An Active-Site Peptide Containing the Second Essential Carboxyl Group of Dextransucrase from *Leuconostoc mesenteroides* by Chemical Modifications

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Received May 3, 1993; Revised Manuscript Received August 23, 1993®

ABSTRACT: The treatment of *Leuconostoc mesenteroides* B-512F dextransucrase with 10 mM 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) and glycine ethyl ester (GEE) inactivated the enzyme almost completely within 24 min where the modification of one carboxyl group/mol of the enzyme by EDC was attained. Though 30 mM diethyl pyrocarbonate (DEP) also inactivated the enzyme, about 35% of the activity remained during a 36-min incubation. When 10 mol of imidazole residues/mol of the enzyme was modified by DEP, 50% of the activity was still retained. The addition of the substrate sucrose greatly retarded the enzyme inactivation by EDC. However, the addition of dextran slightly protected the inactivation of the glucosyl-transferring activity and accelerated the inactivation of the sucrose-cleaving activity. In the case of DEP, the addition of sucrose or dextran gave no influence on the inactivation of the enzyme. Therefore, the carboxyl group seemed to play a more important role in the substrate binding and in the catalytic activity of the dextransucrase than the imidazolium group. Differential labeling of *Leuconostoc* dextransucrase by EDC was conducted in the presence of a sucrose analog, sucrose monocaprate. The fluorescent probe *N*-(1-naphthyl)ethylenediamine (EDAN) was used as the nucleophile instead of GEE. A fluorescent labeled peptide was isolated from a trypsin digest of the EDC–EDAN modified enzyme. The amino acid sequence of the isolated peptide was Leu-Gln-Glu-Asp-Asn-Ser-Asn-Val-Val-Val-Glu-Ala. The sequence had about 58% homology to those of streptococcal glucosyltransferases GTF-S and GTF-I producing soluble and insoluble dextrans. Those peptides were distinguished from the active-site peptides of GTF-S and GTF-I from *S. mutans*, which contained catalytic aspartic acid residues of Asp465 and Asp451, respectively. They were located at 30–45 amino acids toward the amino terminal from the catalytic aspartic acid. Therefore, the isolated peptide seemed to contain the second essential carboxyl group for the catalytic activity.

Dextransucrase (EC 2.4.1.5) is produced by several strains of *Leuconostoc* and *Streptococcus*, and it catalyzes the glucosyl transfer reaction from sucrose to α-D-glucan (Hehre, 1951). Streptococcal glucosyltransferases are classified into two major groups: sucrose:1,3-α-D-glucan 3-α-D-glucosyltransferase (GTF-I)[1] and sucrose:1,6-α-D-glucan 6-α-glucosyltransferase (GTF-S) (Hamada & Slade, 1980). GTF-I synthesizes a water-insoluble α-1,3-D-glucan, and GTF-S synthesizes a water-soluble α-1,6-D-glucan. In contrast, *L. mesenteroides* B-512F produces one type of dextransucrase that synthesizes the water-soluble α-1,6-D-glucan (Sidebotham, 1974; Walker, 1978). Initial velocity measurements of *Leuconostoc* dextransucrase suggested that the ping–pong Bi–Bi mechanism is applicable to the enzyme (Kobayashi & Matsuda, 1978). Fu and Robyt proposed that two histidine residues were essential for the activity of dextransucrase from *L. mesenteroides* B-512F (Fu & Robyt, 1988). Moreover, Mooser et al. determined the sequence of glucosyl peptide isolated from the stabilized glucosyl–enzyme complexes of GTF-I and GTF-S from *Streptococcus sobrinus* (Mooser et al., 1991). They suggested that the glucosyl moiety of sucrose was linked to the catalytic residue of aspartic acid. In this paper, chemical modifications of *L. mesenteroides* B-512F dextransucrase with EDC-GEE and with DEP were conducted. To clarify the contribution of the carboxyl groups and the imidazole groups to the catalytic activity of dextransucrase, the effects of ligand protection with the two substrates, sucrose and dextran, were examined in detail. The findings suggested that the modification of carboxyl groups gave a much larger loss of activity than that of the imidazole groups. Moreover, the active carboxyl group of the *Leuconostoc* dextransucrase was labeled with the fluorescent reagent under the protection with sucrose monocaprate as the sucrose analog, and the sequence of the isolated peptide containing the substrate binding domain was identified.

EXPERIMENTAL PROCEDURES

Materials. Leuconostoc mesenteroides NRRL B-512F was cultured with 2% sucrose according to the method described (Jeanes, 1965). The B-512F dextransucrase was purified as described previously (Kobayashi & Matsuda, 1980). Endodextranase from *Chaetomium gracile* was purchased from Seikagaku Kogyo Co. Immobilized dextranase was prepared as described previously (Kobayashi & Ichishima, 1991).

Bio-Gel P-2 was purchased from Bio-Rad; EDC was from Dojindo Laboratories; sucrose monocaprate was from Mitsubishi-Kasei Food Corp.; trypsin was the sequencing grade

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® Abstract published in *Advance ACS Abstracts*, November 15, 1993.

[1] Abbreviations: DEP, diethyl pyrocarbonate; EDAN, *N*-(1-naphthyl)-ethylenediamine ((*N*-1-ethylenediamino)naphthalene); EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; GEE, glycine ethyl ester; GTF-I, sucrose:1,3-α-D-glucan 3-α-D-glucosyltransferase; GTF-S, sucrose: 1,6-α-D-glucan 6-α- D-glucosyltransferase; TEMED, *N,N,N',N'*-tetramethylethylenediamine.

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(cat. no. 1047841) from Boehringer Manheim Biochemica; GEE, DEP, EDAN, and pullulan were from Wako Pure Chemical Industries Ltd.

Enzyme Assay. The sucrase activity was determined by measuring the release of reducing sugar from sucrose as described previously (Kobayashi & Matsuda, 1980). The transferase activity was determined by measuring the ethanol-insoluble polysaccharide in the reaction mixture. To 50 μL of the reaction mixture were added 50 μL of distilled water, 100 μL of pullulan, and 300 μL of ethanol, and the precipitates were collected by centrifugation. The precipitates were washed twice with 755 μL of ethanol and dissolved with 400 μL of 10 mM phosphate buffer (pH 6.0). Then, 100 μL of 0.01 mg/mL of endodextranase solution was added and incubated at 30 °C for 2 h. The reducing sugar was measured by the Nelson–Somogyi method (Somogyi, 1945) and represented as the amount of synthesized dextran.

Chemical Modification. EDC modification of dextransucrase was performed by incubating the enzyme (0.1–2.0 mg of protein/mL) with 20 mM of EDC and 100 mM of GEE in 20 mM acetate buffer (pH 5.2) at 30 °C. Then, 165 μL of ice-cold 200 mM acetate buffer (pH 5.2) was added to 50 μL of the reaction mixture to stop the reaction. DEP modification was performed by the addition of 30 mM of DEP instead of EDC and GEE and incubated as described above.

Affinity Gel Electrophoresis. Dextran affinity gel electrophoresis with 7.0% acrylamide containing an appropriate amount of clinical dextran was carried out with 50 mM Tris–glycine buffer (pH 8.3) at 4 °C according to Maurer (Maurer, 1968). Protein bands were stained with 0.25% Coomassie brilliant blue.

Protein. Protein was determined by the absorbance at 280 nm. The absorbance coefficient of dextransucrase was 4.52%$^{-1}$ cm^{-1}.

Molecular Weight. The molecular weight of dextransucrase was 170 kDa determined by SDS polyacrylamide gel electrophoresis.

Differential Labeling of Dextransucrase. About 0.4 mg of freeze-dried purified enzyme was incubated with 5 mM EDC, 100 mM GEE, and 50 mM sucrose monocaprate in 1 mL of 20 mM acetate buffer (pH 5.2) at 30 °C for 40 min. Then the mixture was applied to a Bio-Gel P-2 column (1.1 × 30 cm) previously equilibrated with distilled water. After the elution, V_o fractions were pooled and incubated with 6 mg of immobilized dextranase at 30 °C overnight to remove the dextran synthesized from sucrose contaminated in sucrose monocaprate. Then the immobilized endodextranase was removed by centrifugation, and the supernatant was concentrated with the centrifugal concentrator (CC-101 TU-040, TOMY). The dried sample was incubated with 4.8 mM of EDAN and 20 mM EDC in 1 mL of 20 mM *N,N,N',N'*-tetramethylethylenediamine (TEMED)–HCl buffer (pH 4.75) at 30 °C for 4 h. Bio-Gel P-2 gel filtration was performed, and the V_o fractions were pooled. Immobilized dextranase treatment was performed again because even if a small amount of dextran still remained in the enzyme, it would disturb the following trypsin digestion. The fractions were concentrated by the centrifugal concentrator. The dried sample was (*S*)-pyridylethylated by the method of Hermodson et al. (Hermodson, 1973) and digested with 10 μg of trypsin in 500 μL of 50 mM Tris–HCl buffer (pH 8.5) containing 1.5 M urea at 30 °C for 8 h. Peptides were separated by HPLC (JASCO PU-980) with a C_{18} reversed-phase column (Wakosil 5C18, Wako). Absorbance at 220 nm and fluorescence intensity (Ex = 330 nm, Em = 430 nm) were monitored by the UV spectrometer (JASCO UV-970) and fluorospectrometer (JASCO 821-FP), respectively. The EDAN-incorporated peptides were pooled, and the amino acid sequences were determined by the peptide sequencer (TOSOH PI-2020).

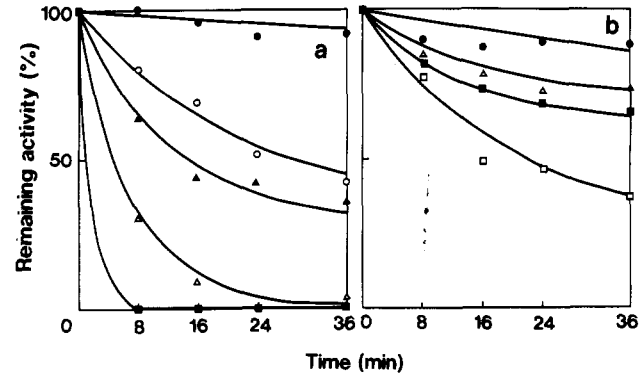

FIGURE 1: Chemical modification of dextransucrase. The enzyme was modified with various concentrations of EDC (a) or with DEP (b) as described in the Experimental Procedures. Dextransucrase (0.33 mg of protein/mL)(131 IU/mg) was subjected to the modification. Concentrations of EDC or DEP (mM) were 0 (●), 2 (○), 5 (▲), 10 (△), 20 (■), and 30 (□). After modification, the reaction mixtures were diluted 12-fold with 200 mM acetate buffer (pH 5.2), and the remaining activity was measured with 10% sucrose in 20 mM acetate buffer (pH 5.2) at 30 °C for 15 min. Sucrase activity was assayed by the Nelson–Somogyi method.

RESULTS

Chemical Modification of Dextransucrase by EDC and DEP. The treatment of the *Leuconostoc* dextransucrase with EDC or DEP led to a loss of enzyme activity. As shown in Figure 1, 10 mM of EDC completely inactivated the enzyme within 24 min of incubation, while 30 mM of DEP inactivated only 65% of the enzyme after 36 min of incubation. EDC modification is performed usually at pH 4.75, and DEP modification is performed at pH 6.0. However, the *Leuconostoc* dextransucrase was unstable at these pHs; *i.e.*, during the incubation at 30 °C for 30 min in the absence of EDC or DEP, about 50% of the enzyme activity was lost in the acetate buffer (pH 4.75), about 15% in the acetate buffer (pH 5.5), and about 35% in the phosphate buffer (pH 6.0). When dextransucrase was incubated in the acetate buffer (pH 5.2), more than 90% of the activity remained under the same conditions. Therefore, the chemical modification was conducted at pH 5.2. In the case of EDC modification, TEMED buffer is preferable rather than acetate buffer to avoid the reaction of carbodiimide with acetate carboxyl groups to form acetylisourea. However, in the present experiment, TEMED buffer (pH 5.2) caused ~45% loss of activity. When EDC modification was done with acetate buffer (pH 5.2), no pH change was observed during the reaction. After the reaction was quenched with an excess amount of 200 mM acetate buffer (pH 5.2), no changes in pH and enzyme activity were detected during the storage at 0 °C for 1.5 h.

Because this enzyme has two substrates of donor and acceptor molecules, *i.e.*, sucrose and dextran, these substrates were added to EDC or DEP, and the effects of these substrates on the chemical modification were examined. *Leuconostoc* dextransucrase cleaves sucrose and releases fructose (sucrase activity), and the glucosyl moiety is transferred to the acceptor molecule and forms a new α-1,6-bond (transferase activity). As shown in Figure 2a, EDC inactivated the transferase activity about twice as much as the sucrase activity, but when 150

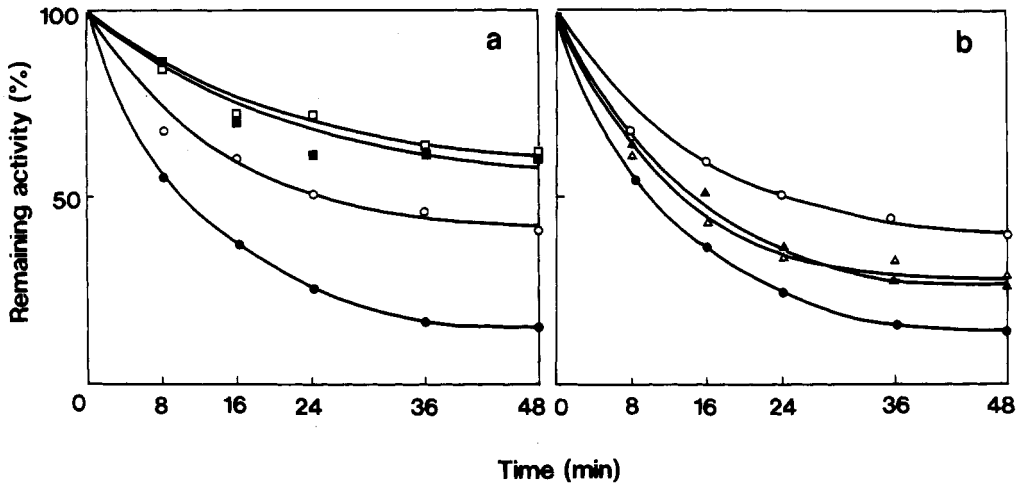

FIGURE 2: Effects of sucrose and dextran on the EDC modification. Dextransucrase (0.33 mg of protein/mL) was modified with 5 mM EDC and 100 mM GEE in the absence of substrates (○, ●) and in the presence of 150 mM sucrose (□, ■) or 1.5% dextran (△, ▲). The enzyme was modified as described in the legend for Figure 1. Sucrase activity (open symbols) and transferase activity (solid symbols) were assayed after a 15- and 30-min incubation, respectively, as described in the Experimental Procedures.

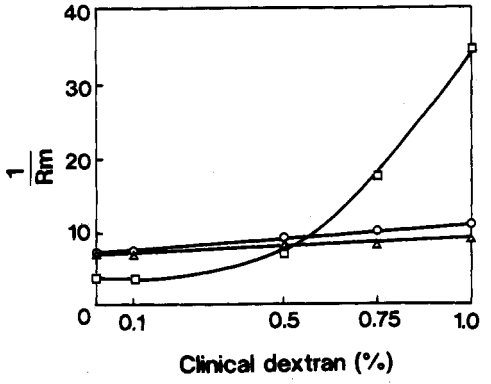

FIGURE 3: Effects of clinical dextran on the mobilities of the modified and native enzymes analyzed by affinity PAGE. In each reaction, 1.1 mg of dextransucrase was used. The EDC-modified enzyme was prepared by the incubation with 20 mM EDC and 100 mM GEE in 20 mM acetate buffer (pH 5.2) at 30 °C for 100 min. The DEP-modified enzyme was prepared by the incubation with 30 mM DEP in the 20 mM acetate buffer (pH 5.2) at the same incubation temperature and period. Native enzyme (○), EDC-modified enzyme (□), and DEP-modified enzyme (△) were applied to affinity gel electrophoresis as described in the Experimental Procedures at 4 °C for 1 h applying 20 mA. Protein bands were stained with 0.25% Coomassie brilliant blue.

mM of sucrose was added to the reaction mixture, both sucrase and transferase activities were retained to the same level. The K_m values for sucrose in the absence and the presence of dextran were 36.6 and 5.4 mM, respectively (Kobayashi et al., 1984). The concentration of 150 mM sucrose for the protection of enzyme from EDC modification was higher than the K_m values in both cases. On the contrary, 1.5% of clinical dextran accelerated the EDC inactivation of the sucrase activity, whereas the transferase activity was slightly protected by the addition of dextran. This protection by dextran did not reach the level attained by that of sucrose (Figure 2a,b). In contrast to this substrate protection for EDC reaction, inactivation by DEP caused no significant change by the addition of sucrose or dextran (data not shown).

To obtain further information on the relationship between carboxyl residues and substrates as well as between imidazole residues and substrates, affinity gel electrophoresis with clinical dextran was done (Figure 3). The mobility of the native enzyme gradually decreased with the increase in the concentration of clinical dextran in the gel. However, the mobility of the DEP-modified enzyme was almost the same as that of the native enzyme. The mobility of EDC-modified enzyme drastically decreased with the increase in the concentration of clinical dextran; that is, the affinity of the enzyme to substrate dextran increased by the blocking of carboxyl residues with EDC.

Characteristics of the Modified Dextransucrase. As shown in Figure 4a,b, the inactivation rates of both sucrase and transferase activities increased in proportion to the concentration of EDC. The relationship between log [EDC (mM)] and log [k (min^{-1})] (Levy et al., 1963) gave slopes with n = 0.84 and 0.87 for transferase activity and for sucrase activity, respectively (Figure 4c), which suggested that the inactivation of dextransucrase was caused by the modification of one essential carboxyl group/mol of enzyme.

We also determined the correlation between the enzyme activity remaining and the number of histidine residues modified (Figure 5). In phosphate buffer (pH 6.0), the modification of three histidine residues gave rise to 97% of inactivation, while in acetate buffer (pH 5.2), modification of 10 histidine residues gave rise to only about 50% of inactivation. Because this enzyme was unstable at pH 6.0 as described above, the synergistic inactivation of enzyme by DEP and pH conditions must have occurred. Fu and Robyt reported that when 14 histidine residues out of the total of about 40 histidine residues per mol of enzyme were modified, enzyme activity was lost completely (Fu & Robyt, 1988). At pH 5.2, the addition of 1.5% of clinical dextran nor 150 mM of sucrose did not cause any change in the inactivation pattern by DEP (Figure 5).

Differential Labeling and Structure of Labeled Peptide. To determine which carboxyl group was modified during the EDC inactivation, the fluorescent reagent EDAN was used as the nucleophile instead of GEE. The stoichiometry of EDAN incorporation per mole of dextransucrase was measured. About 1.18 mol of EDAN per mol of dextransucrase was incorporated (Figure 6). Almost complete inactivation seemed to be attained by the modification of one carboxyl residue of dextransucrase. To isolate the fluorescent labeled peptide, first, EDC–GEE modification was performed in the presence of sucrose monocaprate to protect the sucrose binding site. Since the C-6 hydroxyl group of the glucosyl moiety of sucrose molecule is substituted by caprate, dextran synthesis was restricted during the incubation. After the reaction was completed, the excess sucrose monocaprate was removed by

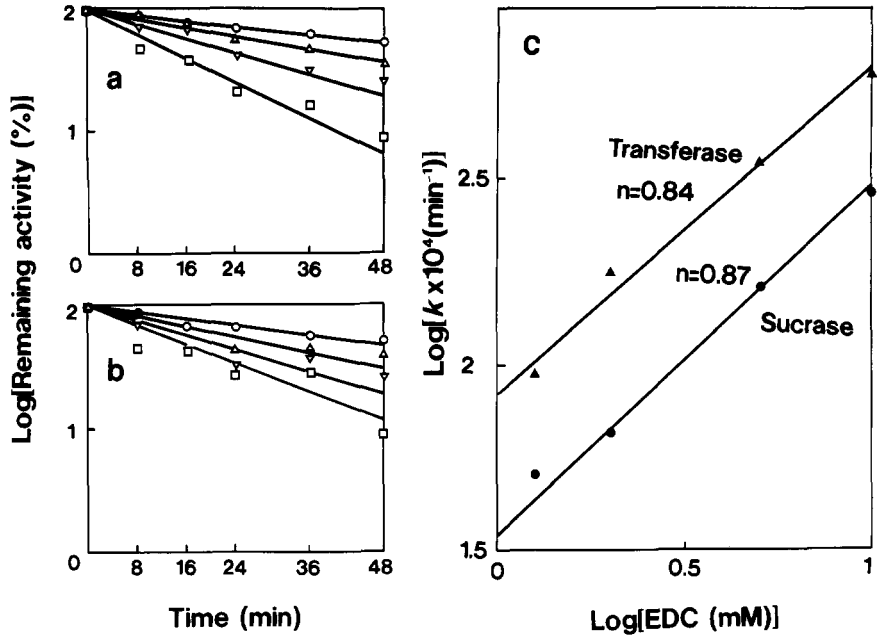

FIGURE 4: Inactivation of dextransucrase by EDC. Dextransucrase (0.33 mg of protein/mL) was modified with 1.25 (○), 2 (△), 5 (▽) and 10 mM (□) EDC as described in the legend for Figure 1. Sucrase (a) and transferase activities (b) were assayed as shown in Figure 2. (c) The relationship between log EDC (mM) concentration and log k (pseudo-first-order rate constant). The k values for sucrase were obtained from graph a, and those for transferase were obtained from graph b. The slope was drawn by the method of least squares.

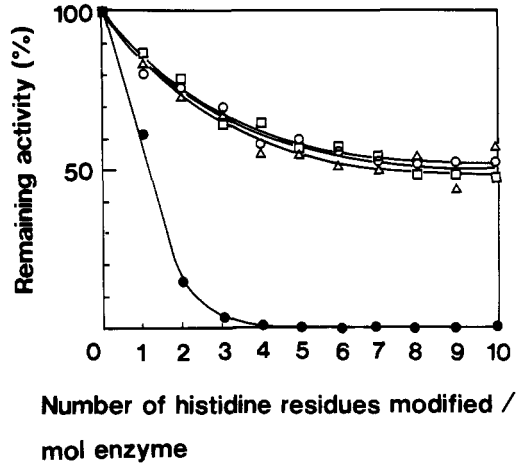

FIGURE 5: Correlation of DEP inactivation of dextransucrase with the number of histidine residues modified. Dextransucrase (0.33 mg of protein/mL) in 0.1 M phosphate buffer, pH 6.0 (solid symbol), or in 0.1 M acetate buffer, pH 5.2 (open symbols), was treated with 11.5 mM DEP without dextran or sucrose (●, ○), with 150 mM of sucrose (△), and with 1.5% of clinical dextran (□). The reaction was monitored continuously by recording the increase in absorbance at 240 nm, and the number of histidine residues was calculated from the absorbance by using extinction coefficients of 5.0×10^3 $M^{-1}\cdot cm^{-1}$ for phosphate buffer and 5.2×10^3 $M^{-1}\cdot cm^{-1}$ for acetate buffer at 240 nm (Roosemont, 1978). Enzyme activity was assayed at each time point.

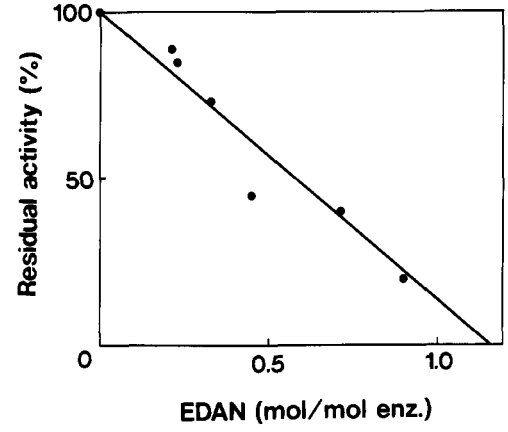

FIGURE 6: Incorporation of EDAN and resulting loss of dextransucrase activity. The enzyme (1.1 mg of protein) was modified with 5 mM EDC and 100 mM GEE in the presence of 50 mM sucrose monocaprate as described in the Experimental Procedures. The modified enzyme was applied to the Bio-Gel P-2 column (1.1 × 30 cm) and eluted with 20 mM acetate buffer (pH 5.2). V_o fractions were pooled and freeze dried. The dried sample was modified again with 5 mM EDC and 10 mM EDAN in 20 mM acetate buffer (pH 5.2) for 0, 5, 10, 15, 20, 30, and 40 min. Each reaction mixture was concentrated with an Ultra-free tube (Millipore UFC 3L GCOO) and washed three times with the same buffer. Fluorescent intensity (Ex = 330 nm, Em = 430 nm) and sucrase activity were measured. The enzyme was inactivated 43% by the modification of EDC-GEE in the presence of sucrose monocaprate. The 100% activity used as that of the enzyme after the EDC modification in the presence of sucrose monocaprate. The amount of EDAN incorporated per mol of dextransucrase was calculated based on the protein concentration and the molecular weight shown in the Experimental Procedures.

gel filtration and then the enzyme was labeled with fluorescent EDAN by the EDC reaction. The modified enzyme was digested by trypsin, and EDAN–peptide was purified using an HPLC/Wakosil 5C18 column, which was subjected to the structural analysis. Only one large fluorescent peak (peptide 1) was obtained (Figure 7). Table I shows the sequence of the fluorescent peptide. This sequence was compared with the deduced amino acid sequences of streptococcal GTF-S and GTF-I (Table I). We found a ~58% homologous region located at ~30–45 toward the amino terminal from the active aspartate residues in the presumed active-site regions of streptococcal enzymes, Arg-Val-Asp-Ala-Val-Asp (Mooser et al., 1991).

DISCUSSION

Active carboxyl groups have been found in various transferases and glycosidases. Mooser et al. reported a catalytic aspartic acid from GTF-S and GTF-I of *S. sobrinus* (Mooser et al., 1991). Carbodiimide modification showed that essential carboxylic acids including Asp176, Glu179, and Glu180 were involved in the catalytic reaction of glucoamylase G2 from *Aspergillus niger*. (Svensson et al., 1990). Site-directed mutagenesis studies on the glucoamylase from *A. awamori*

Table I: Homology of Peptide 1 from the *Leuconostoc* Dextransucrase and Streptococcal Glucosyltransferases

enzyme	source	amino acid sequence
GTF-S	*L. mesenteroides* (peptide 1)	Leu Gln Glu Asp Asn Ser Asn Val Val Val Glu Ala[a] 27 12 13 12 5 3 5 5 8 7 3 3
GTF-S	*S. mutans*	(424)Asn Asp Ile Asp Asn Ser Asn Pro Val Val Gln Ala[b]
GTF-S	*S. downei*	(396)Asn Asp Val Asp Asn Ser Asn Pro Val Val Gln Ala[c]
GTF-I	*S. mutans*	(410)Asn Asp Val Asp Asn Ser Asn Pro Val Val Gln Ala[d]
GTF-I	*S. sobrinus*	(412)Asn Asp Val Asp Asn Ser Asn Pro Ile Val Gln Ala[e]

[a] Approximately 60 pmol of peptide 1 was analyzed. The picomole yield of each Edman cycle is listed below the sequence. [b] From *S. mutans gtfD* gene (Honda et al., 1990). [c] From *S. downei gtfS* gene (Gilmore et al., 1990). [d] From *S. mutans gtfB* gene (Shiroza et al., 1987). [e] From *S. sobrinus* MFe28 *gtfI* gene (Ferretti et al., 1987).

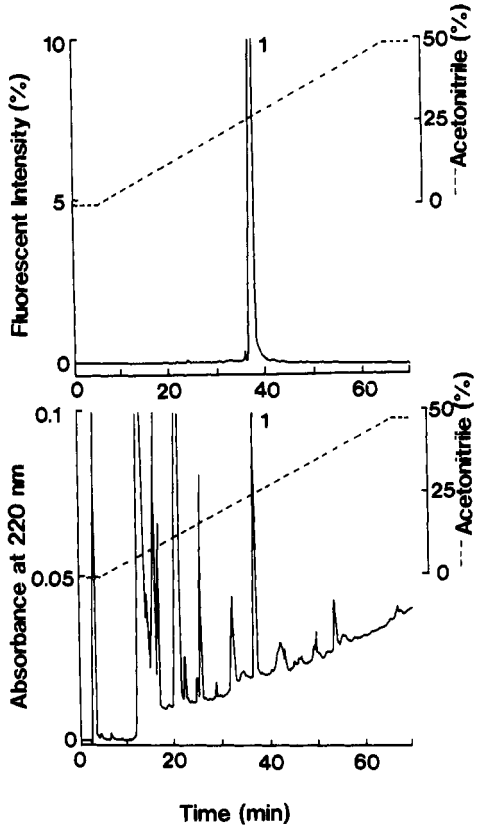

FIGURE 7: HPLC mapping of the trypsin digests of the EDAN-modified dextransucrase. The dextransucrase (1.6 mg of protein) was subjected to the differential labeling method and the trypsin digestion as described in the Experimental Procedures. A portion of the digests was applied to a column (Wakosil 5C18, 4.0 × 200 mm) equilibrated with 0.1% trifluoroacetic acid and eluted with a 60-min linear gradient of acetonitrile concentration from 0 to 48%. The flow rate was 0.8 mL/min. Peptides were monitored by measuring the absorbance at 220 nm and fluorescent intensity (Ex = 330 nm, Em = 430 nm).

indicated that Asp55 and Asp309 also participated in the catalytic reactions (Sierks & Svensson, 1993). Site-directed mutagenesis studies on the pneumococcal CPL1 lysozyme suggested that the Asp9 and Glu36 were essential for catalytic activity (Sanz et al., 1992). Tull et al. showed that Glu274 was the nucleophile in the active site of an exoglucanase from *Cellulomonas fimi* by using a tritium-labeled inactivator of 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside (Tull et al., 1991).

Dextransucrase of *L. mesenteroides* was treated with EDC or with DEP to find the catalytic amino acids. Dextransucrase was more effectively inactivated by EDC than DEP (Figure 1). Addition of sucrose retarded the inactivation of enzyme by EDC but had no effects on the DEP modification (Figures 2 and 5). EDC-modified enzyme increased the affinity to dextran, but DEP-modified enzyme did not change the affinity to dextran (Figure 3). These findings showed that the modification of the carboxyl group was highly regulated by the substrates. The EDC modification of about 1 mol of carboxyl group caused the complete inactivation of the enzyme (Figure 4), which suggested that the catalytic activity was closely related to the carboxyl group. On the other hand, the modification of 10 mol of imidazol groups by DEP in acetate buffer (pH 5.2) inactivated ~50% of the enzyme.

Both the donor substrate (sucrose) and the acceptor substrate (dextran) gave different profiles on the ligand protection between sucrase activity and transferase activity. Sucrose retarded the inactivation of sucrase activity during the modification of carboxyl residues (Figure 2a). By contrast, dextran accelerated the inactivation of sucrase activity during EDC modification (Figure 2b). These different results in the ligand protection indicated that the dextran binding site was isolated from the sucrose cleaving site, while the sucrase activity and the sucrose-binding site were closely related.

In the case of streptococcal GTF-I and GTF-S, an additional glucan-binding domain was identified besides the sucrose-binding and dextran-binding domains in the catalytic site. (Kobayashi et al., 1989; Wong et al., 1990; Mooser et al., 1991; Nakano et al., 1992). The evidences for the substrate-binding sites except for the catalytic sites were demonstrated for several other carbohydrate-metabolizing enzymes. The catalytic domain and the starch-binding domain were identified for glucoamylase-I from *Aspergillus awamori* (Aleshin et al., 1992; Hayashida et al., 1989a,b). Cellulase from *Cellulomonas fimi* had the cellulose-binding domain in addition to the catalytic domain (Gilkes et al., 1988, 1992).

Leuconostoc dextransucrase was modified with EDC more effectively when the reaction was allowed to proceed in the presence of dextran (Figure 2a). The EDC-modified enzyme had a higher affinity to clinical dextran than the native enzyme (Figure 3). On the basis of the results of biphasic kinetics and the action of antagonistic effectors, we previously indicated that *L. mesenteroides* dextransucrase had an allosteric property in the synthesis of dextran and suggested that the transferase activity was regulated allosterically by the interconversion of the aggregated oligomer forms (Kobayashi et al., 1985; Kobayashi & Matsuda, 1986; Kobayashi et al., 1986). The binding of dextran to the enzyme induced conformational changes, and the sucrose binding site might be exposed similarly to the case of the *R*-state conformation and be attacked readily by EDC. The modification of the enzyme by EDC might result in some conformational change of the dextran-binding site to enable an easier binding of dextran. These assumptions are not in conflict with the above model of sucrose-binding site and dextran-binding site, which are located in a different geometry, and the conformational change in one of the substrate-binding sites influences the other site greatly.

The fluorescent probe of EDAN was incorporated into only one peptide fraction (Figure 7). The amino acid sequence of

Table II: Comparison of Amino Acid Sequences of Conserved Regions in Various Sugar-Related Enzymes

enzyme	conserved regions 1	2	3	4	peptide 1
α-amylase (*B. sub.*)[a]	(138)DAVINH	(213)GFRFD*AAKH	(249)EILQ	(305)WVESHD	
α-amylase (*A. ory.*)[a]	(117)DVVANH	(202)GLRIDTVKH	(230)EVLD	(297)FVENHD	
GTF-S (*S. mut.*)[b]		(461)GVRVDAVDN	(503)EAWS	(579)FIRAHD	(424)NDIDNSNPVVQA
GTF-I (*S. mut.*)[c]		(447)SIRVDAVDN	(489)EAWS	(557)FIRAHD	(410)NDVDNSNPVVQA
SPase (*L. mes.*)[d]		(192)LIRLDAFAY			(27)LKEDIGAIGGVH
G6PDH (*L. mes.*)[e]		(346)QTRVDIVFK			(160)LQNDLENAFDDN
DSucrase (*L. mes.*)					LQEDNSNVVVEA

[a] Nakajima *et al.*, 1986. [b] From *S. mutans gtfD* gene (Honda et al., 1990). [c] From *S. mutans gtfB* gene (Shiroza et al., 1987). [d] From *L. mesenteroides* sucrose phosphorylase gene (Kitao & Nakano, 1992). [e] From *L. mesenteroides* glucose 6-phosphate dehydrogenase gene (Lee et al., 1991). [f] Catalytic aspartic acid (Mooser et al., 1991).

the isolated peptide was about 50–58% homologous to both the streptococcal GTF-S from *S. mutans* (residues 424–435)[a] and *S. downei* (396–407)[b], as well as GTF-I from *S. mutans* (410–421)[c] and *S. sobrinus* (412–423)[d]. The isolated peptide fragment was not identical with the glucose-binding active site regions (459–469[a], 437–447[b], 445–455[c], and 448–456[d], respectively) containing essential Asp (465[a], 443[b], 451[c], 453[d]) residues, but corresponded with the region of 30–45 amino acids toward the amino terminal from those regions. This fragment might be long enough to contain the glucose binding site. However, these glucose-binding sites were well conserved in both streptococcal GTF-S and GTF-I. Because the EDAN-modified peptide from *Leuconostoc* enzyme had about 58% homology to the streptococcal enzymes, the glucose-binding region must be very similar. In GTF-S from *S. sobrinus*, the sequence of active-site peptide was Arg-Val-Asp(catalytic residue)-Ala-Val-Asp, and there was an arginine residue two residues ahead toward the amino terminal from the catalytic aspartate residue (Mooser et al., 1991). In this experiment, *Leuconostoc* dextransucrase was digested completely by trypsin. Therefore, the fluorescent-labeled peptide probably did not contain the active aspartate. When the differential labeling was performed, sucrose monocaprate was used to protect the active region. Since less than 5% of sucrose was contaminated in the sucrose monocaprate preparation used, synthesis of a small amount of dextran during the modification reaction could not be excluded, but sucrose monocaprate was not hydrolyzed by the dextransucrase. Mooser and Iwaoka isolated the glucosyl–enzyme complex from a quenched reaction of *S. sobrinus* GTF-S and radiolabeled sucrose (Mooser & Iwaoka, 1989). Because the C-6 hydroxyl group of the glucosyl residue of sucrose monocaprate is substituted by monocaprate, the intermediate complex of the enzyme and glucose monocaprate moiety might be more unstable than the glucose–enzyme complex, and sucrose monocaprate could not work as an effective protector of the catalytic aspartate residue they detected (Mooser, et al., 1991).

In α-amylases, four highly conserved regions were found in the amino acid sequences of all the enzymes from different origins such as bacteria, fungi, plants, and animals (Nakajima et al., 1986). These regions were important for the catalytic activity of α-amylases. The amino acid sequence of the *Streptococcus mutans* GTF-S enzyme was deduced from the *gtfD* gene (Honda et al., 1990). Comparison of the sequence of GTF-S enzyme from *S. mutans* with the four conserved regions of α-amylase from *A. oryzae*, residues 117–122(I), 202–210(II), 230–234(III), and 293–298(IV) (Nakajima et al., 1986), revealed the sequence of a 44% homologous region (461–470) to region 2, a 25% homologous region (503–506) to region 3, a 50% homologous region (579–584) to region 4, and no homologous region to region 1 (Table II). The EDAN modified peptide fragment from *Leuconostoc* enzyme might correspond to region 1 of α-amylase from *A. oryzae* or it might be originated from the region peculiar to *Leuconostoc* enzymes. The EDAN-modified peptide was compared with the other enzymes from *L. mesenteroides*, and we found a 25% homologous region (27–38) for sucrose phosphorylase (Kitao & Nakano, 1992) and a 33% homologous region (160–171) for glucose 6-phosphate dehydrogenase (Lee et al., 1991) as shown in Table II.

The isolated peptide has one aspartic acid and two glutamic acids in the sequence, but we could not determine which one was substituted by EDAN. Amino acid analysis data showed no changes in the retention time of these three amino acids. The amide bond between carboxylic amino acids and EDAN seemed to have cleaved during the Edman degradation cycle. Dextransucrase of *L. mesenteroides* should require two active carboxyl residues as other α-glucan hydrolases, and the isolated peptide might contain the second essential carboxyl group. Since the EDAN labeling was conducted by the differential labeling method with the substrate sucrose analog, the identified peptide would correspond with the substrate binding domain and the carboxyl residues had an important role in the catalytic activity of *Leuconostoc* dextransucrase.

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