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Inhibition of dextran synthesis by acceptor reactions of dextransucrase, and the demonstration of a separate acceptor binding-site*

Aziz Tanriseven and John F. Robyt†
Department of Biochemistry and Biophysics, Iowa State University, Ames, IA 50011 (U.S.A.)

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ABSTRACT

Hanes–Woolf plots of dextransucrase–sucrose digests with methyl α-D-glucopyranoside and with methyl 6-deoxy-α-D-glucopyranoside showed that they were weak, apparent competitive inhibitors with K_i values of 97 and 267mM, respectively; a methyl 6-deoxy-6-fluoro-α-D-glucopyranoside plot showed that it was a very weak, noncompetitive inhibitor with a K_i of 400mM. A Michaelis plot with 6-deoxysucrose, a known competitive inhibitor with a K_i of 1.6mM, converged with a sucrose plot at 500mM sucrose. A Michaelis plot with methyl α-D-glucopyranoside, a relatively good acceptor but poor competitive inhibitor, did not converge with the sucrose plot at 600mM sucrose. Extrapolation of the sucrose and the methyl α-D-glucopyranoside plots showed that convergence would occur at ≈ 2M sucrose. Based on its concentration and its K_i value, the methyl α-D-glucopyranoside plot should have converged with the sucrose plot at 200mM sucrose if it were competing with sucrose for the sucrose binding-sites. From these observations, it is concluded that methyl α-D-glucopyranoside does not bind at the sucrose binding-sites when it acts as an acceptor, but it binds at a separate acceptor binding-site. This is further corroborated by the observation that methyl 6-deoxy- and 6-deoxy-6-fluoro-α-D-glucopyranoside analogues are bound very poorly, whereas the corresponding sucrose analogues, 6-deoxysucrose and 6-deoxy-6-fluorosucrose are bound approximately ten times more tightly than sucrose. The very high concentration of sucrose (≈ 2M) required for the convergence of the methyl α-D-glucopyranoside plot with the sucrose plot indicates that sucrose binds only very weakly to the acceptor binding-site.

INTRODUCTION

Leuconostoc mesenteroides B-512FM dextransucrase (EC 2.4.1.5) catalyzes the polymerization of the glucosyl moiety of sucrose to form dextran, which has 95% α-(1→6) linkages and 5% α-(1→3) branch linkages:

$$n \text{ sucrose} \xrightarrow{\text{Dextransucrase}} \text{Dextran} + n \text{ Fructose}$$

The reaction is essentially nonreversible. α-D-Glucopyranosyl fluoride[1] and *p*-nitrophenyl α-D-glucopyranoside[2] are also glucosyl donors for the synthesis of dextran by this enzyme.

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† To whom inquiries should be directed.

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The reaction mechanism of dextran formation by dextransucrase has been studied by several groups[3-8]. Robyt *et al.*[4] studied the mechanism by pulse and chase techniques with [^{14}C]sucrose and Bio-Gel P-2 immobilized dextransucrase. They found that glucosyl and dextranosyl covalent enzyme complexes were formed and that the glucosyl unit was added to the reducing end of the growing dextran chain.

When various carbohydrates are added to a sucrose–dextransucrase digest, some of the glucosyl groups are transferred to the added carbohydrates[8-11], which are called acceptors. Many different carbohydrates act as acceptors, including monosaccharides, oligosaccharides, and polysaccharides. In some of these reactions, the acceptor products themselves act as acceptors, and a homologous series of acceptor products are formed, whereas other acceptors give only a single acceptor-product[9,10]. Maltose, isomaltose, and methyl α-D-glucopyranoside are examples of acceptors that give a homologous series; D-fructose, lactose, and raffinose are examples of acceptors that give single products[9].

Using radioactively labeled acceptors such as [^{3}H]methyl α-D-glucopyranoside, D-[^{3}H]glucose, D-[^{14}C]glucose, and D-[^{3}H]fructose, Ebert and Schenk[3] showed that a single acceptor molecule was incorporated into a dextran molecule.

Robyt and Walseth[10] studied the mechanism of the acceptor reactions by charging the enzyme with sucrose followed by reactions with ^{14}C-labeled D-glucose, D-fructose, and maltose in the presence and absence of nonlabeled sucrose. Each acceptor gave a high-molecular-weight dextran and a low-molecular-weight saccharide, with the acceptor attached to the products at the reducing end. It was proposed that the acceptor reaction proceeds by displacing glucosyl and dextranosyl groups from the covalent enzyme-complex and that the acceptor reaction terminates dextran polymerization by displacing it from the active site.

Robyt and Walseth[10] also showed that, for the acceptors giving a homologous series of products, the first acceptor product was an acceptor to give the second acceptor product, which was an acceptor to give the third, *etc.*, with each succeeding acceptor product being produced in a smaller amount than was the preceding one.

Robyt and Taniguchi[11] showed that a similar reaction takes place when dextran chains are the acceptor. These investigators showed that exogenous dextran displaces dextran and glucose from the active site to give α-(1→3) branch linkages between the acceptor dextran and the displaced dextran chain and the displaced glucose residue.

To study the mechanism of the acceptor reaction further, we have carried out kinetic studies with methyl α-D-glucopyranoside, methyl 6-deoxy-α-D-glucopyranoside, and methyl 6-deoxy-6-fluoro-α-D-glucopyranoside as acceptors and 6-deoxysucrose as an inhibitor.

MATERIALS AND METHODS

Carbohydrates and reagents. — [U-^{14}C]Sucrose was obtained from Sigma Chemical Co. (St. Louis, MO). 6-Deoxysucrose (β-D-fructofuranosyl 6-deoxy-α-D-glucopyranoside) was synthesized as previously described[12]. Methyl 6-deoxy-α-D-glucopyrano-

side was prepared by the reduction of methyl 6-chloro-6-deoxy-α-D-glucopyranoside[13] with Bu_3SnH. Methyl 6-deoxy-6-fluoro-α-D-glucopyranoside was synthesized by first tritylating and then benzoylating methyl α-D-glucopyranoside, followed by the removal of the trityl group with acid. The free C-6 hydroxyl group was replaced by fluorine by reaction with diethylaminosulfur trifluoride (DAST), followed by the removal of the benzoyl groups with NaOMe in MeOH.

Enzyme. — *L. mesenteroides* B-512FM dextransucrase (EC 2.4.1.5) was prepared as previously described[14]. A radiochemical assay[15] using [U-^{14}C]sucrose was used to determine the activity of the enzyme. Assays were conducted at 25° and pH 5.3 with 25mM acetate buffer. The amount of labeled glucose incorporated in methanol-insoluble dextran was measured. Activity is given in International Units (IU), which is the amount of enzyme incorporating one μmol of D-glucose into dextran per min. The specific activity of the enzyme was 63 IU/mg of protein.

Enzyme digest conditions. — All digests were carried out at 25° in 25mM acetate buffer (pH 5.3) containing mM calcium chloride, 0.1 mg/mL sodium azide, and 0.1 mg/mL Tween 80 (refs. 16, 17).

Digest conditions to obtain Hanes–Woolf plots. — All digests had a total volume of 150 μL. Various sucrose concentrations (5, 10, 15, 20, and 25mM) were used. The concentrations of methyl α-D-glucopyranoside and methyl 6-deoxy-α-D-glucopyranoside were 50 and 100mM, and the concentration of methyl 6-deoxy-6-fluoro-α-D-glucopyranose was 100mM. The amounts of enzyme used with methyl α-D-glucopyranoside, methyl 6-deoxy-α-D-glucopyranoside, and methyl 6-deoxy-6-fluoro-α-D-glucopyranoside were 34, 17, and 34 mIU, respectively. The initial velocities were determined by taking aliquots at various times (8, 16, 24, and 32 min) and the amount of dextran synthesized was measured as a function of time. The slope of the resulting line was determined by a linear least-squares analysis.

Digest conditions to obtain Michaelis plots. — All digests had a total volume of 160 μL. Sucrose concentrations of 25, 50, 100, 200, 300, 400, 500, and 600mM were employed. The concentrations of methyl α-D-glucopyranoside and 6-deoxysucrose were 50 and 2mM, respectively. The amount of enzyme in both digests was 58 mIU. The initial velocities were measured by taking aliquots at various times (10, 20, 30, and 40 min) and determined as already described.

Determination of the amount of dextran synthesized. — Aliquots (25 μL) were taken from each digest and spotted onto Whatman 3MM filter paper squares (1.5 × 1.5 cm). These papers were immediately put into MeOH and stirred for 10 min. Washing in MeOH was repeated five times to remove any labeled D-fructose, D-glucose, unreacted sucrose, and low-molecular-weight, oligosaccharide acceptor-products. A control assay using only sucrose was also carried out. The filter papers were dried, and a liquid scintillation spectrometer was used to count the radioactivity on the papers[15]. For digests with sucrose concentrations 200mM and above, the 25 μL aliquots were added to 0.3M sodium hydroxide, which was neutralized after 60 min to give a 1:5 dilution. This solution (25 μL) was spotted onto paper for counting. The dilution diminishes the relatively high concentrations of unreacted sucrose, increasing the efficiency of the

sucrose removal from the paper. The dilution also keeps the amount of dextran precipitated on the paper in the proper range[15] for a quantitative determination.

RESULTS AND DISCUSSION

The action of dextransucrase can be measured in two general ways: (*1*) by measuring the amount of dextran synthesized, as has been done in this study, and (*2*) by measuring the amount of D-fructose formed. The latter is attained by either measuring the reducing value, as has been used by Koepsell *et al.*[18], Tsuchiya *et al.*[19], Ebert and Schenk[20], and Bovey[21] or by measuring the D-fructose enzymically[22,23]. The reducing-value method has its limitations in that it not only measures the release of D-fructose, it also measures any other reducing compounds that are produced, such as D-glucose and reducing acceptor-products. The enzymic method also has limitations as it measures both D-fructose and D-glucose, and gives low D-fructose values because of the action of D-fructose as an acceptor with dextransucrase to give leucrose. Within these limitations, the measurement of D-fructose gives the approximate determination of the overall reaction of sucrose, measuring both the amount of dextran synthesized and the amount of acceptor products formed.

Because the primary reaction of dextransucrase is the synthesis of dextran from sucrose, we have measured the rate of reaction of dextransucrase by measuring the rate of incorporation of D-glucose into dextran[15–17]. We consider that this is the best and most specific method for measuring the reaction of dextransucrase, and it is especially the best method for determining the effects of acceptors on the synthesis of dextran.

By using the determination of the rate of formation of D-fructose, several investigators[4,18,21] have found that some acceptors (such as methyl α-D-glucopyranoside, maltose, and isomaltose) produce an increase in the overall rate of reaction of sucrose with dextransucrase. In the present study, we have found by measuring the rate of dextran synthesis and using Hanes–Woolf plots (Fig. 1) that methyl α-D-glucopyranoside and methyl 6-deoxy-α-D-glucopyranoside were very weak competitive inhibitors, with K_i values of 97 and 267mM, respectively, and that methyl 6-deoxy-6-fluoro-α-D-glucopyranoside was a very weak noncompetitive inhibitor, with a K_i value of 400mM.

It was expected that because of its structural similarity to the glucopyranosyl moiety of sucrose, methyl α-D-glucopyranoside would bind at the sucrose binding sites. This also was suggested by the binding of α-D-glucopyranosyl fluoride at the sucrose site where it acts as a substrate (namely, a glucosyl donor) for dextransucrase[1].

If an inhibitor is indeed competing with the substrate for the substrate binding site, higher concentrations of the substrate should eliminate the inhibition. Michaelis plots of the initial velocities of the incorporation of D-glucose into dextran *versus* sucrose concentration in the presence and absence of two inhibitors, 6-deoxysucrose and methyl α-D-glucopyranoside are given in Fig. 2. This plot shows that the rate of dextran synthesis by dextransucrase follows the Michaelis–Menten equation up to 200mM sucrose. Above this concentration, the rate of dextran synthesis decreases. This was interpreted by Stringer and Tsuchiya[5] and Bovey[21] to be due to substrate (sucrose)

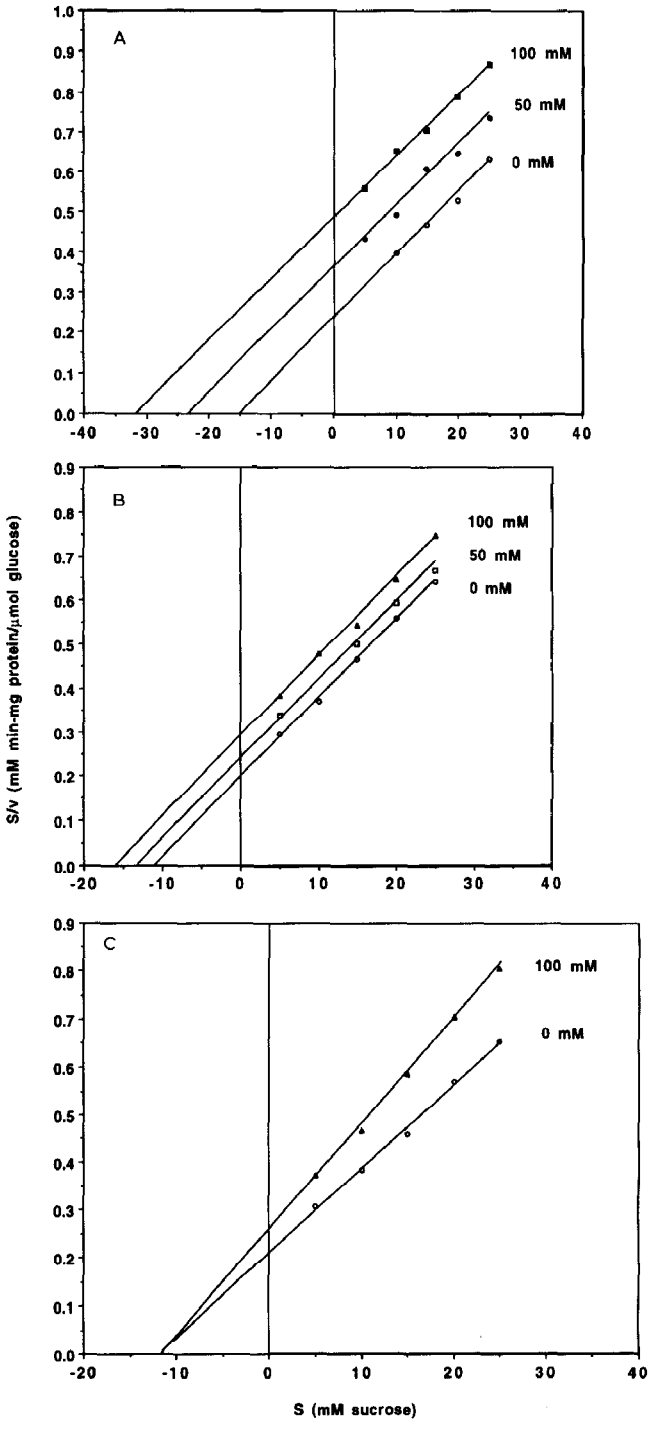

Fig. 1. Hanes–Woolf plots of dextransucrase digests with different concentrations: (A) ■, 100mM and ●, 50mM methyl α-D-glucopyranoside; (B) ▲, 100mM and □, 50mM methyl 6-deoxy-α-D-glucopyranoside; and (C) ▲, 100mM methyl 6-deoxy-6-fluoro-α-D-glucopyranoside. The v on the Y-axis is reported as μmol of D-glucose incorporated into dextran per min.

inhibition, and by Ebert and Schenk[3] to be due to sucrose acting as an acceptor. It is known that as the concentration of an acceptor is increased, the rate and amount of dextran synthesized is decreased[9]. Alsop[24] has shown that as the concentration of sucrose is increased, the concentration of free D-fructose and D-glucose is also increased due to their formation by the reaction of dextransucrase with sucrose. This increase in the concentration of D-fructose and D-glucose produces a concomitant decrease in the rate of dextran synthesized because of the diversion of the D-glucose moiety of sucrose away from its incorporation into dextran, and its incorporation into acceptor products by reaction with free D-fructose and D-glucose acceptors. Thus, it is not only sucrose itself that gives the sucrose-dependent inhibition but it is the formation of acceptor products as well. It has been suggested that sucrose acts as an acceptor and in doing so inhibits the synthesis of dextran[3]. Low-molecular-weight sucrose acceptor products, for example, tri-, tetra-, and penta-saccharides, however, have never been observed.

The Michaelis plot in the presence of 2mM 6-deoxysucrose, a known potent competitive inhibitor with a K_i value of 1.6mM (ref. 25), shows (Fig. 2) that the inhibition is completely eliminated at 500mM sucrose. The Michaelis plot, in the presence of 50mM methyl α-D-glucopyranoside, a relatively good acceptor but a poor competitive inhibitor, shows that the inhibition is not eliminated at 600mM sucrose (Fig. 2). Extrapolation of the methyl α-D-glucopyranoside and the sucrose lines show that they do converge at an extremely high sucrose concentration of ≈ 2M.

A comparison of the inhibition curves of Fig. 2 for the two inhibitors is valid even though the concentrations of the two inhibitors were different by a factor of 25; i.e., 50mM for methyl α-D-glucopyranoside and 2mM for 6-deoxysucrose. The K_i values are

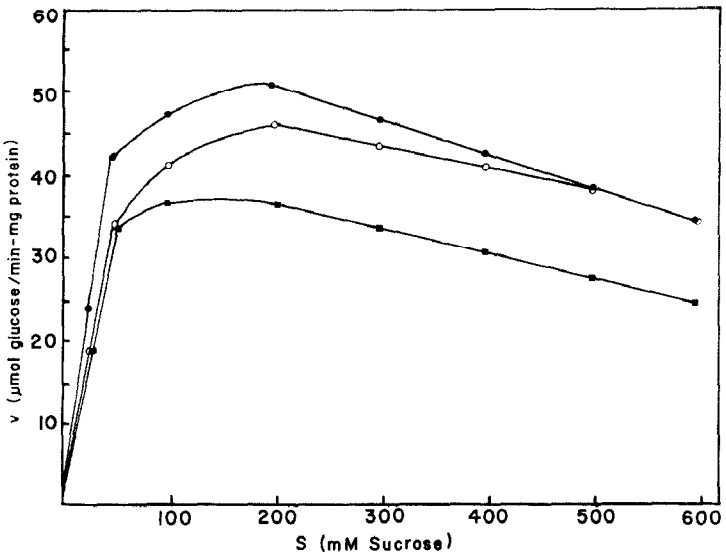

Fig. 2. Michaelis–Menten plots of dextransucrase reaction: ○, sucrose digest; ●, sucrose digest with 2mM 6-deoxysucrose; ■, sucrose digest with 50mM methyl α-D-glucopyranoside. The v on the Y-axis is reported as μmol of D-glucose incorporated into dextran per min.

vastly different — 97mM for methyl α-D-glucopyranoside and 1.6mM for 6-deoxysucrose. The ability of sucrose to relieve the inhibition depends upon the ratio of the inhibitor concentration to the K_i, or $[I]/K_i$. The lower the ratio, the easier it is for sucrose to compete with the inhibitor and to eliminate the inhibition. In the inhibition studies, the ratio for methyl α-D-glucopyranoside was (50:97 = 0.52) and the ratio for 6-deoxysucrose was (2:1.6 = 1.25). Thus, the ratio for methyl α-D-glucopyranoside was 2.5 times lower than the ratio for 6-deoxysucrose. If methyl α-D-glucopyranoside was competing with sucrose for the same binding site, the increase in the sucrose concentration to 200mM (500mM : 2.5) should have eliminated the inhibition as it did for 6-deoxysucrose.

Because an increase in the sucrose concentration effectively eliminated the inhibition by 6-deoxysucrose but had little effect on the inhibition by methyl α-D-glucopyranoside, it is concluded that the sucrose analogue, 6-deoxysucrose, is binding at the sucrose binding site, but the acceptor–inhibitor, methyl α-D-glucopyranoside, is not binding at the sucrose binding site and, therefore, is binding at a separate acceptor binding site. The very high concentrations (≈ 2M) of sucrose required to have the methyl α-D-glucopyranoside and sucrose lines merge further indicates that sucrose is weakly binding at the acceptor binding site as was previously postulated by Ebert and Schenk[3].

The presence of a separate acceptor binding site is corroborated by the very weak competitive inhibition of methyl 6-deoxy-α-D-glucopyranoside (K_i = 267mM) and the strong competitive inhibition of 6-deoxysucrose (K_i = 1.6mM), and the very weak noncompetitive inhibition of methyl 6-deoxy-6-fluoro-α-D-glucopyranoside (K_i = 400mM) and the strong competitive inhibition of 6-deoxy-6-fluoro-sucrose (K_i = 0.8mM). If methyl 6-deoxy-α-D-glucopyranoside and methyl 6-deoxy-6-fluoro-α-D-glucopyranoside were binding at the same site as sucrose, it would be expected that they would be as potent inhibitors as are the 6-deoxy- and 6-deoxy-6-fluoro-sucrose analogues. Because the methyl α-D-glucopyranoside analogues are not strong inhibitors, it can be concluded that they are not binding to the sucrose binding site.

We, therefore, propose that the active-site of *L. mesenteroides* B-512FM dextransucrase has separate sucrose binding sites and acceptor binding site(s). A similar conclusion was reached by Bovey[21] when he observed the activation effects of methyl α-D-glucopyranoside on the reaction of dextransucrase with sucrose, by using a reducing value method to measure the rates. He further concluded that the acceptor does not compete with sucrose but it does compete for some intermediate formed later in the process, a conclusion that we also make from this study. It is reasonable to expect that all of the acceptors should bind at the same acceptor binding site, even though they might bind with different affinities. Ebert and Schenk[3] found that some acceptors increased the rate of reaction of sucrose with B-512F dextransucrase and some decreased the rate. These effects were additive when a mixture of acceptors were used, indicating that all of the acceptors were binding at the same site. Mayer *et al.*[23] found that methyl α-D-glucopyranoside and maltose compete for the same binding site on *Streptococcus sanguis* dextransucrase and suggested that all acceptors are binding at this same site.

A proposed active-site model for dextransucrase is shown in Fig. 3A. In this model there are two sucrose binding sites that are required for the synthesis of dextran[4] and one acceptor binding site. The number of acceptor sites is not known, other than there is at least one, but there may be more than one. The incorporation of a separate acceptor binding site into the model is a refinement of the original acceptor-mechanism proposed by Robyt and Walseth[10]. The acceptor binding site is placed between the two X-glucosyl (or dextranosyl) groups (see Fig. 3B). The binding of the acceptor to the

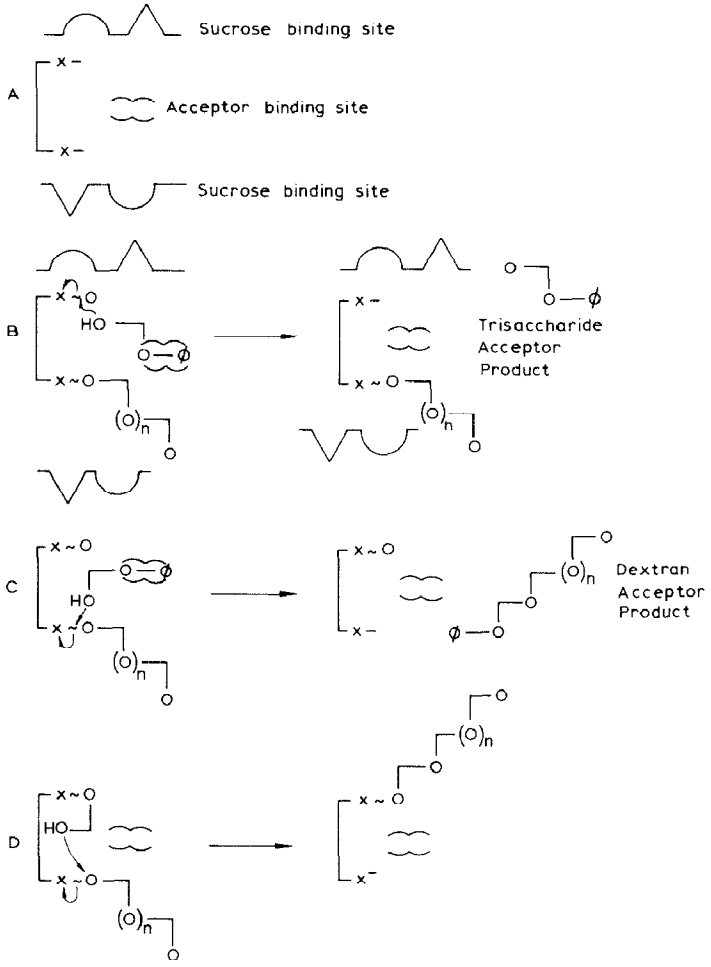

Fig. 3. Model of the active site of *Leuconostoc mesenteroides* B-512FM dextransucrase. Symbols: X^-, catalytic nucleophiles; ○, glucopyranosyl residues; ∅, reducing-end glucopyranose residues;, α-(1→6) glycosidic bond; —, α-(1→4) glycosidic bond. (A) Active site with two catalytic nucleophiles, two sucrose binding sites, and the acceptor binding site. (B) Active site showing the glucosyl and dextranosyl–enzyme complex, an acceptor (maltose) in the acceptor binding site, and its reaction with the glucosyl complex to give a trisaccharide acceptor product. (C) Active site showing the acceptor reaction with the dextranosyl complex to give reducing-end terminated, acceptor dextran product. (D) Active site, without acceptor, showing the reaction of the glucosyl residue with the dextranosyl unit to give the addition of glucose to the reducing end of the growing dextran chain. The sucrose binding sites have been omitted for clarity in C and D.

acceptor binding site, would thus, block the insertion of the glucosyl residue into the growing dextran chain, giving inhibition of the synthesis of dextran and the diversion of the glucosyl residue into acceptor products. Similarly, the acceptor can also react with the dextranosyl chain (Fig. 3C) to release it from the active site and terminate its polymerization[10]. It is further proposed that the acceptor binding site has a minimum of two glucosyl subsites as maltose, which has two glucosyl moieties, is the most effective acceptor[9].

Thus, although such acceptors as methyl α-D-glucopyranoside and maltose are binding at an acceptor binding site rather than at the sucrose binding sites, their kinetic effects on the initial velocity of dextran synthesis, at relatively low sucrose concentrations, appears to be competitive with sucrose in a Hanes–Woolf plot or in a Lineweaver–Burk plot because they are competing with dextran for the glucosyl intermediate that arises from sucrose, instead of competing with sucrose for the same binding site.

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