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EXTERNAL YEAST β-FRUCTOSIDASE: STEREOSPECIFIC LABELING BY [^{3}H]CONDURITOL-B-EPOXIDE AND ISOLATION OF LABELED PEPTIDES FROM THE ACTIVE SITE

HERSZ BRAUN

Laboratorium für Biochemie, Eidgenössische Technische Hochschule Universitätsstr. 16, 8006 Zürich (Switzerland)

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Summary

1. External yeast β-fructosidase (β-D-fructofuranoside fructohydrolase EC 3.2.1.26) was labeled by [^{3}H]conduritol-B-epoxide, an active-site directed inhibitor of this enzyme. During the inactivation 1—2 mol of inhibitor were bound covalently per mol of enzyme. The labeled enzyme was digested with pepsin and ^{3}H-labeled peptides were isolated.

2. Conduritol-B-epoxide is a racemic mixture of 1-D-1,2-anhydro-*myo*-inositol and 1-L-1,2-anhydro-*myo*-inositol, but only the latter one was now shown to be the reactive component.

3. The label was released from the enzyme by mild alkaline treatment and identified by paper chromatography as 1-D- or L-*chiro*-inositol, which suggested that an ester linkage had been formed by reaction of the epoxide with a carboxylate group at the active site of the enzyme. Position and role of this carboxylate group are discussed.

Introduction

Recently we have shown the specific, irreversible inactivation of external yeast β-fructosidase (invertase, β-D-fructofuranoside hydrolase, EC 3.2.1.26) by conduritol-B-epoxide, an active-site directed inhibitor of this enzyme [1]. From the kinetic data obtained it was concluded that one mole of inhibitor reacts with one mole of active site. From the pH-dependence of the inactivation a carboxylate with pK_a 3.05 was suggested to be the reactive group.

The present communication describes the labeling of the enzyme with [^{3}H]-conduritol-B-epoxide, which enabled us to confirm the stoichiometry of the reaction. The enzyme, inactivated with [^{3}H]epoxide, was digested with pepsin and peptides carrying the labeled group were isolated. Conduritol-B-epoxide is a racemic mixture of 1-D-1,2-anhydro-*myo*-inositol and 1-L-1,2-anhydro-*myo*-

inositol, and only the latter one was now shown to be the reactive component. The label could be released from the enzyme by mild alkaline treatment at pH 9.0 and was identified as 1-D- or L-*chiro*-inositol. This further suggests that an ester linkage had been formed between a carboxylate group of the enzyme and the epoxide and allows a decision at which C-atom the epoxide ring of 1-L-1,2-anhydro-*myo*-inositol has been attacked by the enzyme's carboxylate group. The role of this group at the active site is discussed.

Methods and Materials

Materials

Yeast invertase was purchased from Boehringer GmbH Mannheim (Cat. No. 15067/1974/75), purified and the activity measured as before [1]. [^{3}H]Conduritol-B-epoxide was synthesized as described for the non-radioactive material [2,3]. *Myo*-[2-^{3}H]inositol (Radiochemical Centre, Amersham, U.K.) was diluted with *myo*-inositol to a specific activity of 0.166 mCi/mmol. Radioactivities were counted in Aquasol (NEN-Chemical, G.F.R.) in a liquid scintillation counter. The samples were corrected for quenching by adding [1,2-^{3}H]hexadecane in toluene (Amersham) as internal standard. 1-L-1,2-Anhydro-*myo*-inositol was prepared from 1-L-1-*O*-toluene-*p*-sulfonyl-*chiro*-inositol according to the method of Mercier et al. [4]. This compound was a gift from Professor Barnett, Nottingham.

Preparation of labeled enzyme and release of label

20 mg invertase in 0.7 ml sodium acetate (100 mM, pH 4.6) were incubated with 10 mM [^{3}H]conduritol-B-epoxide at 37°C for 7 h. The labeled enzyme was extensively dialysed against distilled water and finally lyophilized. The lyophilized material was incubated in 1.5 ml of 50 mM Na_2CO_3 buffer, pH 9.0, for 24 h at 37°C. A dialysis against 3 changes of 50 ml distilled water followed and the dialysate was desalted on a mixed bed ion-exchanger (Type V, Merck). After concentration by rotary evaporation the released radioactive material was chromatographed on Whatman 1 paper together with *scyllo*-inositol and 1-D-*chiro*-inositol (both generous gifts from Professor Th. Posternak) for 8 h in acetone/water (4 : 1, v/v). The migrations of the inositols were related to that of glucose, incorporated in the chromatogram. The non-radioactive inositols were detected by the silver nitrate/sodium hydroxide reagent according to the method of Anet and Reynolds [5].

Denaturation, reduction, cyanoethylation and pepsin digestion of labeled enzyme

Invertase (29.5 mg) labeled with [^{3}H]conduritol-B-epoxide (10 mM, 7 h at 37°C in 100 mM sodium acetate, pH 4.6) was dissolved in 2.5 ml of a solution containing 6 M guanidine · HCl, 2 mM EDTA and 0.5 M Tris/Cl, pH 7.0 and incubated under nitrogen at 50°C for 30 min. Then 15 mg dithiothreitol were added under nitrogen. After 4 h at room temperature the solution was adjusted to pH 8 with NaOH and reacted with 45 µl acrylonitrile for 20 min. It was then adjusted to pH 3 with formic acid and dialysed against water of the same pH. The solution was then adjusted to pH 2 with formic acid, 0.84 mg of pepsin

were added and the solution left at room temperature for 2 h. Finally the solution was brought to pH 4.5 with dilute ammonia and the peptides were lyophilized.

Isolation of labeled peptides

The residue obtained on lyophilization was dissolved in 1.5 ml 2% acetic acid and fractionated on a Bio-Gel P-10 column (1.6 × 90 cm) with 2% acetic acid as eluent, at a flow rate of 7 ml/h at 4°C. 5 fractions per h were collected and monitored for ^{3}H-content, absorbance at 280 nm and the neutral sugars (by the phenol/sulfuric acid method [6]).

Results and Discussion

Stoichiometry of labeling

Table I shows the correlation between the extent of enzyme inactivation and the incorporation of ^{3}H-labeled epoxide. After complete inactivation 1.44 mol of epoxide/mol of enzyme were incorporated, indicating incorporation of 1—2 mol of inhibitor per mol of enzyme, assuming a molecular weight of 270000 of the latter [7]. Incubation of denatured enzyme with ^{3}H-labeled epoxide under the same conditions gave no incorporation of radioactivity. The above ratio of incorporation could be explained by an additional unspecific reaction of the epoxide with some group(s) in the enzyme, not located at the active site, or by the presence of two active subunits in the enzyme. Since chromatography of native and denatured enzyme (in 6 M guanidine hydrochloride, see Methods) on Sepharose 4B gave the same elution volume in both cases (data not shown), there is no positive evidence for the presence of several smaller active subunits at the moment. This is in agreement with sedimentation velocity studies of external yeast β-fructosidase which gave no evidence for the existence of subunits [7]. Dansylation of the enzyme according to Hartley [8] showed only one NH_2-terminal in our case, namely leucine. This is consistent with but not proof for the absence of subunits.

Isolation of ^{3}H-labeled peptic peptides

Invertase labeled with [^{3}H]conduritol-B-epoxide was prepared and digested by pepsin as described in Methods. Upon fractionation of the peptic peptides

TABLE I

STOICHIOMETRY OF LABELING OF EXTERNAL YEAST β-FRUCTOSIDASE WITH [^{3}H]CONDURITOL-B-EPOXIDE

6.8 mg enzyme were incubated with 6 mM epoxide in 0.23 ml of 100 mM sodium acetate, pH 4.6, at 37°C. After 1, 2 and 7 h aliquots were taken, diluted with distilled water and excess reagent removed by exhaustive dialysis against distilled water.

Time of incubation (h)	Inactivation (%)	Ratio of epoxide to enzyme (mol/mol)
1	43	0.62
2	74	1.06
7	100	1.44

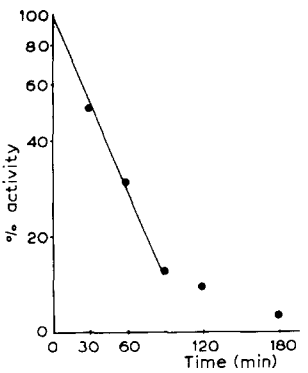

Fig. 1. Inactivation of invertase by 1-L-1,2-anhydro-*myo*-inositol. The incubation mixture at 37°C contained 50 mM sodium acetate buffer, pH 4.6, invertase (8.5 μM) and 1-L-1,2-anhydro-*myo*-inositol (6 mM).

on a Bio-Gel P-10 column, one major radioactive peak was observed, containing about 60% of the total radioactivity, and several minor radioactive peaks in the region, where the glycopeptides were eluted (figure not shown). These labeled peptides could be large peptides from the active site, which were digested incompletely by pepsin and/or they could be the result of some unspecific labeling of additional reactive groups as discussed above.

Inactivation of invertase by 1-L-1,2-anhydro-myo-inositol

A plot of invertase activity versus the time of reaction with this compound is shown in Fig. 1, indicating pseudo-first-order kinetics down to 11% activity. Exhaustive dialysis of the inactivated enzyme gave no reactivation. The apparent second order constants for invertase inactivation under identical conditions by conduritol-B-epoxide (the racemic mixture) and by 1-L-1,2-anhydro-*myo*-inositol were 2.06 and 4.19 $min^{-1} \cdot mol^{-1} \cdot l$, respectively. Since the k_{app} value for inactivation by the racemic mixture is almost exactly half of the value for inactivation by the L-enantiomer alone, we conclude that during inactivation of external yeast β-fructosidase by conduritol-B-epoxide the L-enantiomer is the reactive component and that the presence of the inactive D-enantiomer does not have any (protective) effect on inactivation by the other component.

Contrary to these results the β-glucosidases from almond [4] and *Aspergillus wentii* [9] and α-glucosidase from yeast [4] do not react with 1-L-1,2-anhydro-*myo*-inositol.

When invertase was incubated with epoxides other than conduritol-B-epoxide, only epoxy-cyclohexane gave a rather high rate of inactivation, probably due to its structure, which resembles the glucose moiety of the substrate sucrose more closely than other open-chain epoxides [1]. Now we tried also inactivation of the enzyme with epoxy-cyclopentane, which resembles the fructose moiety of the substrate. At pH 4.6 (100 mM sodium acetate) and 37°C no significant inactivation by 25 mM epoxy-cyclopentane was found.

Release of the label

Treatment of [^{3}H]epoxide-labeled enzyme in 50 mM Na_2CO_3 buffer, pH 9.0,

at 37°C for 24 h released all the radioactivity from the labeled enzyme. Under these conditions native external yeast β-fructosidase rapidly loses activity [10]. The lability of labels linked to peptides or denatured proteins through an ester bond under slightly alkaline conditions was noted before [11,12].

When examined by paper chromatography together with internal standards of *scyllo*-inositol or 1-D-*chiro*-inositol the released compound moved together with 1-D-*chiro*-inositol. All radioactivity was found in this spot. Thus the epoxide-label seems to be linked to the enzyme through an ester bond. Since no 1-L-*chiro*-inositol was available, co-crystallisation of the released compound together with L- and D-*chiro*-inositol could not be performed to decide which of the 2 enantiomers had been released from the enzyme. As 1-L-1,2-anhydro-*myo*-inositol was found to be the reactive component one would expect the released compound to be identical with 1-L-*chiro*-inositol (Fig. 2).

Since 1-L-1,2-anhydro-*myo*-inositol resembles closely the structure of the glucose moiety of the substrate sucrose and epoxy-cyclopentane contrary to epoxy-cyclohexane gave no inactivation, it is reasonable to assume that the L-enantiomer binds at the glucosyl-subsite at the enzyme surface. If the Dreiding model of the L-enantiomer is superimposed on that of sucrose, so that the C_2-C_3-C_4 atoms of glucopyranose correspond to the C_4-C_5-C_6 atoms of the L-enantiomer, it becomes apparent that the epoxide oxygen and the glycosidic oxygen in the substrate are in a position, in which they can be protonated by the same group of the enzyme surface (Fig. 2). In other orientations of bound L-enantiomer the epoxide oxygen would either come in a position between C_1 and C_2 of the glucosyl moiety with only two hydroxyl groups of both compounds being equatorial and the C atoms in identical chain conformation, or it would be on the 'wrong' side of the cyclohexane ring.

The attack of the enzyme's carboxylate group at C_2 of 1-L-1,2-anhydro-*myo*-inositol (corresponding to O_5 in the invertase · substrate complex) would lead to the formation of an ester of 1-L-*chiro*-inositol, while attack at C_1 would lead to an ester of *scyllo*-inositol (Fig. 2). From the results of paper chromatography

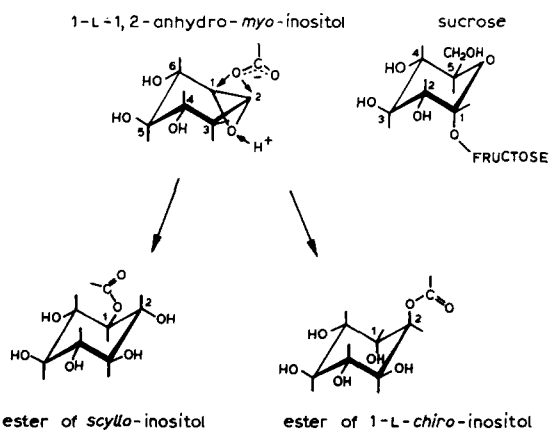

Fig. 2. Reaction of a carboxylate residue of external yeast β-fructosidase with 1-L-1,2-anhydro-*myo*-inositol. For a better presentation the equatorial OH at C_3 in the L-enantiomer is not shown.

of the released label, as discussed before, we conclude that the epoxide ring was attacked at C_2. By analogy one may suggest that in the enzyme-substrate complex the carboxylate group is located nearer to O_5 than to C_5 of the glucose moiety.

When sucrase-isomaltase, an enzyme which also splits sucrose, was affinity-labeled with conduritol-B-epoxide, an aspartate residue at each of the two active sites reacted with the 1-D-1,2-anhydro-*myo*-inositol enantiomer [12,13]. The label was released by treatment of the enzyme with hydroxylamine at pH 9.0 and identified as *scyllo*-inositol [13], which implied, that in sucrase-isomaltase the reactive aspartate residue was located nearer to C_1 than to O_5 of the glucose moiety of the substrate. This agreed well with its suggested involvement in catalysis by stabilizing the oxocarbonium intermediate (ref. 14 and Vanni, P. and Semenza, G., in preparation).

In yeast β-fructosidase rupture of the glycosidic linkage with sucrose as substrate occurs on the fructose side of the glycosidic oxygen [15]. At least two possible roles for the epoxide-reactive carboxylate group in yeast β-fructosidase can be envisaged:

(a) the carboxylate forming the ester bond with the label is also involved in the stabilization of the proposed intermediate oxocarbonium ion [16] (which would however imply a rotation of the carboxylate group to get into the proper position for such a stabilization).

(b) the carboxylate could be involved in substrate binding, e.g. by forming hydrogen bonds to the -OH groups of the glucose or fructose moieties.

Further purification of the ^{3}H-labeled peptides to identify the sequence around the labeled residue is presently in progress.

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