Affinity Labeling of the Active Sites in the Sucrase-Isomaltase Complex from Small Intestine*

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SUMMARY

Conduritol-B-epoxide, a compound structurally related to the substrates of sucrase and isomaltase is an active-site-directed inhibitor. The inactivation of sucrase and isomaltase can be complete, is irreversible, and is prevented by the presence of reversible competitive inhibitors and substrates. During the inactivation 1 mole of inhibitor is bound covalently per mole of subunit. The release of the label by treatment with hydroxylamine and the apparent pKₐ values of the reactive groups suggest that a carboxylate is present in the active sites of both subunits.

Fractionation of the tryptic digest of the sucrase-isomaltase complex labeled with [3H]conduritol-B-epoxide shows that the label is bound to a glycopeptide, thus locating the active sites of sucrase and isomaltase near a hydrophilic region.

The sucrase-isomaltase complex from rabbit small intestine is a pair of digestive disaccharidases which has been localized in the brush border membrane of the enterocytes by different approaches (1, 2) (for review see Ref. 3). It is a glycoprotein consisting of two similar subunits (4), one splitting sucrose and maltose and the other isomaltose, maltose, and palatinose. They can be separated by mild alkaline treatment (4) or by reaction with citraconic anhydride. The principal reason for our interest in this enzymic complex is its role in sugar translocation both in natural (5, 6) and in artificial lipid membranes (7).

In an attempt to identify the groups present in the active sites of the complex, a substrate-like alkylating reagent was sought. Epoxides react with carboxylates in the presence of an acidic catalyst (8). This reaction is in competition with the ring opening to form the corresponding glycol, but the latter reaction is relatively slow above pH 3. Hence an epoxide propens to react with carboxylates carrying the reactive groups. Some data have been presented in a preliminary form (16).

EXPERIMENTAL PROCEDURE

Materials

The sucrase-isomaltase complex was prepared from rabbit small intestine as previously described (17). Conduritol-B-epoxide was synthesized as described by Legler (9) with minor modifications: the intermediate DL-(1,3)-RS-cyclohexentetrol-1,2,5,6-tetraacetate was deacetylated by refluxing in methanol in the presence of catalytic quantities of sodium methoxide; in the step conduritol-B-\(\rightarrow\) conduritol-B-epoxide, \(p\)-nitroperbenzoic acid gave better yields than perbenzoic acid. The final product was recrystallized twice from absolute ethanol. Melting point was 155-157° (uncorrected) (literature 156-159°) (9).

\[\text{C}_\text{H}_\text{O}_\text{O}_\text{O}\]

Calculated: C 44.44% H 6.22%

Found: C 44.38% H 6.14%

To prepare the tritiated compound, 50 mg of conduritol-B-epoxide were irradiated with tritium gas (Wilsbach method; code TR.2/331 (L.O.M31305)) in aqueous solution at the Radiochemical Centre (Amersham). The crude solution was freeze-dried and the residue was purified by paper chromatography on Whatman No. 3MM paper (descending chromatography for 18 hours with pyridine-ethylacetate-water 1:3:6 by volume), using cold conduritol-B-epoxide as a standard. Silver nitrate-NaOH reagent (18) was used for staining on a part of the chromatogram. The radioactive compounds were located on the paper chromatograms using a "Radiochromatogram Spark Chamber" (Birehove Instruments, England). The radioactive compound was eluted with water and rechromatographed under the same conditions. Since it was found that a high molecular weight contaminant, unlabelled by the silver nitrate-NaOH reagent, traveled together with the epoxide, the final purification was obtained by gel chromatography on a Bio-Gel P-30 (Calbiochem) column (90 x 1.6 cm) with water as developer. (Flow rate 5.6 ml per hour; fractions of 2.8 ml were

* This work was supported by Swiss National Science Foundation, Berne. Dedicated to Prof. O. Hoffmann-Ostenhof on the occasion of his 60th birthday.

collected and tested for radioactivity.) The conduritol-B-epoxide was finally obtained by freeze-drying the pooled samples. The specific activity was 0.197 mCi per mg. 

Phytohemagglutinin P (from Phaseolus vulgaris) was purified according to Ref. 19. Erythrina and leuca-phytohemagglutinins were purified as described in Ref. 20. Phytohemagglutinin A4 (from Dolichos biflorus) was obtained from Hyland (Costa Mesa, Calif.). Myo-epoxide,3 prepared according to Ref. 31, was a generous gift of Prof. J. Deshusses (Geneva). CNBr-activated isomaltase activity. When the activity was too low, larger volumes were used for sucrase activity determination and 0.3 ml for tube before and after collection of the sample. Aliquots of 0.1 ml were run at 37°C. At different time intervals 50-μl aliquots were withdrawn and applied to a Bio-Gel P-30 column (0.5 cm X 7 cm). Elution was achieved with 1.2 ml of lithium maleate buffer (50 mM, pH 6.8). The enzymatic activity was determined on the eluate (the volume of which was determined by weighing the test tube before and after collection of the sample). Aliquots of 0.1 ml were used for sucrase activity determination and 0.3 ml for isomaltase activity. When the activity was too low, larger volumes were used.

**Methods**

**Inhibition Studies**—A typical reaction mixture contained in a total volume of 0.4 ml, sodium maleate buffer (100 mM, pH 6.8), 0.5 mg of furanose-isomaltase complex (5 units of sucrase), and conduritol-B-epoxide in varying concentrations. The reaction was run at 37°C. At different time intervals 50-μl aliquots were withdrawn and applied to a Bio-Gel P-30 column (0.5 cm X 7 cm). Elution was achieved with 1.2 ml of lithium maleate buffer (50 mM, pH 6.8). The enzymatic activity was determined on the eluate (the volume of which was determined by weighing the test tube before and after collection of the sample). Aliquots of 0.1 ml were used for sucrase activity determination and 0.3 ml for isomaltase activity. When the activity was too low, larger volumes were used.

**Enzymatic Activity**—Sucrase and isomaltase activities were determined at pH 6.8 as described elsewhere (22) with sucrase and palatinose (33 mM) as the substrate, respectively. The glucose liberated was determined with the hexokinase reagent (Glucose UV Test, Boehringer, Mannheim). Protein was measured according to Lowry et al. (23).

**Neutral sugars** were determined by the phenol-sulfuric acid method (25). Reduction and carboxymethylation of the protein samples after denaturation in 6 M guanidine HCl was performed as previously described (17) with one modification: 0.1 M diithiocrythitol was used in place of 2-mercaptoethanol. Tryptic digestion of denatured, reduced, and carboxymethylated samples and fingerprints of the tryptic peptides were made as described elsewhere (4). Radioactivities were determined in a Beckman LS-335 liquid scintillation system. Liquid samples were counted in a butyl-PBD toluene scintillator (5 g of butyl-PBD, 80 g of naphthalene, 400 ml of 2-methoxyethanol to 1 liter with toluene) or Aquasol (NEN Chemical, Germany). The samples were corrected for quenching by adding [1,2-3H]hexadecane in toluene (Amersham, England) as internal standard. Fingerprints were cut into pieces (2 X 2 cm) and counted in a 0.5 ml, 2.0 mCi beaker.

**Phytohemagglutinin P**—The CNBr-activated Sepharose 4B (6 g dry weight) was suspended in cold 10-3 M HCl and washed with 1 liter of the same solution. The phytohemagglutinin P (70 mg) was dissolved in 0.1 M NaHCO3 buffer containing 0.5 M NaCl (50 ml), mixed with the gel, and shaken mildly overnight at 4°C. Unbound material was washed away with coupling buffer and any remaining active groups reacted with 1 M ethanolamine at pH 8 for 3 hours at 4°C. The gel was then washed with 0.1 M acetate buffer (pH 4), 0.1 M borate buffer (pH 8) (both containing 1 M NaCl), and finally with 10 mM K+ phosphate buffer (pH 6.8). Sepharose-linked A4 lectin from Dolichos biflorus. Lectin (40 mg) was coupled to Sepharose 4B as above.

**Precipitin Reaction**—Lectin (0.05 ml) (1 mg per ml in 100 mM Na+ maleate buffer, pH 6.8) was added to an equal volume of sucrase-isomaltase complex serially diluted with water. The temperature and length of the incubation were different for different lectins: erythro- and leuco-phytohemagglutinins P were incubated for 96 hours at 4°C; concanavalin A at 24°C for 24 hours. At the end of the incubation, in every case, the precipitate formed was spun down and washed twice with 0.9% NaCl. The protein was determined in the precipitate by the Lowry method (23).

**RESULTS**

**Kinetics of Inactivation**—As pointed out by Webb (26), the inactivation of an enzyme by irreversible inactivators can be represented in the following way: $E + I \rightarrow E + I \rightarrow EI$. When the dissociation of the complex can be neglected, the reaction can be simply considered of second order: $E + I \rightarrow EI$ and the extent of inactivation, $i$, is given by: $i = e^{-kIt}$. In all the experiments reported the concentration of inactivator (conduritol-B-epoxide) was much higher than that of the enzyme, hence pseudo-first order kinetics were expected and found. In Figs. 1A and 2A, the plots of ln $A_0/A_1$ (where $A_0$ is the initial

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2 The abbreviations used are: conduritol-B-epoxide, 1,2-epoxy-3,5/4,6 tetrahydroxycyclohexane; myo-epoxide, 2-methylene oxide-1,3,5/4,6 pentahydroxycyclohexane; butyl-PBD, 2-(4'-tert-butylyphenyl)-5-(4'-biphenylyl)-1,3,4-oxadiazol.

3 The abbreviations used are: conduritol-B-epoxide, 1,2-epoxy-3,5/4,6 tetrahydroxycyclohexane; myo-epoxide, 2-methylene oxide-1,3,5/4,6 pentahydroxycyclohexane; butyl-PBD, 2-(4'-tert-butylyphenyl)-5-(4'-biphenylyl)-1,3,4-oxadiazol.

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Fig. 1. A, inactivation of the sucrose subunit in the complex by conduritol-B-epoxide. Each incubation at 37°C contained Na+ maleate buffer (100 mM, pH 6.8), sucrose-isomaltase complex (5 X 10-3 mg), and conduritol-B-epoxide: 1, 10 mM; 2, 20 mM; 3, 30 mM; 4, 40 mM; 5, 50 mM. At the indicated times samples were withdrawn and the enzymatic activity determined as described under "Methods." B, inactivation velocity (mean of 3 sets of experiments ± S.D. calculated from the plots in A) as a function of inactivator concentration.
FIG. 2. A, inactivation of the isomaltase subunit in the complex by conduritol-B-epoxide. Each incubation at 37° contained Na⁺ maleate buffer (100 mM, pH 6.8), sucrase-isomaltase complex (5 × 10⁻³ mM), and conduritol-B-epoxide: 1, 0.5 mM; 3, 2.5 mM; 4, 5 mM. At the times indicated samples were withdrawn for determination of enzymatic activity. B, inactivation velocity (mean of 3 sets of experiments ± S.D. calculated from the plots in A) as a function of inactivator concentration. The linearity of the plots obtained for both sucrase and isomaltase subunits suggests the validity of the above assumptions. The inactivation velocities increased linearly (Figs. 1B and 2B) with the concentration of inactivator and from these plots it was possible to calculate the constants of inactivation.

In order to investigate the specificity of the inactivation, the sucrase-isomaltase complex was incubated with different epoxides not structurally similar to the natural substrates. Very little inactivation was found after 1 hour in every case. Table I reports the second order constants. The effectiveness of conduritol-B-epoxide as an inactivator is demonstrated by its reaction constants being much higher than those of the other epoxides.

Only one other compound structurally related to glucopyranosides, i.e. myo-epoxide, did react at appreciable rates, but still about 3 times (for sucrase) or 35 times (for isomaltase) less efficiently than conduritol-B-epoxide. This high specificity suggested conduritol-B-epoxide to be an active site-directed inhibitor. The same conclusion was reached when different substances, all known to have an affinity for the active sites of the sucrase-isomaltase complex, were added to the reaction mixture (Fig. 3). In all cases the extent of inactivation was markedly reduced and in the presence of 10 mM Tris (a fully competitive inhibitor presumed to bind to the glucosyl subsite (27)) a complete protection of the enzymic activity was achieved, even after three

<table>
<thead>
<tr>
<th>S</th>
<th>k_app (min⁻¹ mol⁻¹)</th>
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<tbody>
<tr>
<td>CONDURITOL-B-EPOXIDE</td>
<td>0.81</td>
</tr>
<tr>
<td>MYO-EPOXIDE</td>
<td>0.20</td>
</tr>
<tr>
<td>EPOXY-PROPANE</td>
<td>0.0055</td>
</tr>
<tr>
<td>1,2-EPOXY-BUTANE</td>
<td>0.01</td>
</tr>
<tr>
<td>EPOXY-CYCLOHEXANE</td>
<td>0.012</td>
</tr>
<tr>
<td>2,3-EPOXY-1-PROPANOL</td>
<td>0.0022</td>
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FIG. 4. A, effect of pH on the rate of inactivation of the sucrase-isomaltase complex by conduritol-B-epoxide. Inactivation velocities were determined at 30 mM (for sucrase) and 5 mM (for isomaltase) conduritol-B-epoxide under the same conditions as in Figs. 1 and 2 and are reported as per cent of the maximal value, observed at pH 9; O—O sucrase; X—X isomaltase. B, semi-log plots similar to Figs. 1A and 2A were drawn for different pH values in the range 4.5 to 9. The inactivation rates derived from them are plotted in Fig. 4A as per cent of the maximal value found at pH 9. For the two enzymatic activities of the sucrase-isomaltase complex, two close sigmoidal curves were obtained, suggesting that identical functional groups are important in the reaction of the two subunits with the epoxide. The apparent dissociation constants of the reactive groups were inferred from the pH values giving half-maximal inactivation rates. From the results obtained in three different sets of experiments, apparent pKₐ values of 5.3 ± 0.1 and 5.5 ± 0.2 were calculated for the isomaltase and sucrase reactive groups, respectively. For comparison, the effect of pH on the enzymatic activities, reported as per cent of the rate at optimum pH, is also shown in Fig. 4B.

Effect of Na⁺ on Inactivation Rates—The reaction mechanism of rabbit sucrase has been identified as Ping pong Bi, reducing to ordered Uni Bi when water alone is the second substrate (27). At pH 6.8, Na⁺ apparently activates the appearance of the first reaction product which results, for a rapid equilibrium, in an increase of kcat with no effect on the apparent Kₐ (22, 27, 28). The effect of this cation on isomaltase, although less extensively investigated, appears to be similar to that on sucrase. K⁺ activates sucrase less than Na⁺, but has a higher affinity for it (29). The affinity of Li⁺ for sucrase is lower than that of either Na⁺ or K⁺. Therefore, the rates of inactivation of both sucrase concentration of K⁺ was maintained constant (100 mM) by addition of KCl.
and isomaltase were studied in the presence of either Na⁺ or Li⁺ buffers. The rate of inactivation of sucrase was higher in the presence of Na⁺ and the degree depended on the pH, ranging from about 10% at pH 6.8 to 35% at pH 5.0, with an almost linear increase. Little or no effect of Na⁺ could be detected on the rate of inactivation of isomaltase at the pH values tested. These results again suggest that the same functional groups are involved in the sucrase activity and in the reaction of the enzyme with conduritol-B-epoxide.

Protection Constants—If an inhibitor acts specifically at the active site(s) of an enzyme, other substances (substrate, competitive inhibitors) can, by competing for the active site(s), reduce the inactivation velocity in a measure which depends on their concentration and their affinities for the active site.

Protection constants were calculated assuming that the protection functions were horizontal hyperbolae. Accurate values were derived from double reciprocal plots of the data shown in Fig. 5.

**Stoichiometry of labeling of sucrase-isomaltase complex by conduritol-B-epoxide**

The indicated amounts of enzyme were incubated in 1 ml of 100 mM Na⁺ maleate buffer, pH 6.8, at 37°C with the reported concentrations of epoxide and competitive inhibitors for the indicated times. Excess reagent was removed by exhaustive dialysis (4 days) against 50 mM Na⁺ maleate buffer, pH 6.8, and then distilled water, both containing a drop of toluene. After freeze-drying, the residues were dissolved in water and percolated through a Bio-Gel P-100 column (0.9 × 60 cm). Elution was achieved with ammonium carbaminate 0.01 M and the protein peak was localized by its optical density at 280 nm. The pooled fractions (corresponding to the void volume) were freeze-dried and their radioactivity was counted using Aquasol as scintillator.

**Fig. 5, A and B.** Determination of the protection constants (for sucrase activity) for glucose and Tris. Each incubation mixture at 37°C contained 100 mM Na⁺ maleate buffer, pH 6.8; sucrase-isomaltase complex, 5 × 10⁻⁶ mol; conduritol-B-epoxide, 10 mM; and the indicated concentrations of glucose or Tris, respectively. The inactivation rates were determined as indicated in Fig. 1. The protection constants indicated in the figures were calculated assuming that the protection functions were horizontal hyperbolae. Accurate values were derived from double reciprocal plots of the data shown in Fig. 5.

**Table II**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Radioactivity</th>
<th>Inactivation</th>
<th>Ratio of epoxide to complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm</td>
<td>%</td>
<td>mg</td>
</tr>
<tr>
<td>1. 10</td>
<td>18076</td>
<td>98%</td>
<td>10</td>
</tr>
<tr>
<td>2. 10</td>
<td>17881</td>
<td>100%</td>
<td>10</td>
</tr>
<tr>
<td>3. 10</td>
<td>2210</td>
<td>21%</td>
<td>10</td>
</tr>
<tr>
<td>4. 10</td>
<td>5430</td>
<td>50%</td>
<td>10</td>
</tr>
</tbody>
</table>

* (1) Conduritol-B-epoxide, 10 mM, 24 hours; (2) conduritol-B-epoxide, 10 mM, 48 hours; (3) conduritol-B-epoxide, 10 mM; Tris, 15 mM; 10 hours; (4) conduritol-B-epoxide, 10 mM; glucose, 200 mM; 10 hours.

**Differential Labeling of Sucrase and Isomaltase Subunits**—The kinetic studies showed a large difference in reactivity between the two subunits of the sucrase-isomaltase complex with conduritol-B-epoxide. To differentially label the sucrase and isomaltase active sites in the complex and to study the stoichiometry of labeling of each subunit, two experiments were performed. In the first, 50 mg of sucrase-isomaltase complex, at higher concentration than usual, were treated with a low concentration of radioactive epoxide (Fig. 6A). In the second experiment, the same amount of enzyme reacted with 5 mM cold epoxide for 1
hour and the reaction was stopped by dialysis overnight. The enzyme was again treated with 4 mM radioactive epoxide (Fig. 6B). Excellent correlation was obtained between the loss of enzymatic activities in the two experiments and the incorporation of radioactivity. Apparently one moiety of epoxide is incorporated per subunit of the sucrase-isomaltase complex. The enzyme obtained from the first experiment was almost exclusively labeled at the isomaltase active site, while the second experiment resulted in a major labeling of the sucrase subunit (rate of sucrase per isomaltase labeling about 3). Hereafter, they will be referred to as lots A and B, respectively.

**TABLE III**

*Treatment of labeled sucrase-isomaltase complex with hydroxylamine*

The labeled enzyme (2 mg per experiment) fully inactivated with radioactive conduritol-B-epoxide and dialyzed for 3 days against distilled water, was freeze-dried, and treated as indicated for 24 hours at 37°. After incubation the solutions were subjected again to exhaustive dialysis against distilled water and the radioactivity counted after recovery of the enzyme by lyophilization.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Radioactivity lost*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₂OH (0.5 M), Na⁺ carbonate buffer (50 mM, pH 9.0)</td>
<td>90 ± 4</td>
</tr>
<tr>
<td>NH₂OH (0.5 M), Na⁺ carbonate buffer (50 mM, pH 9.0), urea 8 M</td>
<td>92 ± 6</td>
</tr>
<tr>
<td>Na⁺ carbonate buffer (50 mM, pH 9.0)</td>
<td>5 ± 10</td>
</tr>
</tbody>
</table>

* Average of 4 experiments ± SE.

**FIG. 6.** A, preferential labeling of isomaltase subunit in the sucrase-isomaltase (S-I) complex. The incubation mixture at 37° contained 100 mM Na⁺ maleate buffer, pH 8.8; sucrase-isomaltase complex, 0.1 mM; [3H]conduritol-B-epoxide, 4 mM. Activities were determined as described under "Methods." At the given times, samples (25 μl) were withdrawn, dialyzed against distilled water, freeze-dried, and counted for radioactivity. After 4 hours solid cold conduritol-B-epoxide was added to bring its concentration to 100 mM. The reaction was stopped after 5 hours by exhaustive dialysis at 4° against distilled water (3 days) and the labeled enzyme was recovered by lyophilization. ○—○, per cent of sucrase activity lost; △—△, per cent of isomaltase activity lost; ▲—▲, moles of inactivator per mole sucrase-isomaltase complex. B, preferential labeling of sucrase subunit in sucrase-isomaltase (S-I) complex. Sucrase-isomaltase complex (0.1 mM) was incubated at 37° in 100 mM Na⁺ maleate buffer (pH 8.8) with cold 3 mM conduritol-B-epoxide for 1 hour. The reaction mixture was dialyzed overnight against 100 mM Na⁺ maleate buffer (pH 8.8) at 4°. A control without epoxide was run simultaneously. No decrease of activity was found. After 40 hours, solid cold inactivator was added to make its concentration 100 mM. The reaction was stopped after 42 hours by dialysis against distilled water. Enzymatic activities, incorporation of radioactivity, and recovery of the labeled sucrase-isomaltase complex as in A. ○—○, per cent of sucrase activity lost; △—△, per cent of isomaltase activity lost; ▲—▲, moles of inactivator per mole sucrase-isomaltase complex.
Fig. 7. A, chromatography of tryptic peptides from the sucrase-isomaltase complex labeled with [3H]conduritol-B-epoxide at the isomaltase site (for details, see “Results”). The Sephadex G-25 column (80 × 1.6 cm) was developed at 4°C with 0.01 M ammonium carbaminate at the flow rate of 5.7 ml per hour. ○—○, optical density at 280 nm; ×—×, neutral sugars (as glucose equivalents); ---, radioactivity. Superposable results were obtained from the sucrase-isomaltase complex preferentially labeled at the sucrase site. B, fractionation of the radioactive peptides derived originally from lot B showed two retarded radioactive peaks, (P₁ and Pᵢ, Fig. 7C), the minor of which had the same Vₑ as the Pᵢ peak derived from lot A.

Since in lot A the isomaltase active site had been labeled, and in lot B the sucrase active site had been preferentially labeled (see previous section), the peptide in peak Pᵢ, was assigned to the active site of the isomaltase subunit and the peptide in peak Pᵢ to that of the sucrase subunit. Both peaks Pᵢ and Pᵢ gave positive tests for neutral sugars (Fig. 7, B and C), strongly suggesting a glycopeptide nature for both of them.

Precipitin Reaction—Some phytohemagglutinins were tested for their ability to interact with the sugar portion of the sucrase-isomaltase complex. While concanavalin A produced unspecific precipitation (Fig. 8) (i.e. the presence of methyl-α-d-mannopyranoside did not interfere with the process), the leuco-phytohemagglutinin P interacted specifically with the sucrase-isomaltase complex (i.e. the presence of N-acetylgalactosamine markedly reduced the amount of precipitate) (32). A strong interaction was found with erythro-phytohemagglutinin P. Its specificity could not be tested, since the only known glycopeptide (33) specifically interacting with that lectin was not available. N-Acetylgalactosamine had no effect.

The A₁ lectin from Dolichos biflorus interacted specifically, although very weakly, confirming the previous observation that
sucrase-isomaltase complex can inhibit the agglutination of blood group A erythrocytes by group A antibodies.4

Affinity Chromatography with Phytohemagglutinin P-Sepharose 4B—Peaks P₁ and P₄, obtained as in Fig. 7, B and C, were chromatographed individually on a column of Sepharose-linked phytohemagglutinin P' (containing both leuco and erythro lectins). The chromatographic pattern (Fig. 9A) showed a front peak, containing sugar-free peptides only, and a retarded peak, containing all radioactivity and all of the neutral sugars. The same pattern was obtained with glycopeptides derived from the unlabeled sucrase-isomaltase complex. When peaks P₁ and P₄ were chromatographed individually on columns of Sepharose-linked A₁ lectin, they gave a single peak which contained peptides, sugars, and radioactivity. The V₅ of this peak (Fig. 9B) was identical with that of the first peak from the Sepharose-linked phytohemagglutinin P. From these results we concluded that the labeled peptides derived from the sucrase and isomaltase active sites are glycopeptides.

DISCUSSION

Conduritol-B-epoxide binds irreversibly to the sucrase-isomaltase complex with a stoichiometry of 1 mole of inhibitor per subunit (Figs. 6, A and B, and Table II). The inactivation follows pseudo first order kinetics and leads to complete inactivation of both sucrase and isomaltase activities. Conduritol-B-epoxide reacts at the active sites of the enzymic complex, as shown by the following observations: (a) epoxides structurally unrelated to the substrates of sucrase and isomaltase have little or no effect (Table I); (b) substrates (34), reversible competitive inhibitors and products protect both sucrase and isomaltase from inactivation by conduritol-B-epoxide (Fig. 3); (c) the radioactive label is incorporated in amounts corresponding stoichiometrically to the active sites inactivated, even after long reaction times (Fig. 6, A and B).

As to the sucrase subsite involved in the reaction with conduritol-B-epoxide, the similarity of the inhibitor with the glucopyranose rather than fructofuranose structure suggests that this inhibitor reacts with the glucosyl rather than with the fructosyl subsite. Some experimental observations support this hypothesis: (a) glucose at a concentration (100 mM) five times larger than its Kᵢ value for sucrase (20 mM) protects from inactivation more than fructose at a concentration (1 M) 10 times larger than its

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4 E. Gershon, unpublished observations.
same site as Tris and glucose do, and that either the dissociation glucose associate with sucrase faster than conduritol-B-epoxide the same, (Equation 12-36), or, perhaps more likely, Tris (and same degree of protection against irreversible inhibition by their Ki values) concentrations of Tris and glucose afford the 12-37 of Ref. 26) that conduritol-B-epoxide competes with the conduritol-B-epoxide. This means (see Equations 1234 through interaction with the fructosyl subsite); (b) both glucose and Tris protect from the inactivation by conduritol-B-epoxide, and the ratio between the apparent protection constants (i.e. 120 from Fig. 5) is very close to the ratio between their Ki values for (glucose equivalents); --- , radioactivity. Identical results were obtained with all the labeled peaks P2 or P3 (obtained as in Fig. 7, B or C) tested. B, chromatography of radioactive peak P1, obtained as in Fig. 7B, on A; lectin-Sepharose 4B column. Experimental conditions as in A.

Ki value (103 mM) (Fig. 3) (actually, the incomplete protection by 1 as fructose may be due in part to reasons other than an interaction with the fructosyl subsite); (b) both glucose and Tris protect from the inactivation by conduritol-B-epoxide, and the ratio between the apparent protection constants (i.e. 120 from Fig. 5) is very close to the ratio between their Ki values for sucrase (i.e. 125, Ref. 27)2; glucose and Tris have been shown to compete for the same subsite (27); (c) sodium increases the apparent affinity of Tris for sucrase (27) and also increases the inactivation rate of sucrase by conduritol-B-epoxide. We conclude, therefore, that conduritol-B-epoxide is an irreversible, active site-directed inhibitor reacting at the glucosyl subsite of sucrase and probably isomaltase (in this case the subsite at which the reaction takes place can only be inferred by analogy).

Over a large pH range around neutrality epoxides must be protonated by an acid before reacting with a nucleophile (8). The selective modification of a single group per subunit, situated in the active sites of the sucrase-isomaltase complex, must be due to the combination of two effects: (a) preferential binding which can be inferred by the similarity between the epoxide and the substrates and (b) the juxtaposition of the epoxide oxygen with an acid and one of the carbons of the oxirane ring with a nucleophile. This nucleophile (to which the inhibitor remains covalently bound) can be identified as a carboxylate because (i) the radioactive label is released by hydroxylamine, which is indicative of an ester linkage (Table III); (ii) the rate of inactivation increases with the pH and apparent pK values of 5.3 and 5.5 were calculated for reactive groups of isomaltase and sucrase, respectively (Fig. 4). Since the sucrase-isomaltase complex is a glycoprotein, but does not contain sialic acid (17), this carboxylate group most probably belongs to a glutamate or an aspartate residue.

Water soluble carbodiimide derivatives also inactivate both sucrase and isomaltase,8 which again points to the presence of carboxyl group(s) in the active sites; these group(s), however, are not identical with those reacting with conduritol-B-epoxide, as shown by double labeling experiments.8 The groups reacting with conduritol-B-epoxide most likely also play a role in the catalytic mechanism of sucrase and isomaltase. In fact: (a) a nucleophilic attack is the rate-limiting step in the reaction mechanism of both sucrase and isomaltase, as shown by the ρ coefficient in the Hammett-Hansch functions; (b) double logarithmic Dixon plots for both sucrase and isomaltase uncover catalytic groups active at the alkaline side of their pK values (which are in the range 5 to 5.5) (36); (c) considerations based on models of conduritol-B-epoxide and α-glucopyranosides suggest that the nucleophile attacking the rear side of the epoxide ring is in the right position to stabilize the carbonium-oxonium ion between C1 and the pyranose oxygen involved as intermediate in the enzymatic reaction.8 Very little can be said at the moment about the nature of the acid catalyst involved in the reaction with conduritol-B-epoxide because the instability of sucrase and isomaltase at alkaline pH values (Fig. 4B) makes it impossible to investigate the pH dependence of the inactivation rate above pH 9, as other authors could do for pepsin (13), phosphoglucose isomerase (15), α-glucosidase (37). However, parallel investigations by Cogoli et al? suggest that participation of histidyl, tryptophanyl, arginyl, and tryosyl groups in the catalysis by sucrase and isomaltase is unlikely. Their role in the reaction with conduritol-B-epoxide is thus improbable, although not entirely ruled out.

Of particular interest is the difference in reactivity between conduritol-B-epoxide and another substrate-like epoxide, myo-

1 Another way of expressing the same results is as follows: from Fig. 5 and from the Ki values for the competitive inhibition by Tris and glucose (27) one can see that identical relative (to their Ki values) concentrations of Tris and glucose affect the same degree of protection against irreversible inhibition by conduritol-B-epoxide. This means (see Equations 12-34 through 12-37 of Ref. 20) that conduritol-B-epoxide competes with the same site as Tris and glucose do, and that either the dissociation constants of the Tris-sucrase and glucose-sucrase complexes are the same, (Equation 12-36), or, perhaps more likely, Tris (and glucose) associate with sucrase faster than conduritol-B-epoxide (Equation 12-37).

8 In principle other protein groups can react with epoxides around neutrality: thiole (native sucrase-isomaltase complex does not carry free SH groups (35) and is not inhibited by SH reagents (see Footnote 7) (21)), tyrosyl and methionyl. Other reagents, known to react with these groups, do not inactivate either sucrase or isomaltase, however (see Footnote 7).

7 A. Cogoli, H. Braun, and G. Semenza, manuscript in preparation.

8 A. Quaroni, unpublished observations.

9 A. Cogoli, and G. Semenza, manuscript in preparation.
epoxide. From a priori considerations the latter, having a structure more closely related to \( \alpha \)-glucopyranoside compounds, would be expected to be a better inhibitor. The experimental data (Table I) show that exactly the contrary is true. There is a conspicuous structural difference between these two epoxides. The most stable form of conduritol B-epoxide approaches the half-chain configuration while that of myo-epoxide is the chair configuration. These observations are paralleled by the fact that gluconolactone is a more effective competitive inhibitor of sucrase and isomaltase than glucose. Isomaltase shows both higher affinity for gluconolactone and greater difference in reactivity with the two above mentioned epoxides. It is possible, therefore, that during the binding of the substrate to the active sites of sucrase and isomaltase a partial chair to half chair transition is imposed on the glucopyranosyl ring. The catalytic mechanism of sucrase and of isomaltase which is emerging is quite similar to that suggested for lysozyme (38).

Finally, some comments should be made on the isolation of the \( [\text{H}] \)conduritol-labeled peptides from tryptic digests. Clearly the label and a sizeable part of the carbohydrate moieties of the sucrase-isomaltase complex are associated (Figs. 7 and 9). Thus the groups present in the active sites in both sucrase and isomaltase reacting with conduritol B-epoxide are located in the proximity of some carbohydrate chains. It follows that the most stable form of conduritol-B-epoxide approaches the half-chain configuration while that of myo-epoxide is the chair configuration. These observations are paralleled by the fact that gluconolactone is a more effective competitive inhibitor of sucrase and isomaltase than glucose. Isomaltase shows both higher affinity for gluconolactone and greater difference in reactivity with the two above mentioned epoxides. It is possible, therefore, that during the binding of the substrate to the active sites of sucrase and isomaltase a partial chair to half chair transition is imposed on the glucopyranosyl ring. The catalytic mechanism of sucrase and of isomaltase which is emerging is quite similar to that suggested for lysozyme (38).

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REFERENCES


The \( K_i \) for glucose is about 20 mm with no difference between \( \alpha \) and \( \beta \) glucose, while the \( K_i \) values for the equilibrium mixture of gluconolactone are 2 and 10 mm for isomaltase and sucrase, respectively; these values reduce to approximately 0.4 and 2 mm if the only inhibiting species is the 1:5 lactone, which amounts to about 20% of the equilibrium mixture.
Affinity Labeling of the Active Sites in the Sucrase-Isomaltase Complex from Small Intestine
Andrea Quaroni, Elaine Gershon and Giorgio Semenza


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