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EXTERNAL YEAST β-FRUCTOSIDASE

AFFINITY LABELING OF THE ACTIVE SITE

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Summary

Conduritol-B-epoxide, a compound structurally related to the substrates of external yeast β-fructosidase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26), is an active-site directed inhibitor of this enzyme. The inactivation is irreversible and first-order with respect to time and inhibitor concentration. From the kinetic data obtained, it is concluded that one molecule of inhibitor reacts with one molecule of the enzyme causing inactivation. The inactivation is prevented by the presence of substrates. The pH-dependence of inactivation shows two dissociating groups in the enzyme with pK_a values 3.05 and 6.8 being involved in the inactivation process. A carboxylate at the active site with pK_a 3.05 is suggested to be the reactive group with conduritol-B-epoxide.

Introduction

Recently a chemical modification study was published on yeast β-fructosidase (intervase) (β-D-fructofuranoside hydrolase, EC 3.2.1.26) employing several selective reagents to investigate the amino acid residues responsible for catalytic activity [1]. No evidence was obtained for tyrosine, histidine, free amino and sulfhydryl groups being essential for activity, whereas oxidation of 5—6 tryptophan residues/molecule with *N*-bromosuccinimide gave complete inactivation of the enzyme. An earlier study showed inactivation of yeast invertase by reaction with iodine, which the authors indicated to be due to the reversible oxidation of a methionine sulphur atom [2].

The presence of a carboxylate at the active site of yeast invertase has been postulated from studies of the pH-dependence of substrate binding [3,4] and from the difference in enzyme inactivation by reaction with iodoacetamide and iodoacetic acid [2]. To our knowledge no modification studies on invertase

with reagents selective for carboxylic groups have been published until now. Recently a successful affinity labeling of the active sites in the sucrase-isomaltase complex from small intestine with conduritol-B-epoxide was reported from our laboratory [5,6]. Conduritol-B-epoxide, a compound structurally related to the substrates of sucrase-isomaltase, reacted with a carboxylate at the two active sites of the complex. Since sucrose is the best substrate for yeast invertase we tried to modify this enzyme with conduritol-B-epoxide. We present some data, which show the specific inactivation of yeast external invertase by this reagent and suggest the presence of one carboxylate at the active site.

Methods and Materials

Inhibitors. Conduritol-B-epoxide was synthesized as described by Legler [7]. The other epoxides were purchased from FLUKA, Switzerland.

Invertase. Yeast invertase (EC 3.2.1.26) was purchased from Boehringer GmbH, Mannheim (Cat. No. 15067/1974/75). The Boehringer preparation was purified by DEAE-Sephadex column chromatography according to the method of Leskovac et al. [1]. The purified enzyme had a specific activity of 1600—1700 units/mg (23°C).

Enzyme assay. Activity of invertase was measured from the rate of glucose formation at 23°C, using the Tris/glucose oxidase/peroxidase reagent [9]. 50 µl of the enzyme (0.5—1 µg) in 0.1 M sodium acetate, pH 4.6, were added to 100 µl H_2O and 50 µl 0.6 M sucrose in the same buffer. The reaction was stopped after 8 minutes by addition of 300 µl of 0.2 M dibasic sodium phosphate and the tubes placed in a boiling water bath for 3 min [8]. Glucose concentration was determined in 10-µl aliquots of the latter solution. After 60 min the reaction with glucose oxidase was stopped by addition of 50% H_2SO_4 and the absorbance read at 546 nm.

Inactivation studies. A typical reaction mixture contained in a total volume of 0.5 ml of 0.1 M sodium acetate buffer, pH 4.6, 0.5 mg of invertase and the inhibitor in varying concentrations. The reaction was run at 37°C. At different time intervals aliquots were withdrawn and diluted with 0.1 sodium acetate buffer, pH 4.6, or applied to small Biogel P-30 columns (0.5 × 7 cm), equilibrated with the same buffer. The enzymatic activity was determined in that part of the eluate, which emerged with V_e, and which contained all the enzyme and no inhibitor.

Results

Kinetics of inactivation

Fig. 1 presents the plots of log invertase activity versus the time of reaction with 4 and 6 mM conduritol-B-epoxide. These plots are linear until the loss of initial activity exceeds 85%, indicating pseudo-first-order kinetics down to 15% activity. The loss of linearity after more than 85% of initial invertase has been inactivated could be due to a side reaction of the epoxide, the enzyme-catalyzed ring opening of the epoxide by water, as observed during the reaction of some glycosidases with conduritol-B-epoxide [10,11]. Exhaustive dialysis of the inactivated enzyme gave no reactivation.

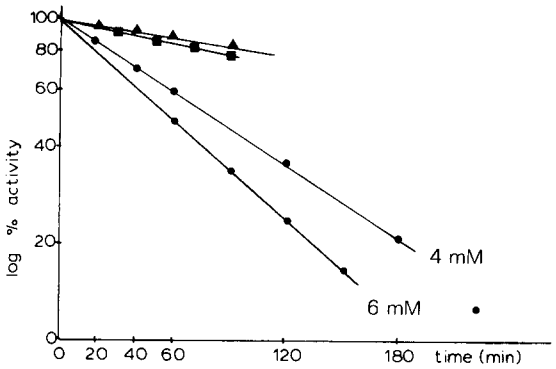
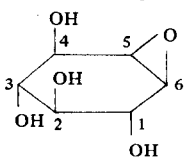
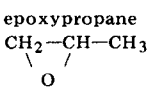
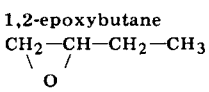
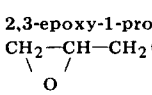
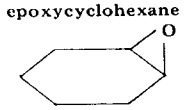


Fig. 1. Inactivation of invertase by conduritol-B-epoxide and protecting action of substrates. Each incubation mixture at 37°C contained 50 mM sodium acetate buffer, pH 4.6, invertase (37 μM) and conduritol-B-epoxide. The incubation time in the presence of substrate could not be extended beyond 90 min because of their rapid hydrolysis. ●, 4 mM epoxide; ▲, 4 mM epoxide + 600 mM raffinose; ■, 4 mM epoxide + 600 mM stachyose.

TABLE I

APPARENT SECOND ORDER CONSTANTS FOR INVERTASE INACTIVATION (k_{app})

A plot of the rate of inactivation as a function of epoxide concentration yielded the apparent second order constant for conduritol-B-epoxide. The k_{app} values for the other epoxides were estimated similarly, using a single concentration of the respective inhibitor (100 mM, in the case of epoxy-cyclohexane 25 mM).

Reagent	k_{app} ($min^{-1} \cdot mol^{-1} \cdot l$)
conduritol-B-epoxide 	2.06
epoxypropane 	0
1,2-epoxybutane 	0.013
2,3-epoxy-1-propanol 	0.020
epoxycyclohexane 	0.51

A plot of the rate of inactivation as a function of different epoxide concentration shows that the inactivation process is pseudo-first-order with respect to inhibitor concentration and yields the apparent second-order constant for conduritol-B-epoxide (Table I). The reaction order with respect to the inhibitor was determined from a plot of log $1/t_{0.5}$ against log [conduritol-B-epoxide] according to the procedure of Levy, Leber and Ryan [12]. This type of plot should give a straight line with a slope equal to n, the apparent number of molecules of inhibitor reacting with each active site of the enzyme to give an inactive enzyme-inhibitor complex. When the data of $t_{0.5}$, obtained for several epoxide concentrations as in Fig. 1, are plotted in this manner, a slope of 0.95 is obtained, suggesting that the reaction of one molecule of conduritol-B-epoxide per active site of yeast invertase is necessary for inactivation.

Specifity of inactivation

When invertase was incubated with epoxides other than conduritol-B-epoxide, which were not related to the natural substrates, little or no inactivation was found. The second-order rate constants are given in Table I. The much higher reaction velocity constant of conduritol-B-epoxide is likely to be due to the similarity of this compound with the substrate, and thus suggested that conduritol-B-epoxide may be an active site-directed inhibitor. The rather high rate of inactivation with epoxy-cyclohexane is probably due to its cyclic structure, which resembles the substrate more closely than other open chain epoxides. Similar observations were made during the reaction of sucrase-isomaltase with cyclic and open chain epoxides [5].

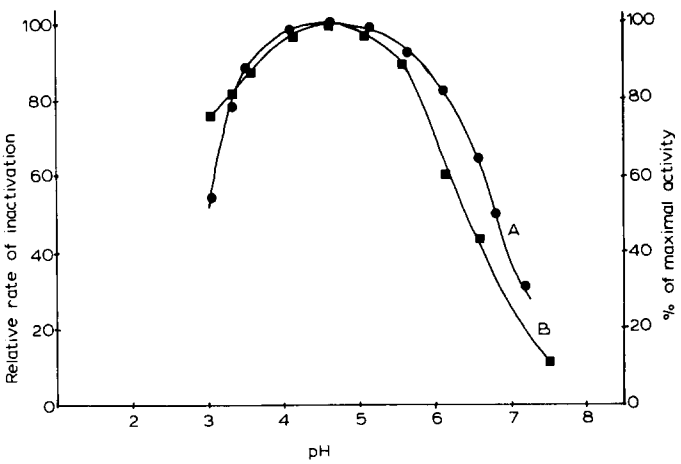


Fig. 2. Effect of pH on the inactivation rate of invertase by conduritol-B-epoxide. Curve A, (●) inactivation velocities were determined in a final volume of 0.13 ml, containing invertase (4 μM) and conduritol-B-epoxide (2.3 mM) at 37°C, and are reported as per cent of the maximal value at pH 4.6. The buffers used (50 mM) were sodium acetate, pH 3.0—5.6, and sodium maleate, pH 6.0 to 7.5. Values were corrected for activity losses due to instability of the enzyme at the respective pH. Curve B, (■) Effect of pH on invertase activity at 37°C, reported as per cent of the rate at the optimum pH 4.6. The buffers used were the same as in the inactivation reaction with the epoxide.

Protection by substrates

When raffinose or stachyose, which are substrates of yeast invertase [13], were added to the reaction mixture, the extent of inactivation was markedly reduced (Fig. 1). Maltose (600 mM), which is no substrate of the enzyme, gave no protection. This protection by substrates also suggested a reaction of conduritol-B-epoxide at the active site of the enzyme. The presence of 15 mM aniline, a non-competitive inhibitor of yeast invertase [3] gave no protection from inactivation.

pH-dependence of the inactivation rate

The pH-dependence of the inactivation rate with conduritol-B-epoxide was compared with the pH-activity curve of the same enzyme under identical conditions (Fig. 2). The close similarities of the two curves suggested the involvement of the same functional groups. Interpretation of the pH-dependence of inactivation by conduritol-B-epoxide according to Alberty and Massey [14] showed two dissociating groups in the enzyme with pK_a values 3.05 and 6.8 being important for the inactivation process.

Discussion

The inactivation of yeast external invertase with conduritol-B-epoxide followed pseudo-first-order kinetics and produced almost complete inactivation of yeast invertase activity (Fig. 1). This inactivation is due to reaction of this compound at the active site of the enzyme, as shown by the following observations:

(i) The data when plotted according to Levy, Leber and Ryan [12] suggested an irreversible binding of one mole of epoxide per active catalytic unit. The inactivation could not be reversed by dialysis of the inactivated enzyme.

(ii) Epoxides structurally unrelated to the substrate had little or no effect on invertase activity (Table I).

(iii) Substrates protected the enzyme from inactivation by conduritol-B-epoxide (Fig. 1).

It is well established that over a large pH range around neutrality epoxides must be protonated by an acid before they react with a nucelophile [15]. The pH-dependence of the inactivation with conduritol-B-epoxide showed two enzyme groups with pK_a 3.05 and 6.8 to be involved (Fig. 2). The enzyme group with pK_a 6.8 could be the acid catalyzer protonating the epoxide oxygen. The similarity with the pH-dependence of the enzyme activity suggests the identity of this group with the weak acid with pK_a 6.8—6.9, which was postulated to protonate the glycosidic oxygen in the substrate during enzymatic hydrolysis and is assumed to be a protonated histidine residue [16]. In view of the present knowledge on the mechanism of lysozyme, this acidic group might be a carboxylic group with an unusually high pK_a [19]. Since conduritol-B-epoxide resembles closely the structure of the glucopyranose part of the substrate sucrose, it is reasonable to assume that this compound binds at the enzyme surface rather at the glucosyl subsite than at the fructosyl subsite. If the Dreiding model of conduritol-B-epoxide is superimposed on that of sucrose, so that the C_2-C_3-C_4 atoms of glucopyranose correspond to the C_1-C_2-C_3 atoms of the epoxide (Table I), it becomes apparent that the epoxide oxygen and the

glycosidic oxygen in the substrate are in an identical position and can be protonated by the same group on the enzyme surface.

The enzymatic group with pK_a 3.05 could be, on the other hand, the nucleophile opening the protonated epoxide ring, which thereby remains covalently bound to the enzyme. This nucleophile could be in principle a sulfhydryl, tyrosyl, lysyl, methionyl group or a carboxylate, all of which were shown to react with epoxides in proteins [17]. In the case of invertase, tyrosyl, lysyl and sulfhydryl groups are not likely to be essential for activity [1] and therefore are probably not involved in the reaction with conduritol-B-epoxide. Although a reaction with a methionine residue cannot be excluded at the moment, we suggest by analogy with other carbohydrases [5,6,18] a carboxylate to be the reactive nucleophile with conduritol-B-epoxide. The group at the active site of yeast invertase with an apparent pK_a of 3 and involved in substrate binding has been supported to be a carboxylate [3,4]. Further studies are presently in progress to confirm the true nature of the modified amino acid residue.

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