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ESSENTIAL HISTIDINE RESIDUES IN DEXTRANSUCRASE: CHEMICAL MODIFICATION BY DIETHYL PYROCARBONATE AND DYE PHOTO-OXIDATION*

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ABSTRACT

Treatment of *Leuconostoc mesenteroides* B-512F dextransucrase with diethyl pyrocarbonate (DEP) at pH 6.0 and 25° or photo-oxidation in the presence of Rose Bengal or Methylene Blue at pH 6.0 and 25°, caused a rapid decrease of enzyme activity. Both types of inactivation followed pseudo-first-order kinetics. Enzyme partially inactivated by DEP could be completely reactivated by treatment with 100mM hydroxylamine at pH 7 and 4°. The presence of dextran partially protected the enzyme from inactivation. At pH 7 or below, DEP is relatively specific for the modification of histidine. DEP-modified enzyme showed an increased absorbance at 240 nm, indicating the presence of (ethoxyformyl)ated histidine residues. DEP modification of the sulfhydryl group of cysteine and of the phenolic group of tyrosine was ruled out by showing that native and DEP-modified enzyme had the same number of sulfhydryl and phenolic groups. DEP modification of the ε-amino group of lysine was ruled out by reaction at pH 6 and reactivation with hydroxylamine, which has no effect on DEP-modified ε-amino groups. The photo-oxidized enzyme showed a characteristic increase in absorbance at 250 nm, also indicating that histidine had been oxidized, and no decrease in the absorbance at 280 nm, indicating that tyrosine and tryptophan were not oxidized. A statistical, kinetic analysis of the data on inactivation by DEP showed that two histidine residues are essential for the enzyme activity. Previously, it was proposed that two nucleophiles at the active site attack bound sucrose, to give two covalent D-glucosyl–enzyme intermediates. We now propose that in addition, two imidazolium groups of histidine at the active site donate protons to the leaving, D-fructosyl moieties. The resulting imidazole groups then facilitate the formation of the α-(1→6)-glycosidic linkage by abstracting protons from the C-6-OH groups, and become reprotonated for the next series of reactions.

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INTRODUCTION

Dextransucrase from *Leuconostoc mesenteroides* B-512F catalyzes the polymerization of the D-glucosyl moiety of sucrose to form dextran with the release of D-fructose. This enzyme has been the subject of several mechanistic studies in this laboratory[1–3]. Robyt *et al.*[1] proposed a mechanism for dextran synthesis in which two nucleophiles at the active site attack two bound sucrose molecules, to give two glucosyl units, covalently linked to the nucleophiles through C-1. In this process, two protons are donated to the leaving D-fructose moieties[3]. The nature of the proton donors is, however, not known. Studies of the pH-dependence of dextransucrase activity suggested that the imidazole moiety of the histidine residue may serve as the proton donor[4]. Because the pKa value of an amino acid residue in a protein depends on its micro-environment, results from pH studies are usually not conclusive. We have therefore studied the question by chemically modifying dextransucrase with diethyl pyrocarbonate (diethyl oxydiformate; DEP) and dye photo-oxidation in order to elaborate further the mechanism of the action of dextransucrase.

DEP has been widely used in the modification of various enzymes[5]. This modification is based on DEP (carbethoxyl)ation of the side chains of amino acid residues in proteins, especially the imidazole group of histidine[5]. At suitable pH values, DEP can also react with other amino acid side-chain groups, such as the hydroxyl group of tyrosine, the sulfhydryl group of cysteine, and the ε-amino group of lysine[6]. However, at pH 7 or below, DEP specifically reacts with the imidazole group of histidine[5]. Essential histidine residues in sheep liver 6-phosphogluconate dehydrogenase[7], *Bacillus amyloliquefaciens* alpha amylase[8], amylolytic enzymes[9], and bovine-heart succinate dehydrogenase[10] have been specifically modified with this reagent.

It is also known that such amino acids as histidine, tryptophan, methionine, and tyrosine in a protein can be photo-oxidized in the presence of a suitable photosensitizing dye. The photo-oxidation of histidine residues in enzymes has been widely used in determining the essential role of the imidazole group in the action of many enzymes[11,12]. Two dyes, Methylene Blue and Rose Bengal, have been the most widely used to photosensitize the oxidation of histidine residues of proteins.

Studies[13,14] have shown that Rose Bengal is more selective for the photo-oxidation of histidine than is Methylene Blue. Both types of photo-oxidation of histidine produce[7,15,16] an increase in absorbance at 250 nm.

In this study, we have chemically modified dextransucrase with DEP at pH 6.0, and have photo-oxidized this enzyme in the presence of Methylene Blue and Rose Bengal. We present evidence that two histidine residues are essential for the activity of dextransucrase from *Leuconostoc mesenteroides* B-512F.

EXPERIMENTAL

Materials. — DEP, L-histidine, N^{α}-acetyl-L-histidine, 1-acetylimidazole, 5,5′-dithiobis(2-nitrobenzoic acid), and dextran T10 and T500 were purchased from Sigma Chemical Company (St. Louis, MO). Sucrose, hydroxylamine hydrochloride, and Rose Bengal were purchased from Fisher Scientific Company (Fairlawn, NJ). Methylene Blue chloride was purchased from Hartman Leddon Co. [U-^{14}C]Sucrose was purchased from ICN Biomedicals, Inc. All other chemical compounds were of analytical grade, and commercially available.

Enzyme. — Dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F, purified through the stage of DEAE-cellulose chromatography, as reported previously[17], had a specific activity of up to 70 IU/mg.

Enzyme assays. — Dextransucrase activity was assayed radiometrically, using [U-^{14}C]sucrose as previously described[17].

Protein and carbohydrate analysis. — Protein was measured by the procedure of Lowry *et al.*[18]. Carbohydrate was measured by the phenol–sulfuric acid method[19].

Chemical modification with DEP. — The enzyme solution (0.3–1.0 mg of protein/mL) in 100mM phosphate buffer, pH 6.0, was incubated at 25° with DEP at several concentrations between 2.8 and 30mM. The concentration of ethanol in the reaction mixture of the enzyme did not exceed 5% (w/v), and was not found to have a noticeable effect on the stability or activity of the enzyme. The reaction with DEP was stopped at different times by transferring one part of an aliquot of the reaction mixture into nine parts of 100mM phosphate buffer, pH 6.0, containing 20mM L-histidine. The enzyme activity was then assayed at 25° and pH 5.2. When the absorbance spectrum of the modified protein was measured, the L-histidine was omitted. The time course of carbethoxylation of imidazole groups was monitored continuously by recording the change in absorbance at 240 nm.

The concentration of DEP in the stock solution was determined by measuring the increase in absorbance[20] at 240 nm when an aliquot of diluted DEP was added to 10mM N^{α}-acetyl-L-histidine in 50mM acetate buffer, pH 6.0. The concentration of DEP was calculated by using an extinction coefficient of $3.2 \times 10^3\ M^{-1}\ cm^{-1}$ at 240 nm.

Protection of enzyme against modification by DEP. — Dextransucrase (75 μg of protein/mL, 70 units/mg) in 50mM phosphate buffer (pH 6.0) was incubated with 5 mg of dextran T10 or T500/mL for 20 min at 25°. The enzyme was then modified with 14.5mM DEP. A control was similarly prepared, except that no dextran was preincubated with the enzyme before modification. The activity was assayed without removal of dextran, which has no effect on the assays[22].

Reactivation of modified enzyme. — Dextransucrase was modified with 2.8, 5.8, or 11.5mM DEP for 0.5 h. Reversal of the modification was achieved by adding hydroxylamine (pH 7.0) to the modified enzyme (and ethanol-treated control) to a final concentration of 100mM in 100mM phosphate buffer, pH 7.0, and incubation for 4 h at 4°. The excess of hydroxylamine was then removed by dialysis against 25mM acetate buffer (pH 5.2) for 20 h at 4° before the enzyme activity was assayed.

Photo-oxidation of enzyme. — Dextransucrase (0.5 mg of protein and 16 IU/mL; 20 mL) in 50mM acetate buffer (pH 5.2) was photo-oxidized with Rose Bengal or Methylene Blue (40 μg/mL) by irradiation with a 200-W floodlamp kept 20 cm above the surface of the sample. The mixture was stirred, and the temperature was controlled at 25° with a circulating-water bath. Aliquots (0.5 mL) were removed at various times between 1 and 7 h, and placed in the dark. The enzyme activity of these samples was assayed without removal of excess dye, which was found not to affect the enzyme activity. Controls were treated similarly, either with 40 μg of dye/mL in the dark, or irradiated in the absence of dye. Spectra of both native and photo-oxidized enzyme were obtained in 30mM Tris·HCl buffer (pH 8.0). Methylene Blue was removed from both the photo-oxidized enzyme and the control by chromatography on Bio-gel P-30 before the spectra were recorded.

RESULTS

Chemical modification with DEP. — Treatment of dextransucrase with 11.5, 19.3, and 27mM DEP led to loss of enzyme activity. Plots of log (residual activity) *versus* time of incubation at the three concentrations of inhibitor were linear, indicating that the inactivation followed pseudo-first-order kinetics (see Fig. 1). A plot of the observed pseudo-first-order rate-constants against the concentrations of DEP gave a straight line passing through the origin (see inset to Fig. 1), from which

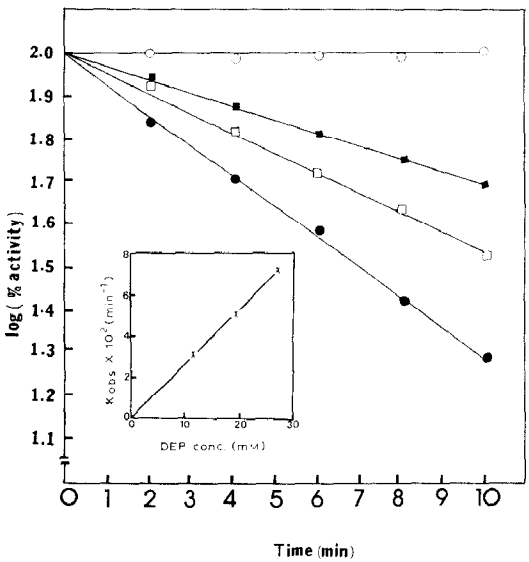

Fig. 1. Kinetics of inactivation of dextransucrase by DEP. [The enzyme (0.5 mg protein/mL, 16 IU/mg) in 100mM phosphate buffer (pH 6.0) was incubated with 0 (○), 11.5 (■), 19.3 (□), and 27mM (●) DEP. At various times, aliquots (0.5 mL) were transferred to 4.5 mL of 100mM phosphate buffer (pH 6.0) containing 20mM L-histidine, and assayed for enzyme activity. Inset: a plot of observed pseudo-first-order rate-constants *vs.* initial DEP concentrations, from which a second-order rate-constant was obtained.]

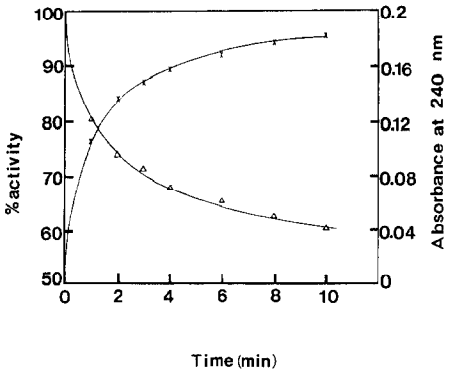

Fig. 2. Correlation of DEP inactivation of dextransucrase with the increase in absorbance at 240 nm. [Dextransucrase (0.3 mg of protein/mL, 23 IU/mg) in 100mM phosphate buffer (pH 6.0) reacted with 17mM DEP at 25°. The reaction was monitored continuously by recording the increase in absorbance (×) at 240 nm, and assaying for enzyme activity (△).]

a second-order rate-constant of 2.65 $M^{-1}.min^{-1}$ was obtained. The modification with DEP led to an increase in absorbance at 240 nm which was proportional to the degree of inactivation (see Fig. 2).

Protection by dextran against DEP modification. — Dextransucrase pre-incubated with dextran T10, or T500, respectively lost 74 and 65% of its original activity when treated with 14.5mM DEP, whereas, in the absence of dextran, the enzyme lost 91% of its original activity (see Fig. 3). This result showed that the product, dextran, which is known to bind at the active site as an acceptor[2], protects against modification of dextransucrase by DEP.

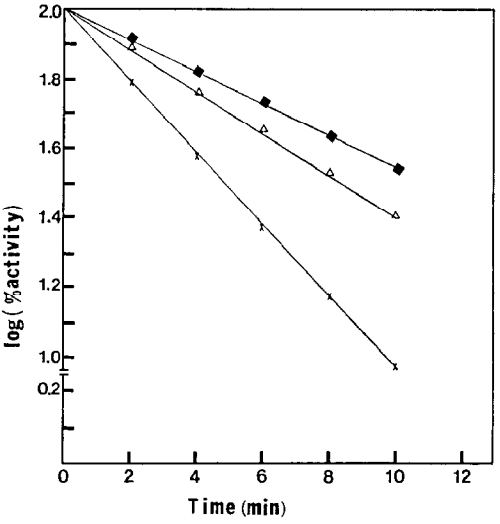

Fig. 3. Protection by dextrans against modification of dextransucrase by DEP. [Dextransucrase (75 μg of protein/mL, 70 units/mg) in 50mM phosphate buffer (pH 6.0) was incubated with 0.0 mg of dextran/mL (×), 5 mg of dextran T10/mL (△), and 5 mg of dextran T500/mL (◆) for 20 min, and then treated with 14.5mM DEP as described in Methods.]

TABLE I

REACTIVATION OF MODIFIED DEXTRANSUCRASE BY HYDROXYLAMINE[a]

DEP (mM)	*Activity (%)*	
	Before	*After*
0.0	100	100
2.8	77	100
5.8	62	95
11.5	29	42

[a]Each of the DEP reactions with dextransucrase was allowed to proceed for 0.5 h, and then incubated (along with an ethanol-treated control) with 100mM hydroxylamine in 100mM phosphate buffer (pH 7.0) for 4 h at 4°. The samples were dialyzed for 20 h against 25mM acetate buffer (pH 5.2) and then assayed for enzyme activity. The control was prepared similarly, and its activity was taken as being 100%.

Reactivation of DEP-modified enzyme. — Modified dextransucrase having 20 to 80% of the original activity regained 40 to 100% of the activity when treated with 100mM hydroxylamine in 100mM phosphate buffer (pH 7.0) (see Table I).

Specificity of inactivation by DEP. — Although DEP at pH 6.0 reacts with the side chain of histidine residues with considerable specificity, possible modification of other residues was studied. Sulfhydryl groups in dextransucrase were titrated at pH 8.1 with 0.3mM 5,5′-dithiobis(2-nitrobenzoic acid)[23]. The native enzyme gave a value of 1.1 sulfhydryl groups per enzyme molecule, for a molecular weight[24] of 158,000. The DEP-modified enzyme having 10% of its original activity gave a value of 1.0. Therefore, it was concluded that sulfhydryl groups had not been modified and that this was not the cause of the enzyme inactivation. Because lysine can only be modified by DEP at pH values higher than 7.0, and (ethoxyformyl)ated lysine residues cannot be de-(ethoxyformyl)ated by neutral hydroxylamine[7], the pH of the reaction and the restoration of activity by hydroxylamine ruled out the possibility of lysine modification. The tyrosine residue modification could also be precluded, as there was no decrease in absorbance at 278 nm when dextransucrase was completely inactivated by DEP. The modification of tyrosine residues with 1-acetylimidazole at pH 7.5 showed that the native and the modified enzyme had an identical decrease in absorbance at 278 nm (ascribable to the *O*-acetylation of tyrosine residues)[25]. This result further excluded the modification of tyrosine by DEP. All of these results, taken together with the proportional increase in absorbance at 240 nm with the inactivation of the enzyme and the relatively high specificity of the DEP reaction at pH 6 for imidazole groups, indicated that the inactivation of B-512F dextransucrase by DEP is due to modification of the imidazole ring of the histidine residues.

Number of essential histidine residues. — Inactivation of dextransucrase by DEP was correlated with the number of modified histidine residues (see Fig. 4). The plot is nonlinear, indicating that not all histidine residues are modified at the same rate. Some histidine residues are more accessible for modification than others.

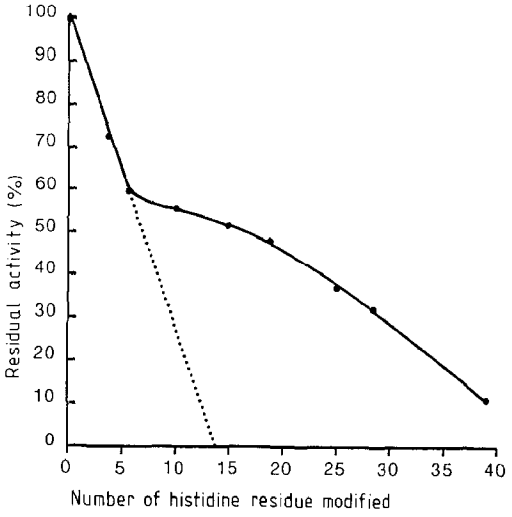

Fig. 4. Correlation of the fraction of enzyme activity remaining with the number of histidine residues modified. [Dextransucrase (0.11 mg of protein/mL) in 0.1M phosphate buffer, pH 6.0, was treated with 2.5 to 6.0mM DEP as described in Methods. After 30 min, when the excess of DEP had been decomposed, the absorbance at 240 nm was measured, and the number of histidine residues was calculated from the absorbance by using an extinction coefficient[21] of 3.2×10^3 $M^{-1}.cm^{-1}$ at 240 nm. Enzyme activity was assayed for each sample.]

Extrapolation of the first phase of the plot to zero enzyme activity showed that, out of a total of ~40 histidine residues per mol. wt. of 158,000, 14 histidine residues were modified. Such an extrapolation does not give the number of histidine residues that are essential for the enzyme activity, but it rather suggests that these 14 histidine residues are modified at a significantly, but not markedly, different rate than are the other histidine residues. Tsou[26] has shown that the number of essential residues modified under conditions where both essential and nonessential residues are modified at significantly but not markedly different rates can be determined by correlating the rate of loss of enzyme activity with the rate of modification of the total number of reactive residues by means of Eq. *1*.

$$m = n(1 - x) = n - p(A/A_0)^{1/i} - (n - p)\,(A/A_0)^{\alpha/i}, \qquad (1)$$

where m is the number of histidine residues modified, n is the total number of histidine residues modified to give complete loss of enzyme activity, p is the total number of histidine residues including i essential residues modified at rate constant K_1, $n - p$ is the number of histidine residues modified at rate constant K_2 ($K_2 = \alpha K_1$), A/A_0 is the fraction of enzyme activity at any time during the modification, and x is the total fraction of unmodified residues remaining at any value of A/A_0. Eq. *1* can be rearranged to give Eq. *2*.

$$\log[nx/(A/A_0)^{1/i} - p] = (\alpha - 1)/i \log(A/A_0) + \log(n - p) \qquad (2)$$

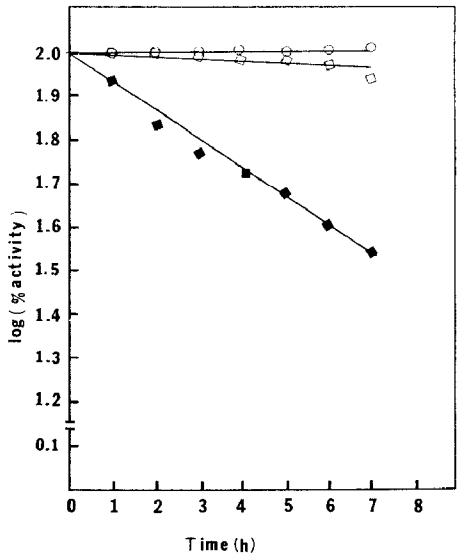

Fig. 5. Time course of photo-oxidation of dextransucrase in the presence of Methylene Blue. [Dextransucrase (0.5 mg of protein/mL) in 50mM acetate buffer (pH 5.2) in the presence of 40 μg of Methylene Blue/mL (◆) was irradiated with a 200-W light. At different times of incubation, aliquots were taken, and assayed for enzyme activity. A control was treated similarly with 40 μg of Methylene Blue/mL in the dark (○), or was irradiated identically in the absence of Methylene Blue (◇).]

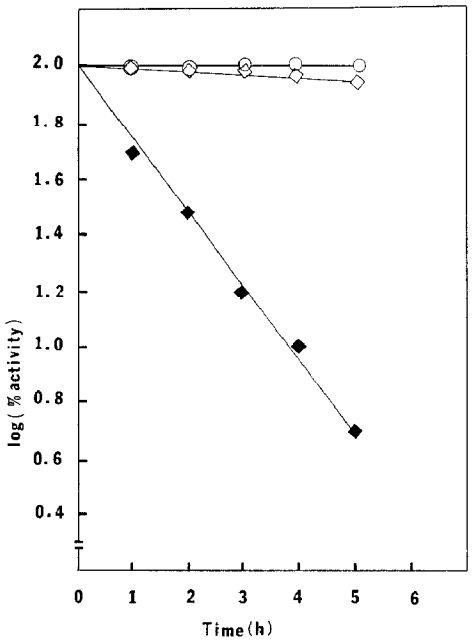

Fig. 6. Time course of photo-oxidation of dextransucrase in the presence of Rose Bengal. [Dextransucrase (0.5 mg of protein/mL) in 50mM acetate buffer (pH 5.2) in the presence of 40 μg of Rose Bengal/mL (◆). At different times of incubation, aliquots were taken, and assayed for enzyme activity. The control was treated similarly in the presence of Rose Bengal in the dark (○), or was irradiated identically in the absence of Rose Bengal (◇).]

Log$[nx/(A/A_0)^{1/i} - p]$ was plotted against log(A/A_0) by using the values of n = 40, p = 14, and i = 1, 2, 3, 4, 5, and 6. When i = 2, the best linear fit to the equation, with a linear regression variance R^2 = 0.94, was obtained. This result suggested that two histidine residues per mol. wt.[17] of 158,000 are essential for enzyme activity.

Photo-oxidation of enzyme. — Irradiation of dextransucrase at 25° in the presence of Methylene Blue or Rose Bengal led to loss of enzyme activity. Inactivation with either dye was found to be dependent on the dye concentration (see Figs. 5 and 6). The loss of activity was linear when log % residual activity was plotted *versus* time, indicating pseudo-first-order kinetics.

Dextransucrase (0.6 mg of protein/mL), photo-oxidized in 30mM Tris·HCl buffer (pH 8.0) in the presence of 80 μg of Methylene Blue/mL, had only 3% of its original activity. After removal of the Methylene Blue by chromatography on Bio-gel P-30, the photo-oxidized enzyme showed an increase in absorbance at 250 nm (see Fig. 7), indicative of the modification of the imidazole ring of histidine[27]. Because a decrease in absorbance at 278 nm was not observed, involvement of tyrosine or tryptophan in this process can be excluded.

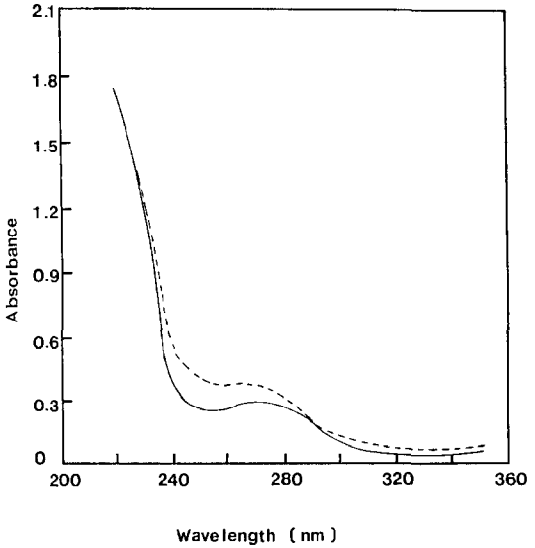

Fig. 7. Absorption spectra of the native and photo-oxidized enzyme. [Dextransucrase (0.6 mg/mL) was photo-oxidized (as described in Methods) in 30mM Tris·HCl buffer (pH 8.0) in the presence of 80 μg of Methylene Blue/mL. The control was treated identically in the dark. The Methylene Blue was then removed by chromatography on Bio-gel P-30. The spectra of the native and of the photo-oxidized enzyme (3% original activity) were taken in 30mM Tris·HCl buffer (pH 8.0). Solid line, native enzyme; broken line, photo-oxidized enzyme.]

DISCUSSION

Robyt and Eklund[3] postulated the role of proton donors at the active site of dextransucrase. The nature of these proton donors is, however, not yet known. The pH-dependence of kinetic parameters suggested the likely involvement of imidazole groups of histidine[4]. Involvement of the imidazole groups at the active site was proposed almost 30 years ago by Neely[28], who showed that irradiation of dextransucrase in the presence of Methylene Blue causes loss of enzyme activity. Studies of the effect of pH are presumptive only for identifying groups at the active site, as the pKa values of amino acid residues of proteins can vary widely in different micro-environments. Photo-oxidation in the presence of Methylene Blue could affect other amino acid residues besides histidine. Therefore, we studied the chemical modification of dextransucrase with DEP, and photo-oxidation with Rose Bengal, a dye that has a higher specificity for oxidizing histidine than does Methylene Blue.

DEP can react with side-chain groups of several amino acids. It has been reported, however, that, at pH 7 or below, DEP specifically (ethoxyformyl)ates the imidazole group of histidine[5,10]. We treated dextransucrase with DEP at pH 6.0, and found that the enzyme was inactivated. The inactivation at three concentrations of DEP followed pseudo-first-order kinetics, which indicated a direct correlation between the inactivation and the modification of the dextransucrase. A plot of observed pseudo-first-order rate-constants against DEP concentrations gave a straight line passing through the origin. After modification with DEP, there was a characteristic increase in the absorbance at 240 nm which was proportional to the degree of inactivation. This indicated that *N*-(carbethoxy)histidine residues were being formed. The characteristic increase in absorbance at 240 nm enabled us to correlate the degree of inactivation to the number of histidine residues modified. Treatment of these kinetic data of inactivation by using Tsou's statistical method[26] suggested the presence of two histidine residues essential for enzyme activity. The role of these two essential histidine residues as proton donors is discussed below.

Because dextran is the product of the action of dextransucrase, and can undergo acceptor reactions to give the formation of α-(1→3)-branched glycosidic linkages[2], dextran binds at the active site. In the presence of dextrans of different molecular weights, the enzyme is protected from DEP inactivation by binding at the active site, supporting the conclusion that histidine residues undergoing modification participate as catalytic groups at the active site of dextransucrase. We also tried the use of sucrose to protect dextransucrase from inactivation, but no conclusion was possible, as sucrose was rapidly converted into dextran by the enzyme. When dextransucrase was inactivated 23% by DEP, addition of 100mM hydroxylamine completely restored its activity. The restoration of dextransucrase activity by hydroxylamine further suggests that the imidazole group of histidine is (ethoxyformyl)ated by DEP, as it is known that hydroxylamine can remove the ethoxyformyl group from (ethoxyformyl)imidazole[5]. It is also known that

prolonged incubation with an excess of DEP can result in the formation of (diethoxyformyl)ated histidine derivatives[5]. Treatment of the disubstituted derivative with hydroxylamine opens the imidazole ring and decomposes the histidine side chain. Our results (see Table I) showed that DEP-modified enzyme having 62 and 29% of the original activity respectively regained 95 and 40% of the original activity upon treatment with hydroxylamine. The incomplete reactivation of the more highly inactivated dextransucrase is most probably attributable to formation of the diethoxyformyl derivative. The reactivation of DEP-modified enzyme upon treatment with hydroxylamine ruled out the possibility of the involvement of lysine, as DEP-modified lysine cannot be reversed by hydroxylamine[7]. Chemical analysis with 5,5′-dithiobis(2-nitrobenzoic acid) provided evidence that cysteine was not modified by DEP. Furthermore, the proportional increase in absorbance of the protein at 240 nm indicated that the imidazole group of histidine had been modified.

Dextransucrase was inactivated by photo-oxidation in the presence of either Methylene Blue or Rose Bengal. Both inactivations followed pseudo-first-order kinetics, and the oxidized enzyme showed an increase in absorbance at 250 nm, indicating that histidine residues had been photo-oxidized[7,27].

Taken together, the following results show that imidazole groups at the active site of *Leuconostoc mesenteroides* B-512F dextransucrase are involved in catalysis: pseudo-first-order inactivation by DEP, and the consequent proportional increase in the protein absorbance at 240 nm; the reversal of the DEP inactivation by hydroxylamine; protection against DEP inactivation by dextran; and the pseudo-first-order inactivation by dye photo-oxidation and the consequent increase in the protein absorbance at 250 nm.

DEP has been used over a wide range of concentrations, from 0.01 to 40mM (the maximum solubility in water[5]). In the present investigation, the concentration of DEP needed for the inactivation of dextransucrase was higher than for some other proteins. This could reflect the possibility that the histidyl residues at the active site are less accessible, which might be wholly, or partially, due to the presence of covalently bound dextran at the active site.

In the synthesis of dextran by dextransucrase, it has been proposed[1,3] that two nucleophiles (X^-) at the active site attack two bound sucrose molecules, to give two D-glucosyl units covalently linked to the nucleophiles through C-1. In this process, we propose that two imidazolium groups of histidine residues at the active site donate protons to the oxygen atom of the leaving D-fructose moieties (see step 1 of Fig. 8). The imidazolium ions are then changed to imidazole groups, and one of the imidazole groups abstracts the proton from the 6-hydroxyl group of one of the covalently linked D-glucosyl units, facilitating nucleophilic attack of the 6-hydroxyl group on C-1 of the other D-glucosyl unit, to form the α-(1→6)-D-glucosidic bond (see step 2 of Fig. 8). As the D-glucosidic linkage is formed, the X group attached to the leaving D-glucose moiety is released, and becomes free to attack another sucrose molecule. The protonated imidazole group that abstracted

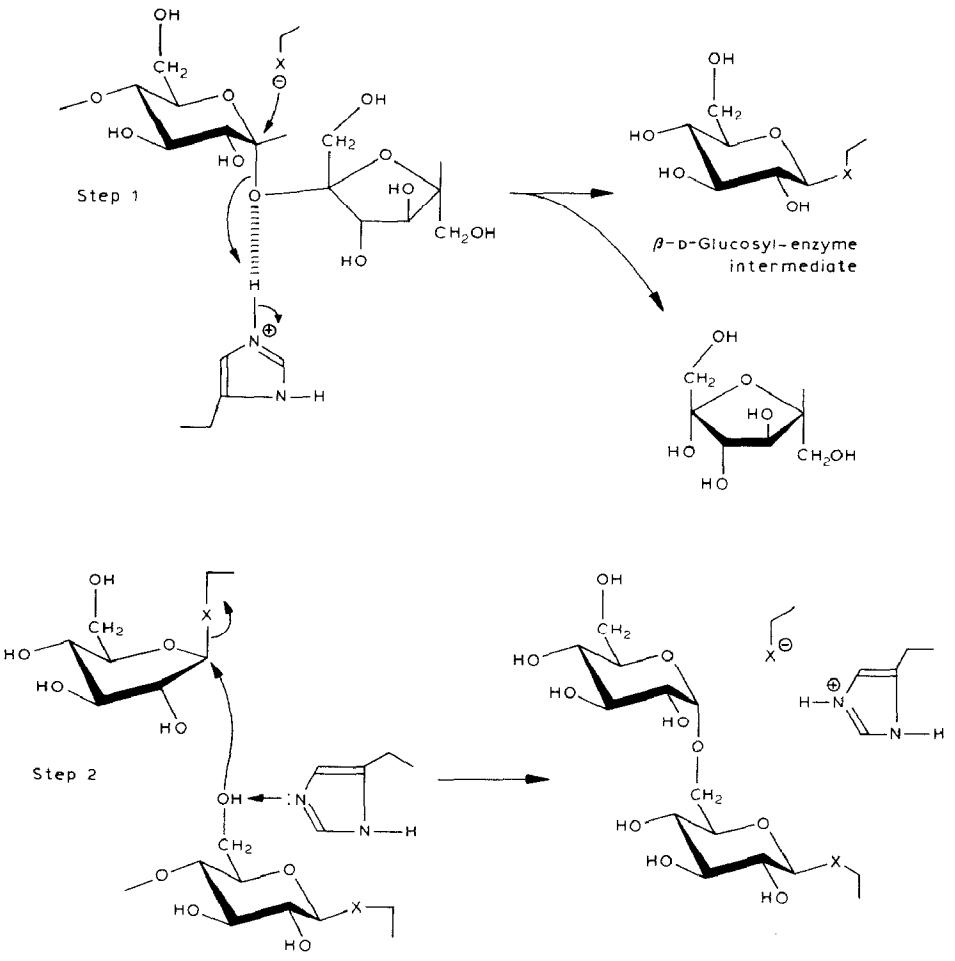

Fig. 8. Proposed mechanism of action of the imidazole group at the active site of dextransucrase. [Step 1. Role involved in the cleavage of sucrose by donation of a proton to D-fructose. Step 2. Role in the synthesis of the α-(1→6)-glucosidic linkage by abstracting a proton from the OH-6 group, facilitating the hydroxyl nucleophilic attack on C-1 of the D-glucosyl–enzyme intermediate.]

the proton from the 6-OH group, to facilitate formation of the D-glucosidic linkage now transfers its proton to the leaving D-fructosyl moiety. This process is then repeated back and forth between the two X groups and the two imidazolium and imidazole groups, to give the synthesis of dextran as shown in Fig. 9.

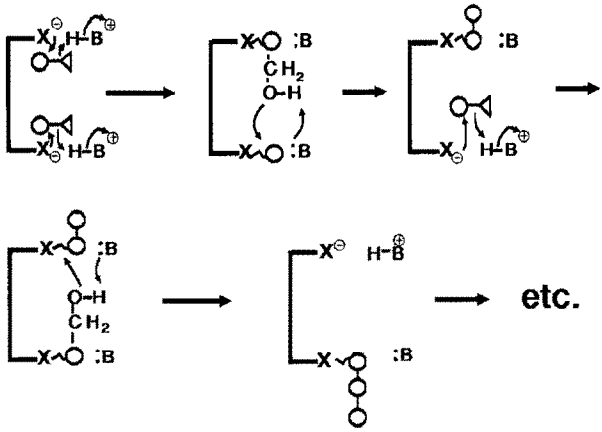

Fig. 9. Dextran synthesis of dextransucrase by the insertion mechanism, using two nucleophile (X^-) and two imidazolium groups (HB^+) as catalytic groups at the active site. [Sucrose (○—◁) reacts with enzyme, D-glucose (○) is transferred to the X-nucleophiles, and the leaving D-fructose moiety (◁) is protonated by the imidazolium group. The resulting imidazole group (:B) then abstracts a proton from the OH-6 group of one of the two D-glucosyl units, facilitating its nucleophilic attack on C-1 of the other D-glucosyl unit, to form an α-(1→6) linkage.]

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