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EVIDENCE FOR THE ESSENTIAL HISTIDINE RESIDUE AT THE ACTIVE SITE OF GLUCOSE/XYLOSE ISOMERASE FROM STREPTOMYCES*

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Modification of glucose/xylose isomerase from Streptomyces sp. NCIM 2730 by diethylpyrocarbonate (DEPC) or its photo-oxidation in presence of rose bengal or methylene blue caused rapid loss in its activity. The inactivation of the enzyme was accompanied by an increase in the absorbance at 240 nm and was reversed by hydroxylamine. Glucose and xylose but not Mg^{++} and Co^{++} afforded significant protection to the enzyme from inactivation by DEPC. Inactivation followed pseudo-first-order kinetics and modification of a single histidine residue per mole of enzyme was indicated.

D-glucose/D-xylose isomerase (GI) (EC 5.3.1.5) catalyses the interconversion of D-glucose and D-xylose to D-fructose and D-xylulose respectively. The enzyme is currently used in the industrial production of high fructose corn syrup from starch and in the conversion of xylose to ethanol. Recently, efforts have been made to identify the catalytic residues of the enzyme (1). Dyson and Noltmann (2) proposed general acid catalysis of ring-opening by a protonated lysine residue and proton transfer by a deprotonated histidine. In contrast, O'Connell and Rose (3) suggested that a glutamate residue mediates the proton transfer in the isomerization process.

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In the present paper, we report on the modification of a histidine residue of the GI from Streptomyces sp. NCIM 2730 by a histidine acylating reagent, DEPC and by its photo-oxidation in the presence of rose bengal or methylene blue. Our results support the involvement of a histidine residue for the catalytic activity of GI.

MATERIALS AND METHODS

DEPC and hydroxylamine hydrochloride were obtained from Sigma Chemical Company, St. Louis MO, USA. Glucose and xylose were from E. Merck, Darmstadt, Germany. Other chemicals were of the highestpurity commercially available.

Microorganisms : Streptomyces sp. NCIM 2730 was isolated in National Chemical Laboratory, Pune, by National Collection of Industrial Microorganisms.

Enzyme assay : GI was estimated by the colorimetric method of Takasaki and Tanabe (4). D-fructose or D-xylose produced in the reaction was determined by the method of Dische and Borenfreund (5) as modified by Marshall and Kooi (6). The enzyme was estimated at pH 7.5 at 70°C for 30 min. The unit of GI is defined as the amount of enzyme that produces 1 µmole of D-fructose or D-xylulose per min under the assay condition.

Protein assay : The protein concentration was measured according to Brandford (7).

Enzyme production and purification : The organism was grown for 96 h on a medium (8) containing 1% xylose as an inducer. The cells were sonicated and extract was heated at 65°C for 15 min. The enzyme was purified by ammonium sulphate precipitation and by preparative gel electrophoresis followed by DEAE cellulose chromatography. The purified enzyme showed a single band on gel electrophoresis (Unpublished results).

Carbethoxylation : The enzyme (400 µg) in 20 mM potassium phosphate buffer pH 7.0 was incubated with indicated concentrations of DEPC diluted in absolute ethanol at 30°C The ethanol at this concentration was found to have no effect on the activity and stability of the enzyme during incubation time. The extent of carethoxylation was monitored by recording the increase in absorbance at 240 nm of the reaction mixture and a control containing an equal amount of the untreated enzyme which was incubated under identical conditions with ethanol but without DEPC. Ten microliter aliquots were withdrawn from the experimental and control at different time intervals and the residual activity was determined.

Decarbethoxylation : The enzyme was treated with 2 mM DEPC for 10 min. The reactivation of the modified

histidine residues was achieved by incubation with 400 mM hydroxylamine hydrochloride at pH 7.0 at 4°C for 16 h.

Photo-oxidation : Photo-oxidation was carried out (9) at room temperature by exposing the enzyme solution in 20 mM phosphate buffer pH 7.0 containing rose bengal or methylene blue to 200 W flood-light bulb held at 10 cm from the sample. After particular time intervals the samples were estimated for the activity. The controls were similarly treated in the dark.

RESULTS AND DISCUSSION

The pH dependence of kinetic parameters for isomerization of glucose was studied in the pH range of 5.0 to 8.5. The plot of log $\left\{\frac{Vmax}{Km}\right\}$ vs pH results in a bell-shaped curve (Fig.1) and the apparent pKa values obtained were 6.8 and 8.4. The former value suggests that imidazole moiety of histidine is involved in catalytic function of the enzyme.

Treatment of glucose isomerase with DEPC led to inactivation and the extent of which was dependent both upon time and reagent concentration. Plot of residual activity versus time at all concentrations of the reagent was linear indicating that the inactivation follows first-order kinetics (Fig.2). A double logarithmic plot of the observed pseudo-first-order

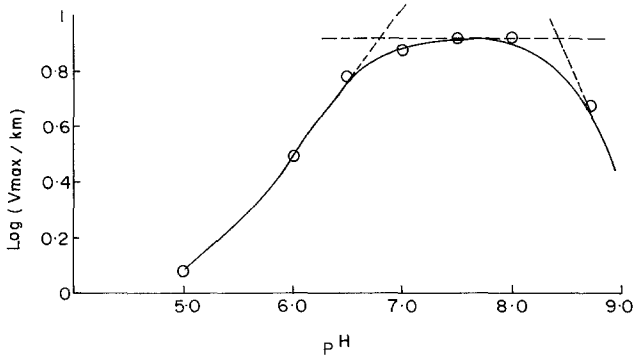

Figure 1. Plot of log $\left\{\frac{Vmax}{Km}\right\}$ vs pH. Km and Vmax at different pH were determined from Lineweaver-Burk plots.

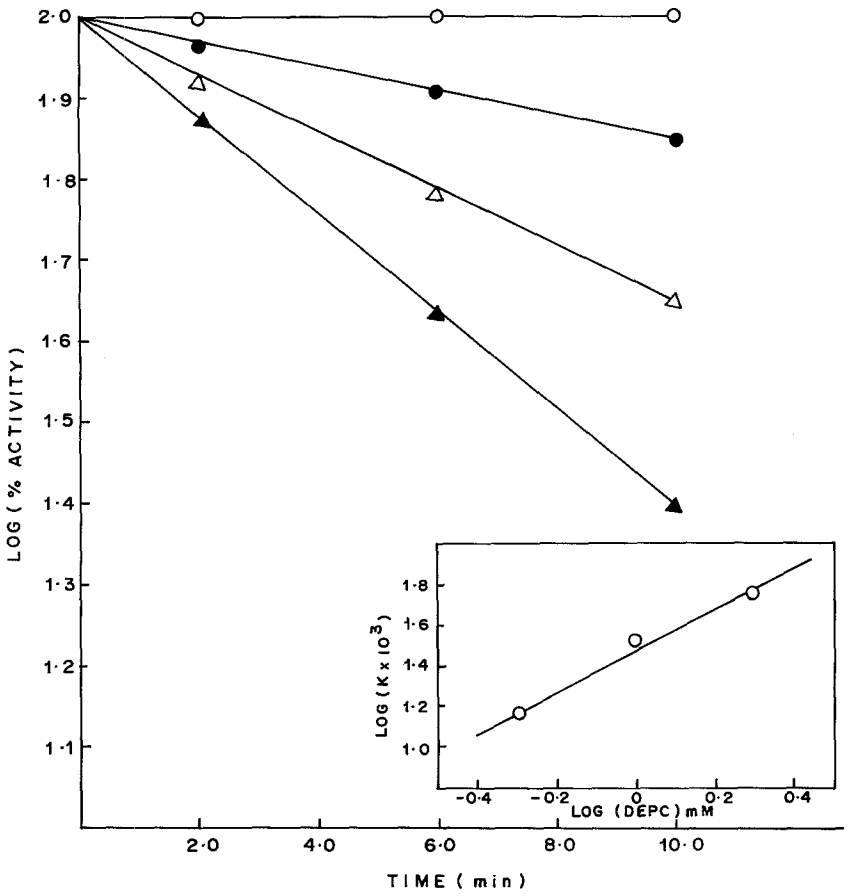

Figure 2. Kinetics of inactivation of GI by DEPC. The enzyme (400 ug) at pH 7.0 was incubated with DEPC 0 mM (o); 0.5 mM (●); 1 mM (△) and 2.0 mM (▲).

Inset - Apparent order of reaction with respect to reagent concentration. The observed pseudo-first-order rate constants calculated from Fig. 2 were plotted.

rate constants against reagent concentrations yielded a reaction order of 1.0 (Fig.2 inset) indicating that the modification of a single histidine residue results in the loss of enzyme activity (10).

The possibility of the inactivation of enzyme due to some non-specific interactions of DEPC with other amino

Table 1 : Protection of GI from DEPC inactivation and its reactivation by hydroxylamine hydrochloride

Additions	Residual activity (%)
None (control)	100
DEPC (2 mM)	26 ± 4
Glucose (100 mM) + DEPC	94
Xylose (5 mM) + DEPC	95
Mg^{++} (5 mM) + Co^{++} (1 mM) + DEPC	26
Hydroxylamine hydrochloride treatment	54

Enzyme (20 μg) was incubated with the reagents at room temperature for 10 min prior determination of enzyme activity. Hydroxylamine treatment was as described in Materials and Methods.

acid residues such as cysteinyl, arginyl or tyrosyl was also considered. GI was not inhibited by pCMB (5 mM) or iodoacetamide (1 mM) ruling out the possibility that the inactivation may be due to the modification of cysteine residues. The inactivation of enzyme due to modification of amino acids other than histidyl or tyrosyl residues cannot be reversed by hydroxylamine. Treatment of carbethoxylated GI with hydroxylamine resulted in 54% reactivation of the enzyme (Table 1) indicating that the inactivation of the enzyme is due to the modification of either histidyl or tyrosyl residues. The possibility of the modification of tyrosyl residues was ruled out by studying the absorbance of DEPC treated enzyme at 278 nm. The modification of the tyrosyl residues by DEPC leads to the formation of 2-O-carbethoxy-tyrosyl which causes a large decrease in the absorbance at 278 nm. No such decrease was observed in the DEPC inactivated enzyme indicating that tyrosyl residues of GI are not modified and the inhibition of enzyme activity

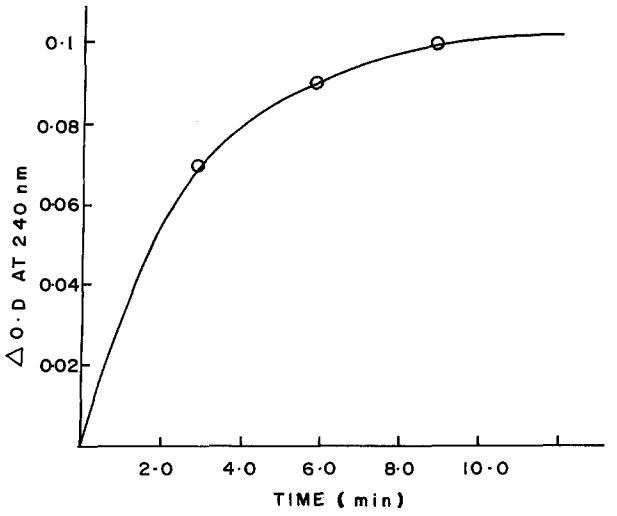

Figure 3. Absorbance of DEPC-treated GI at 240 nm.

was most likely due to specific modification of the histidyl residues.

The inactivation of GI by DEPC was accompanied by a significant increase in absorbance at 240 nm (Fig.3) characteristic for ethoxylcarboxylation of histidine residues which reinforced the evidence of histidine modification at the active site and eliminated the possibility of gross conformational changes.

GI was protected by its substrates glucose and xylose, against inactivation by DEPC (Table 1). However, Co^{++} and Mg^{++} fail to protect the enzyme from inactivation suggesting that the catalytic site is distinct from the cofactor binding site.

Photo-oxidation of the enzyme in the presence of methylene blue or rose bengal caused loss in activity which was completely prevented by shielding from irradiation (Table 2). The inactivation was directly proportional to the concentration of dye and was protected by glucose or xylose. With increase

Table 2 : Photo-oxidation of GI by photo-sensitizing dyes

Photo-oxidizing dye		Conc. (%)	Time (min)	Residual activity (%)
Methylene blue	(pH 7.0)	0.05	10	60
		0.1	10	58
	(pH 8.5)	0.1	10	40
Rose Bengal		0.05	10	76
			20	60
	(pH 7.0)	0.1	10	40
			20	25
		0.2	10	30

in pH the rate of inactivation by methylene blue was increased but that by rose bengal was decreased as observed by Tsai et al. (11).

CONCLUSIONS

pH dependence of kinetic parameters indicated likely involvement of imidazole group at the catalytic site of GI. However, results from such studies are not conclusive since the pKa value of an amino acid residue depends upon its microenvironment. In the present study, the confirmation of active site histidine residue was sought by involving histidine specific reactions. DEPC caused a strong inhibition of the enzyme activity. The inactivation is considered to be specificially due to the modification of the histidine and not of other amino acid residues with which DEPC is known to react because (a) the loss in enzyme activity was associated with an increase in absorbance of the enzyme at 240 nm indicating formation of carbethoxy-imidazole; (b) hydroxylamine treatment which decarboethyoxylates histidine and tyrosine residues only, reactivates the DEPC treated enzyme; (c) no increase in absorbance of the enzyme at 278 nm indicates that carbethoxytyrosine residues are not formed and (d) GI was hot inhibited by pCMB or iodoacetamide. Another confirmatory evidence for the involvement of histidine residue at the active site of GI comes from its inactivation by photooxidation in the presence of a dye. Protection of inactivation by substrates provides convincing evidence that a functional histidyl residue is ncessary for the catalytic activity of the enzyme and most likely is situated at or near the substrate binding.

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