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STEREOSPECIFIC RING OPENING OF CONDURITOL-B-EPOXIDE BY AN ACTIVE SITE ASPARTATE RESIDUE OF SUCRASE-ISOMALTASE

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Summary

Conduritol-B-epoxide inactivates sucrase-isomaltase (sucrose α-glucohydrolase, EC 3.2.1.48 - dextrin 6-α-glucohydrolase, EC 3.2.1.10) irreversibly with incorporation of 1 mol inhibitor/mol subunit, the affinity label being bound in both subunits to a β-carboxyl group of an aspartic acid (Quaroni, A. and Semenza, G. (1976) J. Biol. Chem. 251, 3250—3253). Conduritol-B-epoxide is a racemic mixture of 1-L-1,2-anhydro-*myo*-inositol and 1-D-1,2-anhydro-*myo*-inositol, but only the latter one is the reactive component, since 1-L-1,2-anhydro-*myo*-inositol alone did not inactivate the enzyme. After inactivation by 1-D-1,2-anhydro-*myo*-inositol the label was released by hydroxylamine and identified as *scyllo*-inositol. One can decide now which C atom of the epoxide ring has been attacked by the enzyme's aspartate residue. This explains why only the D-enantiomer is the reactive species and provides further information about the role of the carboxylate residue during enzymic hydrolysis.

Introduction

The sucrase-isomaltase (sucrose α-glucohydrolase, EC 3.2.1.48 - dextrin 6-α-glucohydrolase, EC 3.2.1.10) complex from rabbit small intestine is a glycoprotein of approximate molecular weight 220 000 and consists of two subunits, which can be separated by mild alkaline treatment [1], citraconylation [2] or trypsin digestion [3]. So far, only carboxylic or carboxylate groups have been found to be essential for catalytic activity [4].

Conduritol-B-epoxide, an active site-directed inhibitor, inactivates both subunits irreversibly with incorporation of 1 mol inhibitor/mol subunit [5]. The site of attachment of the affinity label to both subunits is the β-carboxyl group

of an aspartic acid [6]. Conduritol-B-epoxide is a racemic mixture of the two enantiomers 1-L-1,2-anhydro-*myo*-inositol and 1-D-1,2-anhydro-*myo*-inositol. It was shown now that only the latter one is the reactive component. After inactivation of the enzyme complex this label can be removed by treatment with hydroxylamine [5] and was identified in the present communication as *scyllo*-inositol. Thus one can decide at which C atom the epoxide ring of 1-D-1,2-anhydro-*myo*-inositol has been attacked by the enzyme's carboxylate group. This gives further evidence for the participation of this group in the catalytic mechanism of the enzyme.

Materials and Methods

Materials. The sucrase-isomaltase complex was prepared from rabbit small intestine by papain solubilisation as previously described [7]. Conduritol-B-epoxide was synthesized as described by Legler [8] with minor modifications [5]. [^{3}H]Conduritol-B-epoxide was synthesized as described for the non-radioactive material; *myo*-[2-^{3}H]inositol was obtained from the Radiochemical Centre, Amersham and diluted with inositol to a specific activity of 0.166 Ci/mol. 1-L-1,2-Anhydro-*myo*-inositol was a gift of Dr. J.E.G. Barnett, *scyllo*-inositol and 1-D-*chiro*-inositol were a gift from Professor Th. Posternak. Radioactivities were counted in Aquasol (NEN Chemical, Germany) in a liquid scintillation counter.

Preparation of labeled enzyme. 19.3 mg sucrase-isomaltase in 2 ml sodium maleate buffer (100 mM, pH 6.8) were incubated with 40 mM [^{3}H]conduritol-B-epoxide at 37°C for 44 h. At the end of the reaction no enzymatic activity could be detected. The labeled enzyme was extensively dialyzed against several changes of distilled water and finally lyophilized.

Release of label and chromatography. The lyophilized material was incubated in 2 ml 0.5 M hydroxylamine in 50 mM sodium carbonate buffer, pH 9.0, for 24 h at 37°C [5]. The it was dialysed against three changes of 50 ml distilled water. The diffusate was desalted on a mixed bed ion exchanger (Type V, Merck) and then concentrated by rotary evaporation. The released radioactive material was chromatographed on Whatman No. 1 paper together with *scyllo*-inositol and 1-D-*chiro*-inositol for 8 h in acetone/water (4 : 1, v/v). Glucose was incorporated in the chromatogram and the migrations of the inositols related to that of glucose. The non-radioactive inositols were detected by the $AgNO_3$/NaOH reagent according to Anet and Reynolds [9].

Results and Discussion

Conduritol-B-epoxide, as synthesized according to Legler [8], is a racemic mixture of the two enantiomers, 1-D-1,2-anhydro-*myo*-inositol and 1-L-1,2-anhydro-*myo*-inositol (Fig. 1). The latter enantiomer, when tested alone, failed to inactivate either sucrase or isomaltase (30 mM, pH 6.8, 37°C). Under these conditions, the racemic mixture of conduritol-B-epoxide used in the previous [5] and present work totally inactivated isomaltase within 15 min and sucrase within 150 min. We conclude, therefore, that the reactive form must be the 1-D-1,2-anhydro-*myo*-inositol. Due to the very small amount of 1-L-1,2-anhy-

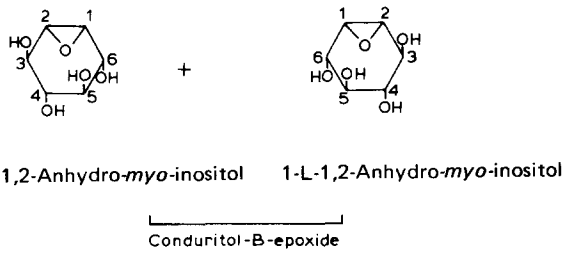


Fig. 1. The two enantiomers of conduritol-B-epoxide.

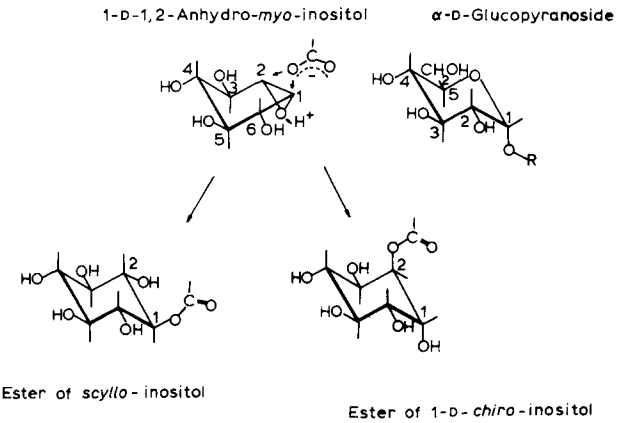
dro-*myo*-inositol available, we have not investigated whether the presence of this inactive enantiomer has any (protective) effect on the inactivation of sucrase or isomaltase by the other component of the racemic mixture of conduritol-B-epoxide.

It has been shown that conduritol-B-epoxide reacts with the glucosyl subsite of sucrose-isomaltase [5]. If the Dreiding model of 1-D-1,2-anhydro-*myo*-inositol is superimposed on that of an α-glucopyranoside, a substrate of the enzyme complex, so that the C_2-C_3-C_4-C_5 atoms of the glucopyranose correspond to the C_6-C_5-C_4-C_3 atoms of the epoxide (Fig. 2A), it becomes apparent that the epoxide oxygen and the glycosidic oxygen in the substrate are in an almost identical position. They could thus be protonated by the same group at the active site. After protonation, an exo-attack by the enzyme's carboxylate group can occur on the epoxide ring either at C_1 or C_2 (Fig. 2A). Attack at C_1 should lead to the formation of an ester of *scyllo*-inositol (Fig. 2A), while attack at C_2 should bring about formation of an ester of 1-D-*chiro*-inositol (formerly D- or (+)-inositol). Sucrase-isomaltase, fully inactivated by [^{3}H]conduritol-B-epoxide (for conditions see Methods and Materials), was treated at pH 9.0 with hydroxylamine. Under the conditions described in Table I 90% of the radioactivity was released from the labeled enzyme. When examined by paper chromatography together with internal standards of *scyllo*-inositol or 1-D-*chiro*-inositol the released compound was identified as *scyllo*-inositol. All radioactivity was found in this compound.

Non-enzymic ring opening of epoxides follows in most cases the rule of Fürst and Plattner [10] to give predominantly the axial isomer. This is also true for hydrolysis of conduritol-B-epoxide with 0.25 M H_2SO_4, which gives more 1-D-*chiro*-inositol and 1-L-*chiro*-inositol than *scyllo*-inositol [11]. With sucrase-isomaltase only the less favourable equatorial opening to *scyllo*-inositol occurs, apparently due to the unique position of the enzyme's aspartate group. We conclude that the epoxide ring was attacked by the aspartate group at C_1 (Fig. 2A).

This conclusion also provides a straight-forward explanation why 1-L-1,2-anhydro-*myo*-inositol failed to inactivate sucrase-isomaltase as mentioned above. If the Dreiding model of this compound is superimposed on that of an α-glucopyranoside, so that the C_2-C_3-C_4 atoms of glucopyranose correspond to the C_4-C_5-C_6 atoms of 1-L-1,2-anhydro-*myo*-inositol (these three OH groups of both compounds being equatorial and the C atoms being in identical chain conformation), the epoxide ring oxygen is situated between O_5 and C_5 of the glucopyranose. In other orientations of bound 1-L-1,2-anhydro-*myo*-inositol the

A



B

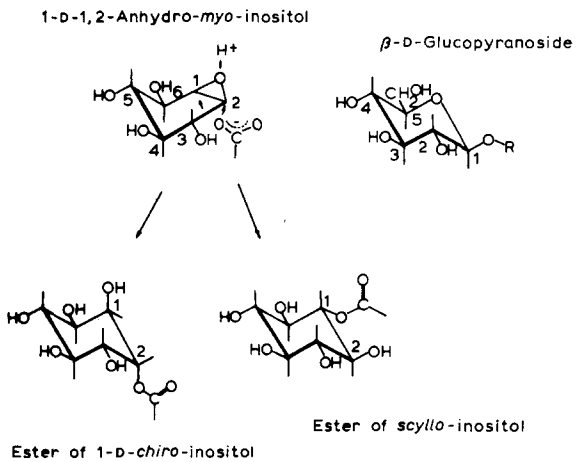


Fig. 2. Reaction of 1-D-1,2-anhydro-*myo*-inositol with the aspartic acid residue of A, sucrase-isomaltase; B, β-glucosidase of *A. wentii*.

TABLE I

TREATMENT OF LABELED ENZYME WITH HYDROXYLAMINE

The labeled enzyme (19.3 mg) fully inactivated with radioactive conduritol-B-epoxide and dialyzed exhaustively against distilled water, was freeze-dried and treated with hydroxylamine as indicated for 24 h at 37°C. After incubation the solutions were dialyzed again against distilled water and the radioactivity bound to the enzyme counted.

Treatment	Radioactivity incorporated (cpm)	Ratio of epoxide to complex (mol/mol)
—	54 164	1.96
NH_2OH (0.5 M), sodium carbonate buffer (50 mM, pH 9.0)	5 400	

epoxide oxygen would either be on the "wrong" side of the cyclohexane ring or it would come in a position between C_1 and C_2 of the glucosyl moiety with only two hydroxyl groups at C_3 and C_4 left for an interaction with the corresponding groups on the enzyme. Even if the epoxide oxygen is now protonated by the same group on the enzyme as the glycosidic oxygen in the substrate, the carboxylate group of the enzyme could not attack the epoxide ring, since apparently it is closer to C_1 in 1-D-1,2-anhydro-*myo*-inositol and is too far from C_1 and C_2 in 1-L-1,2-anhydro-*myo*-inositol. The exact position of the epoxide ring is thus of paramount importance for enzyme inactivation. The fact that it has to be located in a position corresponding to O_5 and C_1 of the glucosyl moiety of the substrate agrees with and supports further the suggestion of an oxocarbonium ion being formed during catalysis in this position [12].

As far as the enzyme-substrate complex is concerned, one may suggest by analogy that the aspartate group is located nearer to C_1 than to O_5 of the glucose moiety of the substrate. However, in the X-ray model of lysozyme with a hexasaccharide bound to its active site, the nearest oxygen of Asp-52 is about 3 Å from the C_1 atom of substrate residue "D" and about the same distance from the ring oxygen O_5 of that residue. It is probably involved in catalysis by stabilizing the oxocarbonium intermediate through electrostatic interaction [13], in a way similar to that subsequently suggested for sucrase-isomaltase [12].

The ion pair oxocarbonium-COO^- is likely to convert reversibly into a covalent intermediate. The suggestive evidence for this is rather strong in the case of *Escherichia coli* β-galactosidase, as pointed out by Sinnott and Souchard [14]. In the case of sucrase-isomaltase it can be calculated that the "glucosyl-enzyme" (the species left after departure of the aglycon) must exist for 10 μs or longer, a time much exceeding the lifetime of non-stabilised carbonium ions (Vanni, P., Luisi, P. and Semenza, G., unpublished).

Conduritol-B-epoxide also inactivates a β-glucosidase from *Aspergillus wentii* with 1 mol epoxide bound per mol of enzyme [15]. In this case the C_2-C_3-C_4-C_5 atoms of a β-glucopyranoside correspond to the C_3-C_4-C_5-C_6 atoms of 1-D-1,2-anhydro-*myo*-inositol (Fig. 2B). The group reacting at the active site of the enzyme was identified as an aspartate residue and the bound inactivator could be released as 1-D-*chiro*-inositol on treatment with hydroxylamine [15,16]. This means that the epoxide ring has been opened at C_2 by the carboxylate group (Fig. 2B). A reaction with diaxial opening of the epoxide ring was also found when β-glucosidase from sweet almonds was inactivated with 6-deoxy-6-bromoconduritol-B-epoxide [17]. Thus both in sucrase-isomaltase (an α-glucosidase) and in β-glucosidase from *A. wentii* and sweet almonds the epoxide-reactive aspartate group is nearer to C_1 than to O_5 in the glucosyl moiety of the substrate. With both enzymes the inhibitor appears to be bound in a way that permits protonation of the epoxide oxygen from the same side as the glucosyl oxygen of the substrate: with α-glucosidase from "below" and with β-glucosidase from "above" the cyclohexane or pyranose ring.

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