Substrate Binding Sites of *Leuconostoc* Dextranucrase Evaluated by Inhibition Kinetics

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Graphical analysis of inhibition kinetics for dextranucrase from *Leuconostoc mesenteroides* was done with typical inhibitors, competitive and noncompetitive. Based on the plots of Yonetani-Theorell and Semenza-Balthazar, mutual competition between the pairs of inhibitors of identical kinetic type was observed, while combination of competitive and noncompetitive inhibitors gave no significant mutual interactions. By the procedure of Nitta *et al.*, binding sites for competitive and noncompetitive inhibitors were shown to be distant from each other. Moreover, two noncompetitive inhibitors competed with each other for a single binding site on the enzyme. Although biphasic reciprocal plots may suggest rather complicated binding of various inhibitors, the results obtained by the three graphical methods are fully explained when competitive and noncompetitive inhibitors for substrate sucrose bind to the so-called donor- and acceptor-sites of dextranucrase, respectively.

Dextranucrase (EC 2.4.1.5) from *Leuconostoc mesenteroides* catalyzes a glucosyl transfer reaction from sucrose to acceptor dextran and produces a high molecular weight dextran as shown in equation (1).¹⁾

In our previous paper,²⁾ we reported the purification of dextranucrase from *L. mesenteroides* strain NRRL B-1416, which contained only a small amount of endogenous dextran. Although the purified enzyme could synthesize dextran without primer, the reaction was accompanied by an obvious lag-period, which was abolished by the addition of exogenous dextran.²⁾ Moreover, the dextranucrase reaction was strongly stimulated by the primer, and the production of reducing sugar from sucrose (sucrase activity) increased 4.4-fold and the synthesis of polymer dextran (transferase activity) increased 8.0-fold.³⁾ The glucosyltransferases from cariogenic *Streptococcus* species show a strict requirement of the

primer dextran,^{4,5)} while our result is the first case for *Leuconostoc* dextranucrase. The amylosucrase from *Neisseria perflava*, which synthesizes a glycogen-like polysaccharide from sucrose, has a similar primer dependency.⁶⁾ Acceptor molecules are therefore essential for polymer synthesis from sucrose by various types of glucosyltransferases.

The reaction of dextranucrase shown in Eq. (1) requires two substrates, sucrose as a glucosyl donor and dextran as a glucosyl acceptor. The binding sites for donor and acceptor molecules are tentatively supposed to be separate sites on the enzyme molecule,⁷⁾ and the results obtained with primer-free dextranucrase of *L. mesenteroides*³⁾ strongly support this. Since inhibition kinetics provide useful information on substrate binding sites, we made some analyses of *Leuconostoc* dextranucrase.

There are many enzymes that catalyze two-substrates reaction.⁸⁾ The locations of substrate binding sites provide useful informations on the catalytic site. Several experimental approaches to distinguish the substrate bind-

ing sites have been tried,⁹⁻¹²⁾ but no such information on the polysaccharide-producing glucosyltransferases is available.

MATERIALS AND METHODS

Materials. A dimeric form of dextranucrase from *Leuconostoc mesenteroides* NRRL B-1416 strain was purified as described previously.²⁾ Preparation of native dextran from *L. mesenteroides* NRRL B-512F has been described.¹³⁾

Enzyme assay. The reaction mixture (125 μ l) contained a suitably diluted enzyme (25 μ l), 1.2~97.4 mM sucrose, 0.05% B-512F native dextran (25 μ l), and inhibitor(s) in 40 mM acetate buffer (pH 5.2). After preincubation of enzyme with inhibitor at 30°C for 10 min, dextran and sucrose were added in this order and incubated 20 min more. Enzyme activity was measured for the reducing sugar released from sucrose by the fluorometric reagent as described previously.¹⁴⁾

Inhibitors. 6'-Amino-6'-deoxy-sucrose was provided by Dr. T. Suami of Keio University.¹⁵⁾ Nojirimycin¹⁶⁾ was provided by Meiji Seika Kaisha, Co., Ltd. D-Glucosylamine¹⁷⁾ and methyl 6-amino-6-deoxy-glucoside¹⁸⁾ were synthesized by the method described. All the other inhibitors were commercially available.

RESULTS AND DISCUSSION

Working hypothesis for inhibition kinetics

Based on our recent studies on *Leuconostoc* dextranucrases,^{2,3,14,19)} we propose a working hypothesis shown in Scheme I, in which the donor (sucrose) and acceptor substrate (dex-

tran) bind to distinct sites on the enzyme. There are several findings for the *Leuconostoc* dextranucrase which support our model. First, we have demonstrated that the inhibition types of various inhibitors were different when assayed for each of the two substrates, for example, D-fructose, maltose, and methyl α -D-glucoside competitively inhibited for sucrose, and mixed and noncompetitive inhibition for dextran.³⁾ This can be explained if sucrose and dextran molecules bind to the donor- and acceptor-sites, respectively. Second, the action of 16 effectors was grouped into six categories by their activation or inhibition of the sucrase and transferase activities of dextranucrase.³⁾ The sucrase activity represents the reaction between the donor sucrose and a catalytic group, and the transferase activity represents the reaction between the acceptor dextran and a catalytic group which retains an intermediate form of the glucosyl residue.²⁰⁾ These two reactions might occur at the intrinsic binding sites for the two substrates. Third, biphasic double reciprocal plots were obtained when the substrate concentrations were varied in the presence or absence of inhibitors.^{3,14,19,21)} This shows that K_m values might change as the sucrose-dextran ratio, and the change in affinity for substrates indicates competition between sucrose and dextran for the acceptor-site as discussed by Chludzinski *et al.*⁴⁾ Therefore, it is possible that an excess of substrate may occupy the two sites, causing a significant change in the reaction velocity. Double reciprocal plots obtained with various sucrose-dextran ratios indicated that the change in Hill coefficients for sucrose and dextran ranged between 0.76~1.1 and 0.44~1.06, respectively.¹⁹⁾ Thus, the activity of *Leuconostoc* dextranucrase would depend largely on the concentration of acceptor dextran. Although all of this strongly supports the idea of independent locations for acceptor- and donor-sites of dextranucrase (Scheme I), further insights for this model may be provided by inhibition kinetic analysis. In this paper, we report a screening of inhibitors

SCHEME I. Proposed Model for Donor- and Acceptor-sites of *Leuconostoc* Dextranucrase.

useful for this approach and some results on the location of acceptor- and donor-sites obtained by the graphical analysis with several inhibitors.

Inhibitors for dextranucrase

Dextranucrase from *L. mesenteroides* NRRL B-1416 was inhibited by various compounds when the residual activity was measured by the sucrase activity (Table I). Among 14 inhibitors tested, EDTA was a potent inhibitor and only 14% of residual activity was detected. Although EDTA is known to inhibit dextranucleases from various strains of *Leuconostoc*,^{3,22,23} it causes almost no inhibition of the Streptococcal enzymes.^{24,25} 6'-Amino-6'-deoxy-sucrose and Nojirimycin caused about 70% inhibition of the enzyme, and the former compound also inhibited the enzyme from *S. mutants*.²⁶ Nojirimycin was a strong inhibitor of α -glucosidase.²⁷ Several amino derivatives with D-glucose residues at C-1 or C-6, such as glucosylamine, *N*-methylglucamine, methyl 6-

amino-6-deoxy-glucoside, and glucuronamide, were effective inhibitors. Moreover, D-glucose analogs of lactone or uronated forms, such as glucono- δ -lactone, D-gluconic acid, and D-

TABLE II. INHIBITION TYPES AND K_i VALUES FOR DEXTRANSUCRASE BY VARIOUS COMPOUNDS

Compound	Higher sucrose ^a		Lower sucrose ^a	
	Type ^b	K_i (mM)	Type ^b	K_i (mM)
6'-Amino-6'-deoxy-sucrose	C	6.6	NC	18.0
EDTA	NC	1.1	NC	0.2
D-Gluconic acid-Ca ²⁺	NC	7.5	NC	6.3
Glucono- δ -lactone	C	9.4	—	—
Glucuronic acid (reduced)	NC	13.9	C	9.5
Me ^c -6-amino-6-deoxy-glucoside	C	12.0	NC	27.3
Me ^c - α -D-glucoside	C	82.4	—	—
N-Me ^c -D-glucamine	C	30.0	NC	68.3
Nojirimycin	NC	1.5	U (M)	—

^a Higher sucrose, 12.2~97.4 mM; lower sucrose, 1.2~4.9 mM.

^b Type of inhibition: C, competitive; NC, noncompetitive; M, mixed type; U, uncompetitive.

^c Methyl-. For the reaction mixture, see MATERIALS AND METHODS.

TABLE I. INHIBITION OF DEXTRANSUCRASE BY VARIOUS COMPOUNDS

Inhibitor	mM	Res. act. (%) ^a
None	—	100
6'-Amino-6'-deoxy-sucrose	10	31
6-Deoxy-glucose	12.5	95
EDTA	1	14
D-Fructose	12.5	82
D-Gluconic acid-Ca ²⁺	12.5	81
Glucono- δ -lactone	12.5	76
Glucuronamide	12.5	85
Glucosylamine	12.5	80
Glucuronic acid (reduced)	12.5	63
Maltose	12.5	78
Me ^b -6-amino-6-deoxy-glucoside	12.5	76
Me ^b - α -D-glucoside	50	84
N-Me ^b -D-glucamine	12.5	67
Nojirimycin	5	30

^a Residual activity. The reaction mixture contained 25 μ l of the diluted enzyme (0.02 units), 25 μ l of inhibitor, 25 μ l of 292 mM sucrose solution, and 0.125% of B-512F native dextran in 40 mM acetate buffer (pH 5.2).

^b Me, methyl.

FIG. 1. Yonetani-Theorell Plots of Dextranucrase Inhibition.

The reaction mixture contained 25 μ l of the diluted enzyme (0.02 units), 25 μ l each of two inhibitors, 25 μ l of sucrose solution (40 mM), 0.25% B-512F native dextran in 40 mM acetate buffer (pH 5.2).

(a) Action of two competitive inhibitors, methyl 6-amino-6-deoxy-glucoside and glucono- δ -lactone. Concentrations of glucono- δ -lactone were 0 mM (∇), 13 mM ($\blacktriangledown$), 27 mM ($\circ$), or 40 mM ($\bullet$).

(b) Action of two noncompetitive inhibitors, EDTA and glucuronic acid (reduced). Concentrations of glucuronic acid (reduced) were 0 mM (∇), 6.7 mM ($\blacktriangledown$), 13.3 mM ($\circ$), or 20 mM ($\bullet$).

glucuronic acid, were also inhibitory.

Of the inhibitors shown in Table I, nine potent inhibitors were analyzed kinetically. The type of inhibition and K_i values were measured by s/v vs. s plots with varying concentrations of substrate sucrose (Table II). Similar to our previous data,¹⁴⁾ the reciprocal plots were biphasic, upwardly convex at about 10 mM sucrose. At higher concentrations of sucrose (12.2~97.4 mM), four inhibitors including 6'-amino-6'-deoxy-sucrose were competitive for sucrose, and three inhibitors, including EDTA, were noncompetitive. For the graphical analysis of inhibition kinetics, couples of competitive and noncompetitive inhibitors having rather small K_i values were selected. Although noncompetitive inhibition was predominant at lower concentrations of sucrose (1.2~4.9 mM), detailed examination is required for the lower half of the limb.

Yonetani and Theorell plots for dextranucrase

A graphical method for the analysis of multiple inhibition of liver alcohol dehydrogenase by two competitive inhibitors was established by Yonetani and Theorell.²⁸⁾ This method was applied to the *Leuconostoc* dextranucrase with two competitive inhibitors, methyl 6-amino-6-deoxy-glucoside and glucono- δ -lactone (Fig. 1a). Although the plots were again biphasic, parallel lines were given by the different concentrations of inhibitors, indicating the mutual competition of the two competitive inhibitors at the sucrose binding site. When the two inhibitors interact with different sites of the enzyme, lines having different slopes should converge in the second quadrant. A combination of EDTA and reduced glucuronic acid was tested for the noncompetitive inhibitors of dextranucrase. Parallel lines for 1~12 mM EDTA suggested the mutual competition of two inhibitors at the dextran binding (donor) site (Fig. 1b). However, the converging lines for less than 1 mM EDTA may indicate no interaction at the same site. Although this corresponds to the noncompetitive (EDTA) and competitive (reduced glucuronic acid) inhibition for the lower

sucrose concentration (Table II), more study is needed.

Semenza and Balthazar plots for dextranucrase

A simple kinetic analysis for detecting or ruling out mutual competition between inhibitors of different or identical kinetic types was described by Semenza and Balthazar.²⁹⁾ They extended the rate equations described by Webb³⁰⁾ and Yonetani and Theorell²⁸⁾ to all combinations of competitive and noncompetitive inhibitors. Therefore, attempts were made to obtain information on the substrate binding sites of dextranucrase by the method of the

FIG. 2. Semenza-Balthazar Plots of Dextranucrase Inhibition.

For the reaction mixture, see the legend to Fig. 1. Action of two noncompetitive inhibitors (a), and two competitive inhibitors (c). Action of noncompetitive and competitive inhibitors (b), and vice versa (d). None ($\square$); 2 mM Nojirimycin ($\blacksquare$); 20 mM glucuronic acid (reduced) ($\blacktriangledown$); 8 mM 6'-amino-6'-deoxy-sucrose ($\circ$); 10 mM glucono- δ -lactone (∇); 0.16 mM EDTA ($\bullet$). The x-axis for (a) and (b) was EDTA, and for (c) and (d) was methyl 6-amino-6-deoxy-glucoside.

Semenza group.

In this study, 6'-amino-6'-deoxy-sucrose, glucono- δ -lactone, and methyl 6-amino-6-deoxy-glucose were selected as competitive inhibitors, and EDTA, Nojirimycin, and reduced glucuronic acid as noncompetitive inhibitors. Pairs of noncompetitive (Fig. 2a) and competitive inhibitors (Fig. 2c) gave parallel lines at the upper half of the limb, indicating mutual competition between the inhibitors. In contrast, a combination of competitive and noncompetitive inhibitors gave lines having different slopes (Fig. 2b, d). Except for the case of glucono- δ -lactone, the extrapolated lines for the upper half of the limb converged with those of the controls (none) at the x -axis. Since the graphical analysis is only applicable to inhibitors having straight lines in Dixon plots, these results may be more carefully explained based on the change of cooperativity shown in our previous paper.¹⁹⁾ Together with the results shown in Fig. 1, Fig. 2a, c supported the hypothesis of the existence of a mutual competition between inhibitors of identical kinetic type. Moreover, no mutual competition was observed between inhibitors of different kinetic types (Fig. 2b, d).

Nitta's plots for dextranucrase

Nitta *et al.* reported inhibition kinetic analysis on the hydrolysis reaction of Takamylase A.³¹⁾ They derived kinetic equations to estimate the location of binding sites for competitive and noncompetitive inhibitors,³¹⁾ i.e., the relationship between the inhibitor binding sites and number of binding sites for noncompetitive inhibitors were closely examined. We applied the kinetic approaches of Nitta *et al.* to dextranucrase.

Binding sites for competitive and noncompetitive inhibitors are termed site C and site N, respectively. In the presence of competitive (6'-amino-6'-deoxy-sucrose) and noncompetitive (EDTA) inhibitors, a plot of s/v vs. s plot gave biphasic lines (Fig. 3a, closed circle). At high sucrose concentrations, the theoretical line (open circle) agreed very well with the experimental data, which indicate that site C and N

FIG. 3. Nitta, Hiromi, and Ono Plots of Dextranucrase Inhibition.

For the reaction mixture, see the legend to Fig. 1. (a) s/v vs. s Plot for the inhibition by a mixture of competitive (5 mM 6'-amino-6'-deoxy-sucrose, ∇) and noncompetitive (1 mM EDTA, $\square$) inhibitors. Experimental ($\bullet$) and theoretical ($\circ$) values. Without inhibitor ($\blacktriangledown$). (b) s/v vs. s Plot for the inhibition by a mixture of two noncompetitive inhibitors (1 mM EDTA and 5 mM gluconic acid- Ca^{2+}). Experimental ($\bullet$) and theoretical ($\circ$) values. Without inhibitor ($\blacktriangledown$).

are distant from each other and there is no detectable interaction between the two sites. Then the number of sites N was kinetically evaluated. In this case, two noncompetitive inhibitors (EDTA and gluconic acid- Ca^{2+}) were both included in the reaction mixture. As shown in Fig. 3b, experimental results (closed circle) agreed well with the theoretical plot, which was derived from the equation representing two inhibitors competing with each other for a single site N .³¹⁾ The results obtained by the procedure of Nitta *et al.* do not conflict with those of Yonetani-Theorell and Semenza-Balthazar, and all of these results indicate an independent location of binding sites for competitive and noncompetitive inhibitors of dextranucrase.

Concluding remarks

Graphical analysis of inhibition kinetics for dextranucrase was done by the procedures of the Yonetani group, the Semenza group, and the Nitta group. Competitive and noncompetitive inhibitors for sucrose had no mutual competition with each other, while there was obvious mutual competition between the inhibitors of the same kinetic type. These re-

sults might substantiate our working hypothesis (Scheme I), where we postulated a separate location of acceptor- and donor-sites. When competitive and noncompetitive inhibitors for sucrose bind to the donor- and acceptor-sites of dextranucrase, respectively, our kinetic results are fully explained. Although these results were derived from the enzyme having no cooperativity (Hill coefficients were close to 1), the biphasic reciprocal plots suggest more complicated binding of various inhibitors.

Further insights on the reaction mechanism of dextranucrase may be provided by the amino acid sequence of the catalytic site. Chemical modification and affinity labeling of the catalytic residue of enzyme are useful for this. The proposed location of acceptor- and donor-sites of dextranucrase is essential to construct a strategy of affinity labeling where the labeling reagent should be specific to either acceptor- or donor-sites of the enzyme.

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