon and co-workers[13] that there would have to be two sets of the conserved amino acids to have participation in a two-site mechanism.

2. Experimental

2.1. Materials

2.1.1. Chemicals

(a) [^{14}C]-D-glucose (1 mCi/mL) was purchased from New England Nuclear in 1975 and kept frozen in the laboratory, until needed; (b) [^{14}C]-UL-sucrose (0.1 mCi/mL) was obtained from Sigma Chemical Co. (St. Louis, MO, USA); (c) isomaltodextrin standards were obtained by acceptor reaction of 75 U of *L. mesenteroides* B-512FMC in 10 mL of 200 mM sucrose and 200 mM D-glucose incubated for 24 h at 23 °C. Excess D-glucose was removed by immobilized yeast;[14] 5 mL of ethanol was added to precipitate dextran, the mixture was centrifuged, and the supernatant was concentrated to 2 mL; the individual isomaltodextrins were purified on BioGel P-2 and P-4 (fine) columns (2.5 × 120 cm), using water as an eluant; (d) ^{14}C was counted in Bray's dioxane cocktail for aqueous samples, and toluene cocktail was used to count the radioactivity of carbohydrates adsorbed onto Whatman 3MM paper.[15]

2.1.2. Enzymes

(a) *L. mesenteroides* B-512FMC dextransucrase (hereinafter B-512FMC dextransucrase) was obtained from *L. mesenteroides* B-512FMC constitutive mutant.[3,16] The culture supernatant was concentrated and dialyzed against 20 mM pyridinium acetate buffer (pH 5.2) on an ultrafiltration, hollow-fiber cartridge (10 cm × 1 m, H5P100-43, Amicon, Inc., Beverly, MA).[17] The B-512FMC dextransucrase activity was 520 IU/mg, using the ^{14}C-sucrose assay, described below, where 1.0 IU = 1.0 μmol of D-glucose incorporated into dextran per min; (b) invertase from baker's yeast was obtained from Sigma Chemical Co.

2.2. Methods

2.2.1. Assay of dextransucrase

B-512FMC Dextransucrase (100 μL) is added to 100 μL of 250 mM (0.1 μCi) UL-^{14}C-sucrose in 20 mM pyridium acetate (Py/Ac) buffer (pH 5.2, the optimum pH for the enzyme); the reaction was allowed to proceed at 23 °C, with 25-μL aliquots removed every 5 min for 20 min and put onto 1.5 × 1.5 cm Whatman 3MM papers, which were immediately placed in 300 mL of 40% v/v methanol–H_2O, with stirring for 15 min to stop the reaction and precipitate the dextran on the papers; the papers were washed 2 times in 40% v/v methanol–H_2O, dried, and placed into toluene liquid scintillation cocktail and the ^{14}C counted. One unit is that amount of enzyme that will give the formation of 1.0 μmol of glucose incorporated into dextran per min.

2.2.2. Kinetic study of the amounts and the number-average molecular weights of dextran synthesized by B-512FMC dextransucrase

B-512FMC dextransucrase (750 units) was added to 100 mL of 200 mM sucrose in 20 mM Py/Ac buffer (pH 5.2) and incubated at 23 °C; 10-mL aliquots were taken at various times from 1 to 1440 min, and the reaction was stopped by heating in boiling water for 10 min; 30 mL of ethanol was added to each aliquot and kept at 4 °C for 24 h; the dextran was recovered by centrifugation and then dissolved in 5 mL of water and precipitated again by adding 15 mL of ethanol; this was repeated 3 times; the dextran was treated 5 times with 10 mL anhyd acetone and once with 10 mL of anhyd ethanol, and then dried in a vacuum oven for 24 h at 40 °C. The reducing value and total carbohydrate of each dextran sample (5–10 mg/mL) were measured by the micro Cu-bicinchoninate method and micro phenol–sulfuric acid method, respectively.[18] The number average DP and number average molecular weights of the synthesized dextrans were obtained from the following determinations: (DP_n) = [(μg of total carbohydrate) ÷ (μg of maltose, determined by measuring the reducing value)] × 1.9, and the number average molecular weight = M_n = $[(DP_n) \times 162] + 18$.

2.2.3. Examination of whether D-glucose and sucrose are initiator primers for the synthesis of dextran by B-512FMC dextransucrase

To test whether sucrose was incorporated in dextran as an initiator primer, the presence of D-fructose at the putative reducing-end of dextran was analyzed; 400 μg of sucrose, leucrose, and dextran (DP 521) was each dissolved in 1.0 mL of water, and 5 μL (2000 ng) was added to a Whatman K5 TLC plate; 200 μg of the compounds was dissolved in 100 μL 0.01 M HCl and incubated at 80 °C for 90 min to liberate D-fructose; 200 μg of each compound was also added to 100 μL of 20 mM Py/Ac buffer (pH 5.0), containing 20 units of invertase, which was incubated at 50 °C for 24 h; 10 μL (2000 ng) of each of the compounds for the two reactions was added to a Whatman K5 TLC plate, which was irrigated 20-cm 3 times with 85/15 volume proportions of acetonitrile–water; and the TLC was dried and rapidly dipped into methanol, containing 0.3% (w/v) *N*-(1-naphthyl)ethylenediamine and 5% concd sulfuric acid (v/v), dried and then placed in an oven at 120 °C for 10 min. Carbohydrates appeared as black spots on a white background, with a sensitivity of 2 ng.[19,20]

2.2.4. Fluorescence-assisted capillary electrophoretic analysis (FACE) of the products formed during the reaction of B-512FMC dextransucrase

Aliquots (40 μL) of the B-512FMC dextransucrase digest, as described in Section 2.2.2, containing 40 μg of carbohydrate were taken to dryness, and 2-μL (5 mg) of 8-amino-1,3,5-pyrenetrisulfonic acid (APTS) in 15% v/v aqueous acetic acid and 2-μL of 1 M sodium cyanoborohydride in tetrahydrofuran were added, and the mixture was allowed to react for 15 h at 42 °C; then 46 μL of Milli-Q pure water was added, and the solution was centrifuged for 2 min; 5 μL of the supernatant was diluted with 195 μL of Milli-Q pure water, and transferred to a 0.5-mL tube for capillary electrophoresis for 60 min, using a P/ACE MDQ Glycoprotein Electrophoresis System (Beckman Coulter, Fullerton, CA).

2.2.5. Demonstration of the formation of covalently linked D-glucose and dextran at the active site of B-512FMC dextransucrase during catalysis of the synthesis of dextran

2.2.5.1. Immobilization of dextransucrase. B-512FMC Dextransucrase (DS) (600 mU in 600 μL of 20 mM Py/Ac buffer, pH 5.2) was added to 600 μL of 25% (w/v) hydroxyapatite (HA) in Py/Ac buffer and stirred for 30 min. The mixture was centrifuged, and the immobilized DS (HA–DS) was resuspended in 1200 μL of buffer. The assay indicated that 100% of the enzyme activity had been immobilized.

2.2.5.2. Reaction of the HA–DS. The substrate solution contained 1.0 μCi of 300 mM ^{14}C-Sucrose in 1.0 mL of 40 mM Py/Ac buffer (pH 5.2), and the reaction was initiated by the addition of 60 mg of HA–DS having 10 units of dextransucrase. The reaction was allowed to proceed for 15 min (0.5 conversion period) at 23 °C, then centrifuged for 1 min, and the supernatant was removed; the HA–DS was washed 4 times with 1 mL of pH 5.2 buffer by suspension and centrifugation. The HA–DS was then suspended in 1 mL of 0.01 M trifluoroacetic acid and incubated for 60 min at 50 °C; the HA–DS was centrifuged, and the supernatant was saved. The HA–DS was then washed 2 times with 500 μL of water, which was added to the supernatant and taken to dryness under vacuo; then 500 μL of water was added to the dried sample, which was then added to a Bio-Gel P2 (fine) column (1.5 × 50 cm) and eluted with water; 500 μL fractions were collected, and 250-μL aliquots were added to 10 mL of Bray's dioxane cocktail for liquid scintillation counting.

2.2.6. Determination of the effect of the concentration of dextransucrase on the molecular size of the synthesized dextran

To 10 mL of 20 mM sucrose solution in 20 mM Py/Ac buffer (pH 5.2), 0.1 unit, 1.0 unit, and 10.0 units of B-512FMC dextransucrase were added and incubated at 23 °C for 200 min, 20 min, and 2 min, respectively. Adjusting the pH to 2 with 1 M HCl stopped the reaction, and the addition of 3 vol of ethanol precipitated the dextran. The precipitated dextran was dissolved in water 3 additional times and was precipitated with ethanol as described above. The dextrans were treated with anhyd acetone several times and once with anhyd ethanol, and then dried in a vacuum oven for 15 h at 40 °C. The degree of polymerization of the dextrans was determined by measuring their reducing value and total carbohydrate as described in Section 2.2.2.

2.2.7. Pulse and chase studies of B-512FMC dextransucrase, using ^{14}C-sucrose and nonlabeled sucrose, respectively

(1) B-512FMC dextransucrase (10 units in 100 μL) was added to 2.0 mL of 1.0 μCi, 100 mM ^{14}C-sucrose in 40 mM Py/Ac buffer (pH 5.2). The reaction was allowed to proceed at 23 °C for 15 min (0.75 conversion period).
(2) The reaction was placed into an ice bath and divided into two 1.0-mL parts.
(3) Two volumes of −10 °C ethanol was immediately added to one of the parts (the pulsed reaction) to stop the reaction and precipitate the dextran; 100 μL of 2.5 M sucrose was

immediately added to the second part, which becomes the chase reaction.

(4) The chase reaction is immediately removed from the ice bath and allowed to proceed for 30 min (1 CP) at 23 °C.

(5) The chase reaction was stopped by placing it in an ice bath and immediately adding 2 vol of −10 °C ethanol to stop the reaction and precipitate the dextran.

(6) The two parts were centrifuged to obtain the precipitated dextrans, which were washed 5 times with 1.0 mL of 67% (v/v) aqueous ethanol to remove any unreacted sucrose and other LMW products that might be present, such as glucose, fructose, leucrose, and LMW isomaltodextrins, by suspending them in the ethanol solutions and centrifuging them.

(7) The pulsed- and chased-dextrans were dehydrated by treating them 5 times with 1.0 mL of anhyd acetone and once with 1.0 mL of anhyd ethanol and placed under continuous vacuum for 30 min.

(8) Equal amounts (10 mg) of the two dextrans were dissolved in 1.0 mL of 20 mM pyridine (pH 10.5), containing 2 mg of $NaBH_4$, which was allowed to react at 70 °C for 1 h.

(9) Five hundred microliters of 12 M TFA were added to 1.0 mL of the two dextrans, and the mixture was allowed to stand at 23 °C for 10 min to destroy the $NaBH_4$. It was then placed in an autoclave at 121 °C for 30 min to completely hydrolyze the dextrans.

(10) Five hundred microliters of methanol were then added to each part, and the two parts were taken to dryness under vacuo 3 times to remove the boric acid and TFA.

(11) A 22 cm × 56 cm length of Whatman 3MM paper was prepared for each sample for descending paper chromatography. The dried samples were dissolved in 210 μL of water and 2 μL of each sample was placed 7.5 cm down from the top of the paper and 1 cm on each side of the paper and 200 μL of the sample was streaked along 16 cm in the center of the paper.[15] The papers were irrigated using 8:1:3:2 volume proportions of nitromethane–acetic acid–ethanol–water–saturated boric acid for 15 h at 23 °C.

(12) The positions of D-glucose and D-glucitol on the paper were determined by cutting 2.0 cm strips from each side of the paper and cutting the strips into 1 cm pieces for heterogeneous liquid scintillation counting in 10 mL of toluene cocktail. From the test strips, D-glucitol and D-glucose were located (it is known that D-glucitol migrates faster than D-glucose), and the paper was sectioned on the 18 × 50 cm piece of paper for heterogeneous liquid scintillation counting in 15 mL of toluene cocktail.[15]

3. Results and discussion

3.1. *Leuconostoc mesenteroides* B-512FMC dextransucrase used in the study

L. mesenteroides B-512FMC is a constitutive mutant that had been obtained by mutation of *L. mesenteroides* B-512F, and was therefore selected for the constitutive production of dextransucrase. It does not require sucrose in the culture medium to induce the production of the enzyme,[3,16] and it can be grown on a glucose medium; it therefore gives an extracellular dextransucrase that has no dextran or isomaltodextrins in the culture supernatant. The mutant also was selected for the production of a significantly higher yield of enzyme, namely 20 units/mL of culture supernatant. The enzyme is hereinafter designated B-512FMC dextransucrase, and it synthesizes a dextran that has the same structure and properties as the wild-type *L. mesenteroides* B-512F dextransucrase.

3.2. Kinetic study of the amount, DP, of dextran formed, and the effect of the enzyme concentration on the synthesis of dextran by B-512FMC dextransucrase

The results of the kinetic study of the amount and number average molecular weight (M_n) of B-512F dextran show that both increase with time (Table 1). The maximum amount (325.3 mg) of dextran was obtained in 24 h (48 CP). The maximum M_n (240,912 ± 4068 Da) was obtained in 60 min or 2 CP, which is twice the amount of time necessary for the amount of dextransucrase present to theoretically convert all of the sucrose into dextran. The relatively low, non-optimum, concentration of sucrose that remains after 2 CP, continues, however, to synthesize dextran, although the chains are of much lower M_n due to the low concentration of sucrose, giving a bimodal distribution of dextran MWs that contribute to the lowering of the number-average molecular weight that is finally obtained (see Table 1).

Table 1
Kinetic study of the amount and number-average molecular weight of dextran synthesized by B-512FMC dextransucrase with 200 mM sucrose at various times

Reaction time[a] (min)	Dextran dry wt.[a] (mg)	Number of conversion periods[b]	Number avg. degree of polymerization DP[c] ± SD[e]	Number avg. molecular weight[d] M_n ± SD[e]
1	2.7	0.033	521 ± 13	84,420 ± 2124
3	24.3	0.100	685 ± 17	110,988 ± 2772
5	45.1	0.167	714 ± 27	115,686 ± 4392
10	101.2	0.333	1038 ± 38	168,174 ± 6174
20	222.8	0.667	1009 ± 10	163,476 ± 1638
30	310.5	1.000	2169 ± 29	239,778 ± 5526
60	310.8	2.000	2206 ± 31	240,912 ± 4068
120	321.1	4.000	1885 ± 53	205,758 ± 6336

[a] Reaction conditions: 100 mL of 200 mM sucrose, 20 mM pyridinium acetate buffer (pH 5.2), 23 °C, 750 units of B-512FMC dextransucrase; 10-mL aliquots taken at various times (1–120 min) and 30 mL of 5 °C anhyd ethanol were added to precipitate the dextran. The precipitate was centrifuged and dissolved in 10 mL of water and precipitated three times with 30 mL of anhyd ethanol, followed by treating five times with 10 mL of anhydrous acetone and once by 10 mL of anhydrous ethanol and then placed under vacuum at 40 °C for 15 h to obtain anhydrous dextran.

[b] One conversion period = the amount of time to theoretically convert sucrose into dextran according to the amount of enzyme added.

[c] Number average degree of polymerization (DP_n) determined by measurement of the total carbohydrate, using the phenol–sulfuric acid method, and measurement of the reducing value by the copper bicinchonate method, using maltose as the standard; DP_n = [(total carbohydrate in μg of glucose) ÷ (reducing value in μg of maltose)] × 1.9.

[d] Number average molecular weight = M_n = [(DP_n) × 162] + 18.

[e] SD = standard deviation.

Table 2
The effect of the decrease of B-512FMC dextransucrase concentrations on the size of dextran synthesized

Sucrose concd (mM)	Dextransucrase activity[a] (U/mL)	Reaction time of 1.0 CP at 23 °C[b] (min)	Dextran obtained from 1.0 L (mg)	Dextran molecular size number avg. DP DP_n[c] ± SD[d]	Dextran number average MW M_n ± SD[d]
20	10.0	2	2200	306 ± 3	49,590 ± 504
20	1.0	20	5890	1182 ± 22	191,502 ± 3582
20	0.1	200	6800	2473 ± 37	400,644 ± 6012

[a] One unit (U) = 1 μmol of Glc incorporated into dextran per min.

[b] Reaction time of 1.0 conversion period (CP) = the theoretical amount of time necessary to convert 20 mM sucrose into dextran for the amount of enzyme present.

[c] Number-average degree of polymerization (DP_n) was determined by measurement of the reducing value, using the copper bicinchonate method,[18] and the measurement of the total carbohydrate, using the phenol–sulfuric acid method;[18] DP_n = [(total carbohydrate in μg of D-glucose) ÷ (reducing value in μg of maltose)] × 1.9; number average molecular weight = M_n = [(DP_n) × 162] + 18.

[d] SD = standard deviation.

When the concentration of the dextransucrase was decreased to 10- and 100-fold, the avg. M_n significantly increased from 49,590 ± 504 Da to 191,502 ± 3582 and 400,644 ± 6012, respectively (Table 2). This result shows that the M_n is inversely proportional to the concentration of the enzyme, and indicates that the polymerization of the dextran chain is highly processive, in contrast to that stated by Remaud-Simeon and co-workers[13] that the reaction was nonprocessive or only semi-processive.

3.3. Results of the study of the Remaud-Simeon and co-workers[13] hypothesis that D-glucose and sucrose are initiator primers for the synthesis of dextran by dextransucrase

The enzyme used by Remaud-Simeon and co-workers[13] was a N- and C-terminal truncated B-512F dextransucrase that was cloned in *E. coli*.[21,22] They proposed that the initial step in the synthesis of B-512F dextran by dextransucrase uses sucrose as an initiator primer in which D-glucose is added to the C6–OH of the D-glucose moiety of sucrose, which is then elongated by the addition of D-glucose to the nonreducing-ends, giving a series of isomaltosyl sucrose homologues, ultimately resulting in sucrose-terminated HMW dextran. Dextransucrase is also known to slowly hydrolyze sucrose to give D-glucose, which they proposed also acts as an initiator primer by adding the D-glucose moiety of sucrose to the C6–OH of D-glucose to form isomaltose, which is then elongated by the addition of D-glucose to the nonreducing-ends to give a series of isomaltodextrin homologues that ultimately results in HMW dextran.

The evidence for these hypotheses was quite minimal and based primarily on a questionable interpretation of an HPLC–PAD analysis of the products at the end of the reaction in which isomaltose and isomaltotriose were the major products. In the present study, we synthesized a dextran with number average DP = 521, giving M_n of 84,420 Da, by reacting B-512FMC dextransucrase with 200 mM sucrose for 1 min. Taking 300 μg of this dextran and treating it with 0.01 M HCl for 90 min at 80 °C resulted in no D-fructose being observed by TLC, whereas an equivalent amount of sucrose was completely hydrolyzed to D-glucose and D-fructose (see Fig. 1). Taking another 300 μg of the dextran and treating it with 10 units of invertase at pH 5 and 50 °C for 24 h, there also was no D-fructose released from the dextran, while a similarly treated sucrose was completely converted to D-glucose and D-fructose.

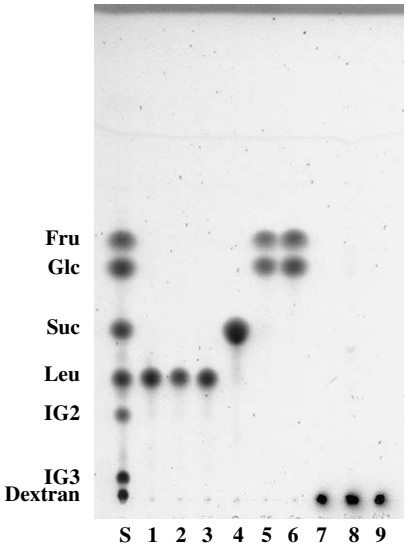

Figure 1. TLC analysis of the treatment of dextran with 0.01 M HCl at 80 °C for 90 min and the treatment of dextran with invertase (10 U/mL) at 50 °C for 24 h. Lane 1, leucrose std; lane 2, leucrose treated with 0.01 M HCl at 80 °C for 90 min; lane 3, leucrose treated with invertase at 50 °C for 24 h; lane 4, sucrose std; lane 5, sucrose treated with 0.01 M HCl at 80 °C for 90 min; lane 6, sucrose treated with 20 units of invertase at 50 °C for 24 h; lane 7, untreated dextran; lane 8, dextran treated with 0.01 M HCl at 80 °C for 90 min; lane 9, dextran treated with 20 units of invertase at 50 °C for 24 h. Fru = D-fructose; Glc = D-glucose; Suc = sucrose; Leu = leucrose; IG2 = isomaltose; IG3 = isomaltotriose.

In 300 μg or 300,000 ng of dextran, there are 300,000 ÷ [(521 × 162) + 18)] or 3.55 nmoles of dextran, avg. DP 521. If there is one sucrose unit at the reducing-end of every dextran chain of avg. DP 521, there should have been an avg. of 3.55 nmoles of D-fructose released in the above treated dextrans, or [3.55 nmoles × 180 ng/nmole] = 639 ng of D-fructose should have been released from the dextran; if 10% of the dextran chains have sucrose at their ends, 63.9 ng of D-fructose should have been released from the dextran chains; and if 1.0% of the dextran chains have sucrose at their ends, 6.39 ng of D-fructose should have been released from the dextran. The limit of detection of D-fructose by TLC is 2 ng, using our method of detection.[20] The TLC (Fig. 1) shows that no D-fructose was released from the treated dextran, thus showing that sucrose is not a primer for the synthesis of dextran by B-512FMC dextransucrase.

We also synthesized a DP 521 dextran by reacting B-512FMC dextransucrase with 200 mM sucrose in the presence of 0.1 μCi (2.2×10^5 dpm) of 0.2 mM ^{14}C-D-glucose. Samples were taken at time periods from 10 to 120 min (data not shown). A maximum of 40 dpm of D-glucose was found in HMW dextran after 1 conversion period or less than 0.02% of the D-glucose in the digest. This very miniscule amount of D-glucose could not have been a primer for the amount (350 mg) and size (DP 521) of dextran obtained in the reaction. D-Glucose is known to be a relatively poor acceptor,[7] and it is most likely that this very small amount of ^{14}C-D-glucose found in the dextran after 120 min, occurred by D-glucose acting as an acceptor that released a small amount of dextran from the active site, as originally shown in 1979 by Robyt and Walseth[6] in their study of the mechanism of the acceptor reactions by B-512F dextransucrase.

3.4. Kinetic analysis of the low-molecular-weight products (D-glucose, D-fructose, leucrose and isomaltodextrins) formed during the synthesis of dextran by B-512FMC dextransucrase

Fluorescence-assisted capillary electrophoresis (FACE),[23] which is a very sensitive method for separating and analyzing the relative quantitative amounts of oligosaccharides[24] from DP 1 to DP 100, was used to study the amounts of D-glucose and isomaltodextrins, formed during the reaction of B-512FMC dextransucrase with sucrose. Samples were taken at various degrees of conversion of sucrose, where one conversion period (CP) equals the amount of time that is theoretically required to convert the sucrose into dextran for the amount of enzyme present. In the early stages of the reaction (Fig. 2A) (0.2 CP) the products were D-glucose, D-fructose, leucrose, and isomaltodextrins in low, exponentially decreasing amounts from DP 2–5, with minuscule amounts of DP 6–12; Figure 2B (0.5 CP) gave the same products, but with exponentially decreasing amounts of isomaltodextrins, down to minuscule amounts of DP 20–25; Figure 2C (1.00 CP) gave the same products, with minuscule amounts of isomaltodextrins, DP 15–25; and Figure 2D (2.00 CPs) gave the same products, with minuscule amounts of DP 25–30. Isomaltose was formed by the well-known 'acceptor reaction' of D-glucose (the hydrolysis products of sucrose) by displacing the covalently linked D-glucose from the catalytic sites at the active site of dextransucrase.[6–8,25,26] Isomaltotriose was formed when isomaltose acted as an acceptor and displaced D-glucose from the active site. Thus, higher isomaltodextrin homologues were each formed by an isomaltodextrin, with one less D-glucose unit, acting as an acceptor to form the next higher homologue. The formation of each subsequent isomaltodextrin, with the addition of a single D-glucose, gives a product with a lower

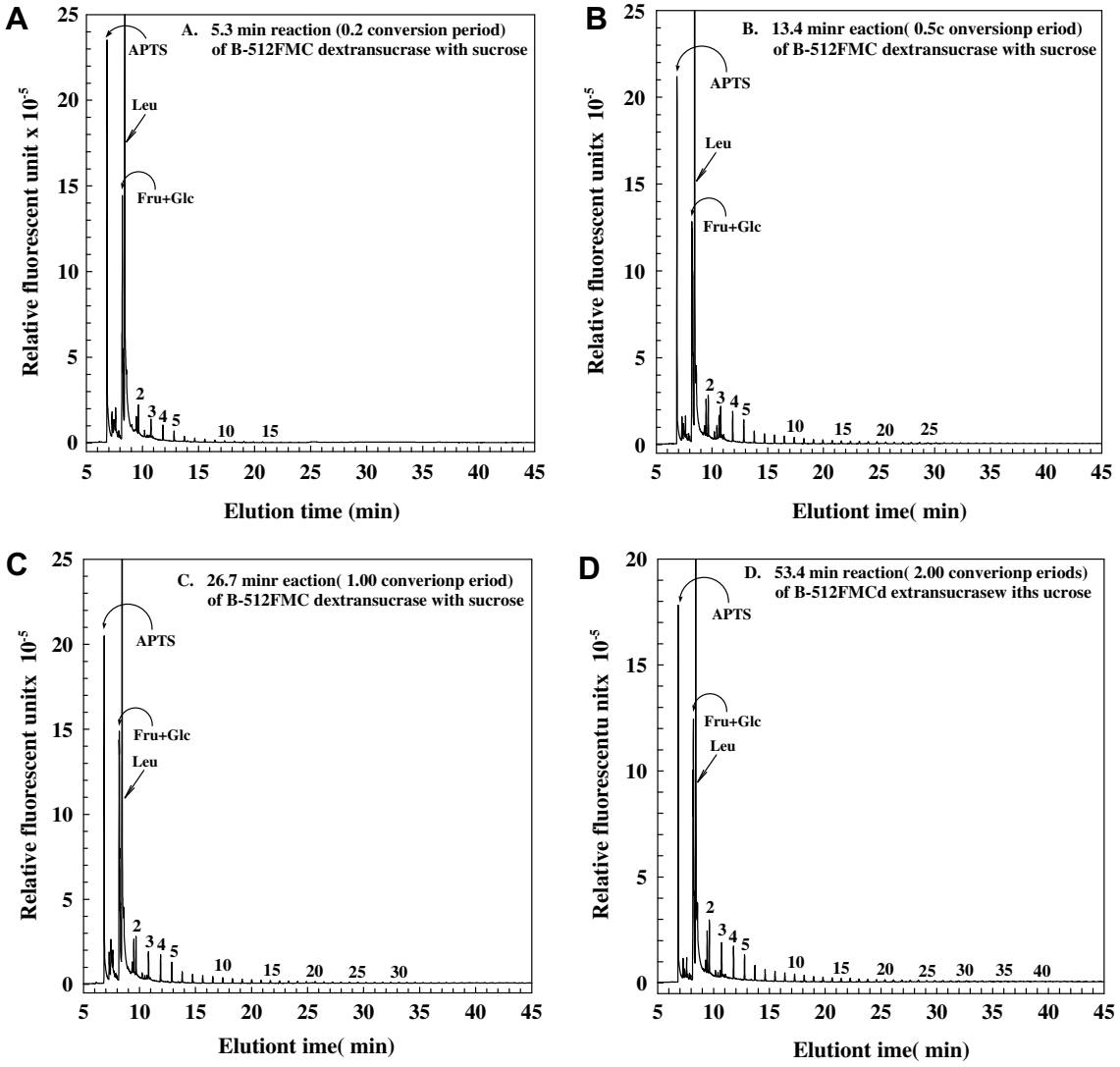

Figure 2. Fluorescence-assisted capillary electrophoresis (FACE) analysis of the formation of products, during the reaction of B-512FMC dextransucrase with sucrose. A. 0.2 conversion period; B. 0.5 conversion period; C. 1.0 conversion period; and D. 2.0 conversion periods. APTS = fluorescent derivatizing reagent; Leu = leucrose; Fru = D-fructose; Glc = D-glucose; numbers 2, 3, 4, ... = isomaltose, isomaltotriose, Isomaltotetraose, and so forth.

concentration than its predecessor, resulting in the exponential decrease in the number and amounts of the isomaltodextrin acceptor products.

The M_n of the dextrans for these same conversion periods were high: 0.20 CP, $M_n = 172{,}000 \pm 1500$ (DP ~1000); 0.5 CP, $M_n = 178{,}000 \pm 2000$ (DP ~1100); CP = 1.00, $M_n = 239{,}000 \pm 3500$ (DP ~1475); and 2.00 CPs $M_n = 240{,}000 \pm 3500$ Da (DP ~1480), respectively, showing that HMW dextran is rapidly produced by dextransucrase. These experiments definitely show that (a) the polymerization of dextran does not occur by the addition of D-glucose from sucrose to the nonreducing ends of isomaltodextrin acceptors, as postulated by Remaud-Simeon and co-workers,[13] and (b) HMW dextran is produced in the very early stages of the reaction and could not have been formed by the addition to nonreducing-ends of primers. If the polymerization was occurring by the primer mechanism, just the opposite result should have been observed; namely, there should have been exponentially increasing amounts of higher DP isomaltodextrins, going up to and including, DP 100–1000 or higher, and the amounts should have increased, either linearly or exponentially.

The FACE analysis of the LMW products formed during dextransucrase synthesis of dextran definitively shows that the formation of isomaltodextrin products by the secondary acceptor reaction was very small in number and amount and could not have contributed to the synthesis of dextran.

The addition of the putative primers, isomaltose and isomaltotriose, in increasing amounts, to the dextransucrase–sucrose digest, shows that they are potent inhibitors of dextran synthesis (see Fig. 3). At 5 mM, isomaltose gave 42% inhibition and isomaltotriose gave 58% inhibition, and at 20 mM isomaltose gave 84% inhibition and isomaltotriose gave 94% inhibition. The inhibition occurs by the isomaltodextrins displacing D-glucose and/or the growing dextran chain from the active site of dextransucrase, thereby inhibiting dextran synthesis by preventing D-glucose from being incorporated into the dextran molecules or by terminating its polymerization by releasing it from the active site.

3.5. Demonstrating that D-glucosyl- and dextranyl-covalent intermediates are formed during the synthesis of dextran catalyzed by B-512FMC dextransucrase

In a ^{14}C-sucrose pulse reaction of B-512FMC dextransucrase, it is shown that both D-glucose and a dextran chain are released from the enzyme when it is heated at pH 2 and 50 °C for 60 min (Fig. 4).

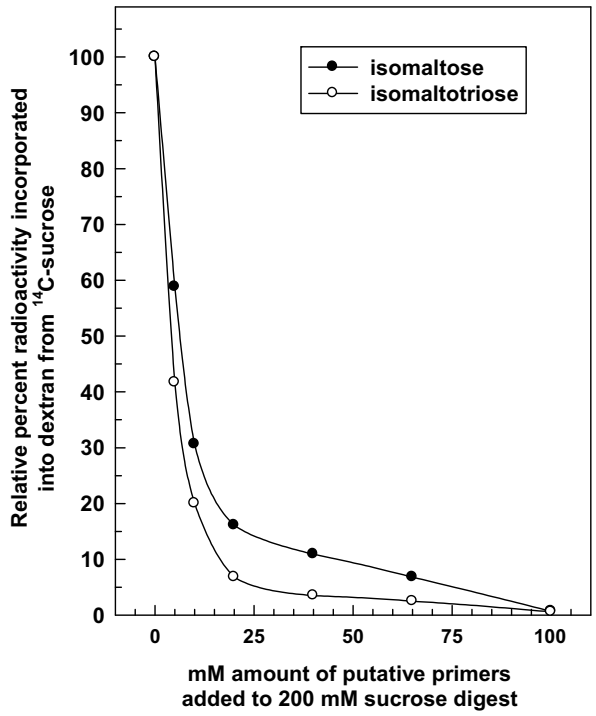

Figure 3. Inhibition of B-512FMC dextransucrase synthesis of dextran by the putative primers, isomaltose and isomaltotriose.

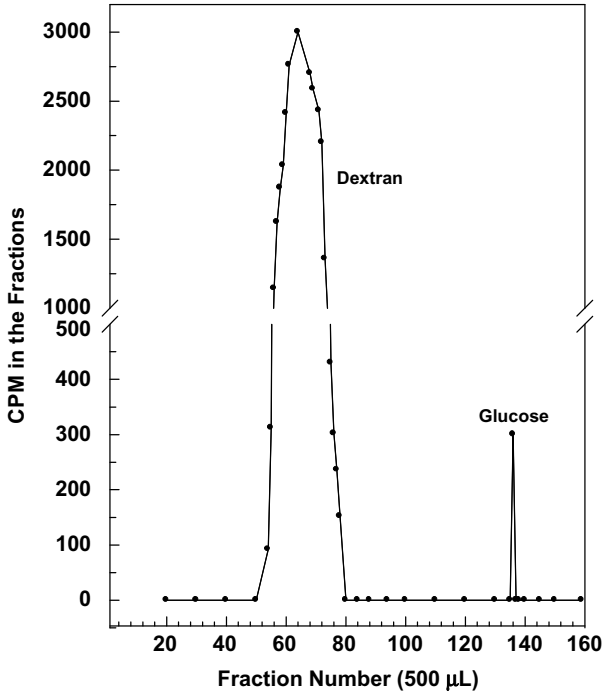

Figure 4. Bio-Gel P2 elution of ^{14}C-dextran and ^{14}C-D-D-glucose released from pulsed immobilized-dextransucrase by treatment with 0.01 M trifluoroacetic acid (pH 2) at 50 °C for 60 min.

Thus, both a D-glucosyl and a dextranyl chain are covalently linked to the active site of dextransucrase during catalysis. Robyt and Walseth[6] had previously shown that both a low-MW (LMW) product (isomaltose) and an HMW product (dextran) were released from an immobilized pulsed dextransucrase on the addition of D-glucose; similarly, an LMW product, leucrose [α-D-glucopyranosyl-(1→5)-D-fructopyranose], and an HMW product (dextran) were released when D-fructose was added; and an LMW product, panose, [α-D-glucopyranosyl-6^{II}-maltose] and an HMW product (dextran) were released when maltose was added, also indicating that both D-glucose and dextran were covalently attached to the active site of the enzyme during reaction with sucrose and were released from the enzyme by action of the acceptors, D-glucose, D-fructose, and maltose. Likewise, Robyt and Taniguchi[8] showed that exogenous dextran chains released D-glucose and dextran chains from the active site of B-512 F dextransucrase to give D-glucosyl α-(1→3) branches and α-(1→3) dextran chain branches, indicating that D-glucose and a dextran chain were covalently linked to the active site of B-512F dextransucrase. Parnaik et al.[27] also have shown that when a 10-s pulsed reaction of *S. sangius* dextransucrase was heated to 93 °C, D-glucose and a dextran chain of DP 17 were released from the enzyme. All of these studies definitively show that both a D-glucosyl unit and a dextranyl chain are covalently linked to the active sites of the glucansucrases during the dextransucrase-catalyzed elongation of dextran.

3.6. Pulse and chase study of the synthesis of dextran by hydroxyapatite-immobilized B-512FMC dextransucrase

The results of three sets of pulse and chase experiments of B-512FMC dextransucrase (Table 3) show that after isolation, reduction, and hydrolysis, a significant amount of ^{14}C-D-glucitol was obtained, and after the nonlabeled-sucrose chased dextran was isolated, reduced, and hydrolyzed, there was a significantly decreased amount of ^{14}C-D-glucitol. These results would have been impossible to obtain, if the synthesis of dextran had been the addition of D-glucose from sucrose to the nonreducing-ends of D-glucose and sucrose initiator primers, followed by the addition of D-glucose to the nonreducing-ends of the putatively growing isomaltodextrins. These experiments definitively show that D-glucose from sucrose is added to the reducing-end of a growing dextran chain and not to the nonreducing-ends of primers, as proposed by Remaud-Simeon and co-workers[13] This reaffirms the original study of Robyt et al.[10] on the mechanism of B-512F dextransucrase synthesis of dextran and the study by Robyt and Martin[11] on the mechanisms of *S. mutans* 6715 dextransucrase and mutansucrase, and the study by Ditson and Mayer[12] on the mechanism of *S. sangius* dextransucrase that the D-glucose moiety of sucrose is added to the reducing-end of the growing dextran chains by dextransucrases and mutansucrase.

3.7. Additional rebuttals of the Remaud-Simeon and co-workers[13] arguments against the two-site insertion mechanism for the synthesis of dextran by dextransucrase

Remaud-Simeon and co-workers[13] made two further hypothetical arguments against the two-site, insertion mechanism in favor of their one-site, primer-dependent mechanism for the addition of D-glucose from sucrose to the C6-OH of the nonreducing-ends of isomaltodextrins for the synthesis of dextrans. The first argument was based on the reports of Mooser and Iwaoka[28] and Mooser

Table 3
Results of the pulse and chase experiments for B-512FMC dextransucrase

Experiment	Pulse D-glucitol (cpm)	Chase D-glucitol (cpm)	Pulse D-glucose (cpm)	Pulsed dextran (M_n)
I	2485	1269	188,778	12,490
II	2535	1352	166,312	10,872
III	2658	1671	169,607	10,550

et al.,[29] who reported the isolation of only one covalent D-glucosyl-denatured enzyme complex for *S. sobrinus* dextransucrase.

It should be pointed out that the only time there are two D-glucosyl intermediates is in the initiation Step III in Figure 5 in the polymerization process. The majority of the time there is only one D-glucosyl intermediate and one dextranyl intermediate during the polymerization process. To trap the intermediates, the enzyme has to be denatured. Mooser et al.[28,29] used pH 2 with either 1.0% (w/v) SDS or 0.6 M urea. They stated that dextransucrase was stable under these conditions, but as the pH was increased, it became increasingly labile and all of the activity was lost. Activity was also lost by heating above 90 °C. The D-glucosyl- and dextranyl-covalent enzyme complexes themselves are of high energy and rapidly undergo reaction, either polymerization or hydrolysis, and are therefore quite labile. Under the conditions used by Mooser et al.[28,29] the dextranyl intermediate may have been more labile than the D-glucosyl intermediate and hydrolyzed, and they failed to obtain it in their denaturation and pepsin hydrolysis experiments at pH 2. Alternately, the pepsin hydrolysis of the denatured protein used to obtain the glucosyl-peptide might not have been produced as a small enough peptide–dextranyl fragment, because of the pepsin specificity or because pepsin was not able to bind close enough to the polypeptide–dextranyl complex due to steric effects of the relatively large dextran chain that was covalently linked to the protein. Therefore, it was not isolated with the LMW glucosyl-peptide that was obtained. In the previous studies of Robyt and Walseth,[6] Robyt and Taniguchi,[8] and Parnaik et al.,[27] and in this study (Section 3.5), it has been shown that both D-glucosyl- and dextranyl-covalent intermediates were present at the active site of dextransucrase, during catalysis, in spite of Mooser et al.[28,29] finding only a single D-glucosyl-peptide from their denatured *S. sobrinus* dextransucrase.

The second argument made by Remaud-Simeon and co-workers[13] for their single-site, primer-dependent mechanism was based on the fact that there is only one set of the three conserved amino acids (Asp-551, Glu-589, and Asp-662) found at the active sites for all glucansucrases in GH-family 70 enzymes, and there were not two sets, as they anticipated there would be, if there were two catalytic sites, or as they have stated, two active sites.

We now show here how one set of the three conserved amino acids (Asp551, Glu589, and Asp662), at a single active site of the glucansucrases, can participate in the catalysis of dextran biosynthesis by the two catalytic-site insertion mechanism. In this mechanism the carboxyl group of Glu589 acts as an acid–base catalyst and the carboxylate groups of Asp551 and Asp662 act as the two catalytic-site nucleophiles that attack C1 of the D-glucosyl moieties of sucrose, making carboxylate acetal-esters with the D-glucoses and with the growing dextranyl chain. Glu589 carboxyl group alternately donates protons to the leaving D-fructosyl moieties of sucrose. The resulting Glu589 carboxylate group becomes reprotonated when it abstracts a proton from the C6–OH group of the D-glucosyl group covalently linked to the carboxyl group, allowing the C6–O^- anion to make a nucleophilic attack onto C1 of the dextranyl-chain, forming an α-(1→6) linkage and giving the addition of D-glucose to the reducing-end of the chain, with its apparent insertion between the carboxyl group and the dextran chain. The reactions continue going back and forth alternately between the two catalytic-site carboxyl groups of Asp551 and Asp662, giving the polymerization of dextran by the two-site, insertion mechanism (see Figs. 5 and 6).

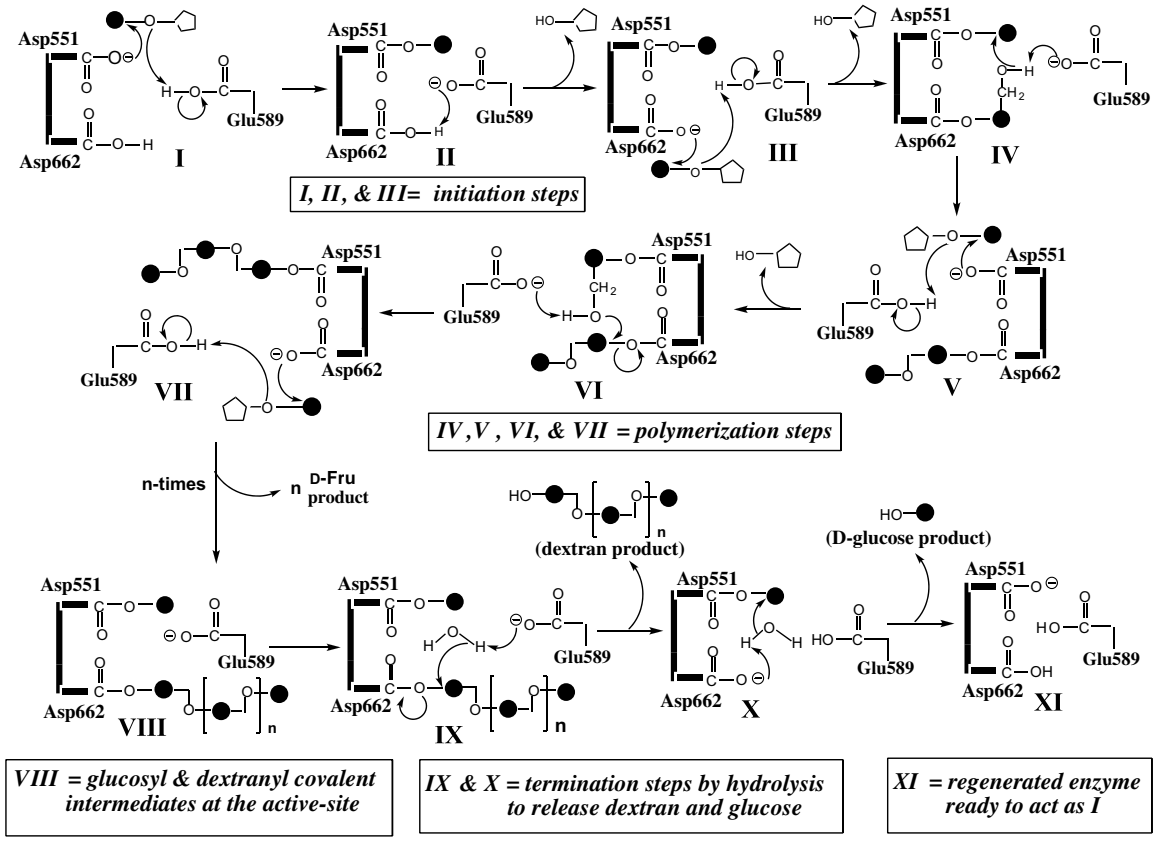

Figure 5. Insertion mechanism for the biosynthesis of dextran by the addition of D-glucose to the reducing-end of a growing dextran chain. ●–△ = sucrose, and ●, is glucose. The carboxylate groups of Asp551 and Asp662 act as nucleophiles that attack sucrose to alternately form covalent D-glucosyl- and dextranyl-intermediates, and Glu589 that alternately acts as an acid–base catalyst.

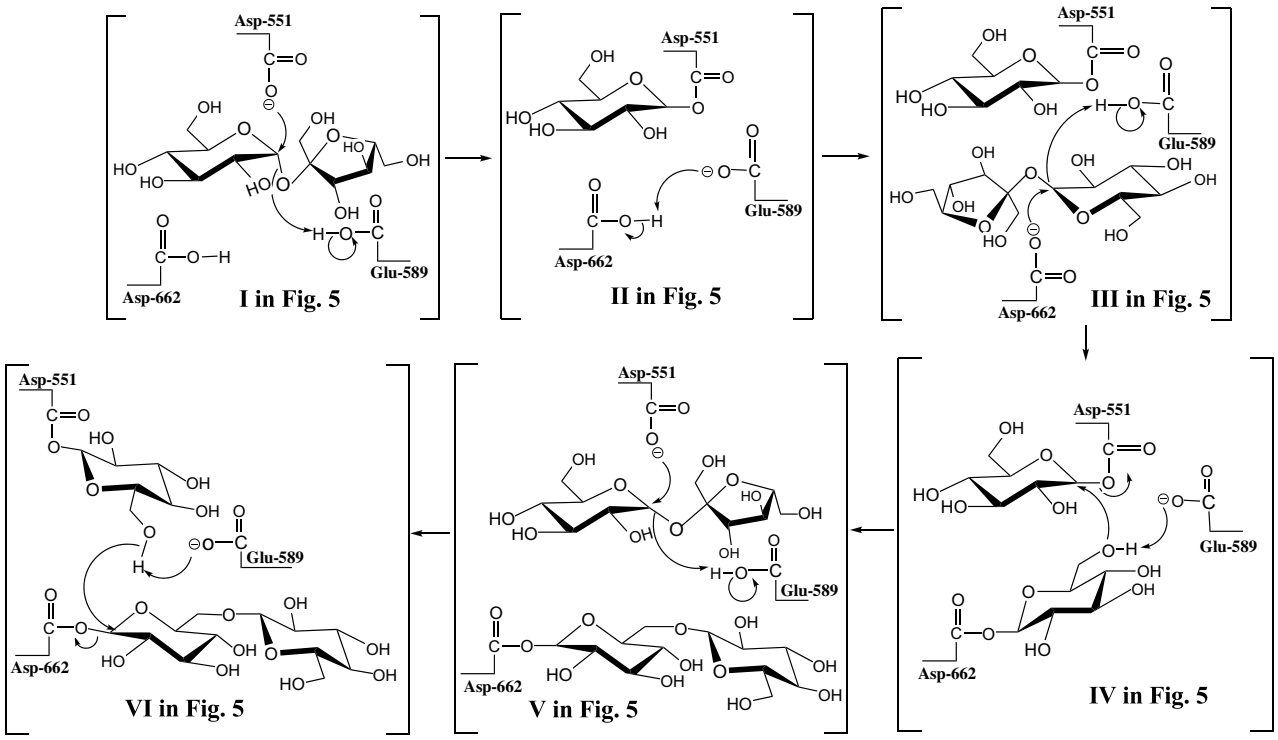

Figure 6. Involvement of the three conserved amino acids (Asp551, Glu589, and Asp662) in the organic reaction enzyme mechanism for the biosynthesis of dextran at the active site of dextransucrase by the insertion mechanism addition of D-glucose to the reducing-end of a growing dextran chain.

3.8. Structure and function of the truncated B-512 F dextransucrase cloned in *E. coli* and of *L. mesenteroides* B-512FMC dextransucrase

Besides having relatively large peptide sequences missing at both ends of the truncated protein, the *E. coli* cloned enzyme used by Remaud-Simeon and co-workers[13] is a protein that is not glycosylated, as *E. coli* is incapable of glycosylating proteins.[30–32] Robyt and Walseth[33] have shown that the *L. mesenteroides* B-512 F dextransucrase has a relatively high degree of glycosylation with high-D-mannose saccharides, and that contrary to common belief, many bacterial proteins are glycosylated.[30] The lack of glycosylation of the cloned dextransucrase could very well have resulted in an altered tertiary structure, due to incorrect folding of the peptide chain, and, therefore, have resulted in an enzyme with low specific activity, as well as, altered enzymatic properties.

The addition of dextran to purified *L. mesenteroides* B-512 F and *Streptococcus* sp. dextransucrases has been found to stimulate the synthesis of dextran; both enzymes, however, could synthesize dextran without its addition to the digests.[34–38] Germaine and co-workers[34–36] and Kobayashi and Matsuda,[37,38] however, interpreted this stimulation to be evidence for a primer-based mechanism for dextran synthesis. This interpretation was based primarily on studies in the 1940s on the action of glycogen- and potato starch-phosphorylases,[39,40] in which it was reported that a primer was required for synthesis. In the presence of inorganic phosphate, glycogen- and starch-phosphorylases catalyze the removal of D-glucose from the nonreducing-ends of glycogen and starch, giving α-D-glucopyranosyl 1-phosphate (α-Glc-1-P) and glycogen or starch chains that in the reverse reaction reacts as acceptors for D-glucose from α-Glc-1-P and is required for the synthetic reaction. It, hence, was called a 'primer'. The synthetic reaction, thus, was the reverse of the degradative reaction, which produced α-Glc-1-P and a partially degraded starch or glycogen chain that became the required putative primer when the reaction was run in the reverse direction. Phosphorylases were later shown to be exclusively degradative enzymes and not synthetic enzymes, as the in vivo concentration of inorganic phosphate was 20–40 times that of α-Glc-1-P. In 1977 Robyt and Corrigan[41] blocked the nonreducing-ends of dextran and showed that dextran was an activator and not a primer, as was assumed, for *S. mutans* OMZ 176 dextransucrase. The nonreducing-end blocked chains still gave activation, but no addition of D-glucose to the nonreducing-end of the blocked chains could occur, thus demonstrating that the dextran was not acting as a primer, but as an activator. In 1995 Robyt et al.[42] also showed that the initial velocities of dextran-free *L. mesenteroides* B-512FMC and *S. mutans* 6715 dextransucrases gave sigmoidal shaped curves when the initial velocities were plotted against the concentration of added dextran. The increase in the reaction rate and the decrease in the sigmoidal curve with increasing dextran concentrations indicated that dextran was binding at a noncatalytic or allosteric site to give a more active enzyme. A positive allosteric effect of a polymer often involves the coupling of two or more active enzyme units.[43] It was further observed that dextransucrase binds to lightly cross-linked dextran (Sephadex G-200), and the binding was inhibited when exogenous dextran was present.[44,45] When dextran binds to this noncatalytic, dextran-binding domain of dextransucrase, the dextran apparently induces a favorable conformation for the synthesis of dextran. There is evidence that the favorable conformation involves the formation of multiple subunits of enzyme. Very high molecular weight aggregates are formed when dextransucrase is elaborated in a sucrose medium,[33,45] where dextran is synthesized from sucrose by secreted dextransucrase from the bacterial cells. These aggregates often precipitate from the culture supernatant and do not migrate on polyacrylamide gels, indicating that the presence of HMW dextran acts as a cross-linking matrix that joins many enzyme units together.[45,46]

A dextran-binding domain was found in the C-terminal domains of *L. mesenteroides* strains and *Streptococcus* sp.

dextransucrases (glucansucrases).[47–49] The C-terminal domains of these enzymes were similar and have a series of four repeating sequences that effectively bind glucan (dextran).[50–55]

In addition to the cloned enzyme, Remaud-Simeon and co-workers[13] also produced a number of successive deletions of the repeating dextran-binding domain, the A-sequences at the C-terminal domain of the cloned enzyme. As the repeating sequences were removed, the enzyme lost activity when compared with the original cloned enzyme. With the removal of two repeating sequences, the enzyme lost $\sim$ 49% of its original activity, but there was no apparent change in the product(s) formed. With the deletion of three repeating sequences at the C-terminal domain, however, there was a dramatic loss of activity of 99.3%. The remaining activity produced primarily a LMW dextran that had a MW of 10,000 Da (DP 62). When four repeating sequences were removed, the enzyme had only 0.1% of the original activity, and produced exclusively LMW dextran with an avg MW of 13,000 (DP 80). From this, Remaud-Simeon and co-workers[13] concluded that the A-repeating sequences were playing a crucial role in the elongation of dextran.

Cheetham et al.[56] have reported a naturally occurring *S. sobrinus* dextransucrase, GTF-S3, that only formed a LMW linear dextran of DP 20–30 with the chains terminated by a sucrose unit at the reducing-end. This enzyme was a naturally occurring dextransucrase mutant that does not have any A-repeating, dextran-binding sequences, and therefore, could not bind dextran and give a branched dextran, but could bind sucrose, which released the LMW dextran from the active site in an acceptor reaction with sucrose, giving a sucrose-terminated dextran. It would thus appear that the A-repeating sequences are very likely dextran-binding sites that allow exogenous dextran chains to act as acceptors, giving α-(1→3) branch linkages for B-512F dextran, and a method for forming HMW dextran by forming 5–6% branched dextran chains, as shown by Robyt and Taniguchi.[8] Cheetham et al.[56] further indicated that the synthesis of the linear, LMW, sucrose-capped dextran could still be synthesized by the two-site insertion mechanism, originally proposed for several of the wild-type dextansucrases.[10–12,25]

4. Summary and conclusions

It has been shown experimentally that (a) the mechanism for the B-512F dextransucrase-catalyzed synthesis of dextran proposed by Remaud-Simeon and co-workers[13] in which sucrose and D-glucose act as initiator primers and that elongation proceeds by the addition of the D-glucose moiety of sucrose to the nonreducing-ends of isomaltodextrins in a one-site, nonprocessive mechanism is not tenable; (b) the presence of both a D-glucosyl- and a dextranyl-covalent intermediate occurs during the polymerization process; (c) pulse experiments with [^{14}C]-sucrose and chase experiments with nonlabeled sucrose definitively demonstrate and reaffirm that the D-glucose moiety of sucrose is added to the reducing-ends of the growing dextran chains during the synthesis of dextran; (d) decreases in the dextransucrase concentrations in the sucrose digests give an increase in the M_n, indicating that the polymerization reactions are highly processive; (e) the pulse and chase experiments and the formation of covalently linked D-glucose and dextran at the active site, during catalysis, indicate that the mechanism for dextransucrase synthesis of dextran is a two catalytic-site, insertion mechanism at the active site; and (f) it is shown how the three conserved amino acids, (Asp551, Glu589, and Asp662) at the active sites of glucansucrases mechanistically participate in the synthesis of dextran and in the secondary acceptor reactions by a two catalytic-site, insertion mechanism, occurring at one active site.

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