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INACTIVATION OF *LEUCONOSTOC MESENTEROIDS* NRRL B-512F DEXTRANSUCRASE BY SPECIFIC MODIFICATION OF LYSINE RESIDUES WITH PYRIDOXAL-5'-PHOSPHATE

ARUN GOYAL and SARVAGYA S. KATIYAR*

Department of Chemistry, Indian Institute of Technology Kanpur, Kanpur 208 016, India

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Dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F was inactivated by pyridoxal-5'-phosphate (PLP). The inactivation was reversible in as much as the loss of enzyme activity was completely reversed by prolonged dialysis. PLP-modified dextransucrase after reduction with sodium borohydride showed a characteristic fluorescence emission maximum at 397 nm when excited at 325 nm. The stoichiometric results indicated that four lysine residues are modified by PLP under the experimental conditions. These results established for the first time that lysine residues are essential for the activity of dextransucrase.

KEY WORDS: *Leuconostoc mesenteroides* NRRL B-512F, dextransucrase, pyridoxal-5'-phosphate, active site, chemical modification

INTRODUCTION

Dextransucrase (Sucrose: 1,6-α-D glucan 6-α-D glucosyl transferase, EC 2.4.1.5) from *Leuconostoc mesenteroides* NRRL B-512F has been purified by various methods and its properties investigated.[1-5] However, little information is available in respect of the amino acid residues present at the active site involved in catalysis. The mechanism for dextran synthesis was postulated to involve two nucleophiles at the active site that attack two bound sucrose molecules to give two covalent D-gluosyl-enzyme intermediates.[6] Subsequently, Fu and Robyt[7] showed the presence of two histidine residues at the active site and proposed that the imidazolium groups of histidine residues donate protons to the leaving D-fructosyl moieties and the resulting imidazole groups facilitate the formation of α-(1→6)-glucosidic linkage by abstracting protons from the C-6-OH groups. Recently, Su and Robyt[8] confirmed the proposed two-site mechanism for dextran synthesis and further proposed that two sucrose binding sites and one acceptor binding site constitute the active site of dextransucrase.

In the present, study we have modified chemically dextransucrase with pyridoxal-5'-phosphate (PLP), a lysine-specific reagent. The enzyme was inactivated by the reaction

*Correspondence: Professor S.S. Katiyar, Fax: 0091–512–250260 and 0091-512-250007 Telex: 0325–296

of PLP at pH 5.2, which became irreversible after reduction with sodium borohydride. The results demonstrated, for the first time, the requirement of lysine residues for the activity of the enzyme.

MATERIALS AND METHODS

Dextransucrase purified to homogeneity as described previously[5] was used in the present study. Pyridoxal-5'-phosphate and L-lysine were obtained from Sigma Chem. Co., (St. Louis, MO, USA). All other chemicals used were of highest purity grade commercially available.

In the reaction of pyridoxal-5'-phosphate with the enzyme, an enzyme solution (0.6 mg protein/ml, 30 U mg^{-1}) was treated with indicated concentrations of PLP in 0.2 M acetate buffer (pH 5.2) at 30°C. The reaction with PLP was stopped by transferring two parts of an aliquot from the incubation mixture into eight parts of 0.2 M acetate buffer, pH 5.2 containing 10 mM L-lysine. An aliquot from the lysine mixture was assayed for the enzyme activity. The enzyme activity was determined by measuring the rate of production of reducing sugar. The assay mixture (1.0 ml) contained 10% substrate sucrose in 0.2 M acetate buffer, pH 5.2 and the enzyme solution. The reaction mixture was incubated at 30°C for 20 min. Aliquots (0.1–0.2 ml) from the reaction mixture were analyzed for reducing sugar by the method described by Nelson[9] and Somogyi.[10] Control mixtures without PLP were run concurrently.

The enzyme (1.2 mg/ml) in 0.2 M acetate buffer (pH 5.2) was incubated with 30 mM PLP for 1 h at 30°C. The enzyme-inhibitor reaction was terminated by reduction with 50 mM sodium borohydride. The reaction mixture was kept at 0°C for 30 min and then dialyzed extensively against 0.2 M acetate buffer (pH 5.2). The resulting N^{ϵ}-phosphopyridoxyllysine complex was characterized by fluorescence emission spectrum with excitation at wave length 325 nm. The number of mole of PLP incorporated per mole of enzyme was determined by taking the ratio of concentrations of N^{ϵ}-phosphopyridoxyllysine and enzyme using the absorbance coefficient of 9700 $M^{-1}cm^{-1}$ at 325 nm[12] and Lowry's method,[13] respectively. This value was taken for stoichiometric calculations where the molecular weight of dextransucrase was taken as 188,000.[5]

RESULTS AND DISCUSSION

The treatment of dextransucrase with PLP led to the loss of enzyme activity (Figure 1). The overall enzyme activity decreased to 25% of the control activity in a period of 60 min. The rate of inactivation increased with the increase in the concentration of PLP but the activity loss did not exceed 80%. The inactivation of dextransucrase by PLP was reversible since the loss of activity was reversed completely by prolonged dialysis. However, reduction with sodium borohydride after PLP treatment rendered the inactivation irreversible. These observations indicated that the inactivation of enzyme by PLP was due to the specific modification of ϵ-NH_2 groups of lysine residues and not due to the non covalent binding of PLP with the enzyme.

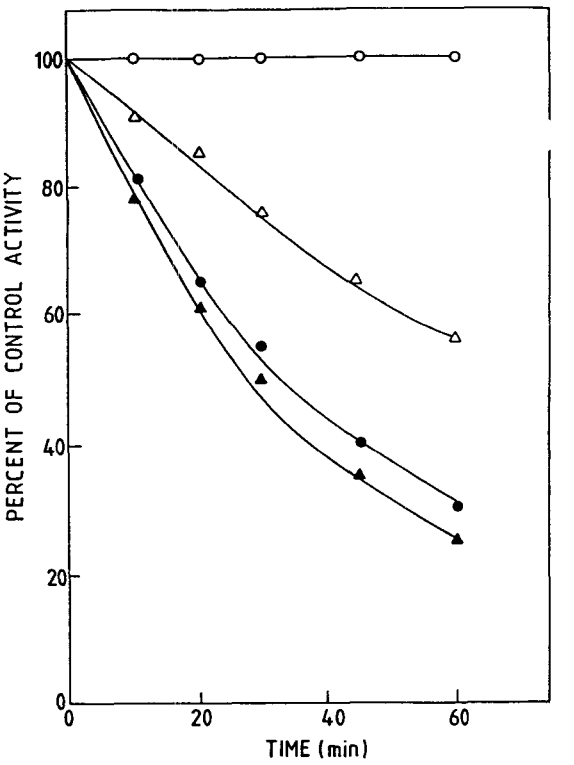

FIGURE 1 Time dependent inactivation of dextransucrase by pyridoxal 5'-phosphate. The enzyme (0.6 mg protein/ml, 30 U mg^{-1}) in 0.2 M acetate buffer (pH 5.2), was incubated with 0 (○), 20 (△), 30 (●), and 35 mM (▲) PLP at 30°C. Aliquots were withdrawn at the indicated time intervals and the residual activity was determined as described in "Materials and Methods".

PLP has been used in others' reports over a wide range of concentrations, from 0.1 to 12.5 mM. In the present investigation, the concentration of PLP required for inactivation of dextransucrase was higher than for some other proteins. This could reflect the possibility that the lysyl 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.

The fact that the inactivation of dextransucrase by PLP was indeed due to the formation of Schiff base and not due to nonspecific effects, was ascertained by spectroscopic methods. The reaction of PLP with ε-NH_2 group of lysine results in the formation of Schiff base which on reduction with sodium borohydride gives N^{ε}-phosphopyridoxyllysine. This product shows an absorption maximum at 325 nm[11] and a fluorescence maximum at 395 nm (excitation wave length, 325 nm).[12] In the present case, too, the reduction of dextransucrase-PLP complex by sodium borohydride resulted in the formation of N^{ε}-phosphopyridoxyllysine, which gave a fluorescence maximum at 397 nm (Figure 2).

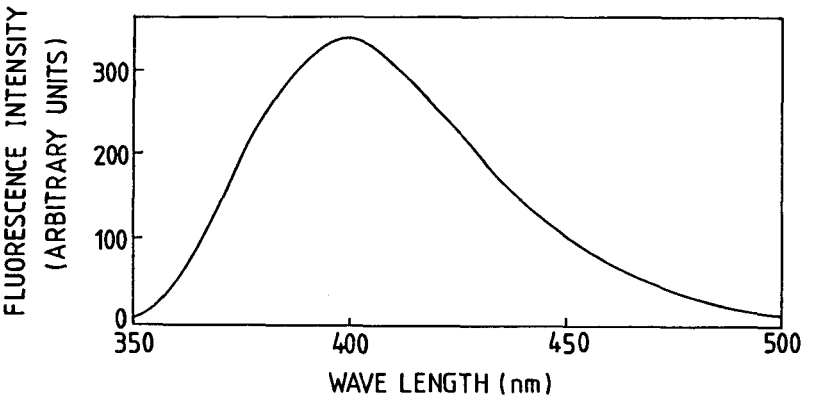

FIGURE 2 Fluorescence emission spectrum of dextransucrase-PLP complex after reduction with sodium borohydride. The enzyme (1.2 mg/ml) in 0.2 M acetate buffer (pH 5.2) was treated with 30 mM PLP and reduced with 50 mM sodium borohydride. This mixture was dialyzed extensively. The resulting N^{ϵ}-phosphopyridoxyllysine complex was characterized by fluorescence emission spectrum with excitation at wave length, 325 nm. For details, see "Materials and Methods".

The stoichiometry of inactivation by PLP was determined by absorbance enhancement at 325 nm after reduction with sodium borohydride (Table 1). The results showed that modification of dextransucrase by 30 mM PLP resulted in about 45% inhibition of the enzyme with concomitant modification of four lysine residues per mole of the enzyme. An increase in the enzyme concentration also increased the absorbance linearly which further confirmed the above stoichiometry. These results support the view that chemical modification of four lysine residues per mole of enzyme resulted in loss of enzyme activity and one or more lysines affected the enzyme substrate reaction.

TABLE 1
Quantitative analysis of binding of pyridoxal-5'-phosphate to dextransucrase

Concentration of dextransucrase	Absorbance at 325 nm	Number of mole of PLP bound/mole enzyme
2 μM	0.08	4.1
4 μM	0.15	3.9
6 μM	0.25	4.3
8 μM	0.31	4.0

Different concentrations of dextransucrase were incubated with 30 mM pyridoxal-5'-phosphate for 30 min, which resulted in 45% of inactivation of the enzyme. The number of moles of PLP incorporated per mole of dextransucrase were calculated as described in "Materials and Methods".

The results presented in this communication clearly demonstrated for the first time the requirement of lysine residues for the activity of dextransucrase. Taken together, these results show that PLP reacted with four lysine residues, resulting in inactivation of the enzyme. It seems unlikely that all the four lysine residues are present at the active site and are essential for catalytic activity of the enzyme. Further studies are in progress, to determine the location of these lysine residues.

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