Rat intestinal trehalase

Studies of the active site

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Rat intestinal trehalase was solubilized, purified and reconstituted into proteoliposomes. With octyl glucoside as the solubilizing detergent, the purified protein appeared as a single band on SDS/polyacrylamide-gel electrophoresis with an apparent molecular mass of 67 kDa. Kinetic studies indicated that the active site of this enzyme can be functionally divided into two adjacent regions, namely a binding site (with pK_a 4.8) and a catalytic site (with pK_a 7.2). Other findings suggested that the catalytic site contains a functional thiol group, which is sensitive to inhibition by *N*-ethylmaleimide, Hg^{2+} and iodoacetate. Substrate protection and iodoacetate labelling of the thiol group demonstrated that only a protein of 67 kDa was labelled. Furthermore, sucrose and phlorizin protected the thiol group, but Tris-like inhibitors did not. Structure-inhibition analysis of Tris-like inhibitors, the pH effect of Tris inhibition and Tris protection of 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide inactivation permitted characterization and location of a separate site containing a carboxy group for Tris binding, which may also be the binding region. On the basis of these findings, a possible structure for the active site of trehalase is proposed.

INTRODUCTION

Trehalose (α -D-glucopyranosyl- α -D-glucopyranose) is a sugar abundant in Nature. The sugar is often found in yeast, young mushrooms, the *Trehala manna* plant and seaweeds (Birch, 1963). In insects, trehalose is the most important sugar for transporting energy from body fat-stores to their flying muscles (Jutsum & Goldsworthy, 1976). Trehalose-containing foods are also an important part of the diet of the oriental population; in fact, trehalose may actually have been more important than sucrose in the diet of ancient man (Semenza, 1968).

The enzyme trehalase, which hydrolyses trehalose into its two glucose molecules, is also widespread in plants and animals. The enzyme has been reported in yeast (White *et al.*, 1985), the haemolymph of insects (Sumida & Yamashita, 1983; Counter & Vega, 1985; Ogiso & Takahashi, 1985) and the epithelium of the mammalian small intestine and renal tubules (Galand, 1984; Yoneyama & Lever, 1984; Nakano & Sacktor, 1985; Lemboke *et al.*, 1985). As with many other hydrolases, intestinal trehalase is a membrane-bound protein present only on the microvilli of enterocytes (Eichholz, 1969). However, unlike most other brush-border enzymes, trehalase is not solubilized by papain digestion (Auricchio *et al.*, 1965). Its purification has been reported with butanol/Triton X-100 extraction (Saseyima *et al.*, 1975), enzyme digestion followed by Triton X-100 and sodium deoxycholate extraction (Nakano *et al.*, 1977), and Emulphogen solubilization (Galand, 1984). The molecular mass of the purified enzyme has been variously reported to range from 30 to 90 kDa (by SDS/polyacrylamide-gel electrophoresis). In the present paper we describe the purification of trehalase by the use of octyl glucoside extraction and demonstrate the recon-

stitution of the functionally active purified enzyme into proteoliposomes.

Many inhibitors of trehalase have been previously identified. These include the fully competitive inhibitors such as sucrose (K_i 3.1 mM), Tris (K_i 3.7 mM) and phlorizin (K_i 1.1 mM), as well as thiol-blocking agents such as $HgCl_2$, *N*-ethylmaleimide and *p*-chloromercuribenzoate (Saseyima *et al.*, 1975; Nakano *et al.*, 1977). The enzyme has also been shown to be affected by acetate buffer (Nakano *et al.*, 1977) and Mg^{2+} ions (Saseyima *et al.*, 1975). However, very little is known about the interactions of these different inhibitors and the structure of the active site of the enzyme has not been previously characterized. We have probed the active site of trehalase with iodoacetate and studied the interactions with substrate and various inhibitors. Three separate regions were identified, and a hypothetical distribution of these regions is proposed for the active site of trehalase.

MATERIALS AND METHODS

Materials

Tris, trehalose, ethanolamine, *NN*-dimethylethylene-diamine, methylamine, choline, serine and δ -hydroxylysine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 2-Amino-2-methylpropane-1,3-diol and 2-amino-2-methylpropan-1-ol were from Eastman Kodak Co. (Rochester, NY, U.S.A.). Phosphate and citrate were purchased from Fisher Scientific (Medford, MA, U.S.A.). Bovine brain phospholipid (acetone/diethyl ether-washed) was obtained from Avanti Polar-Lipids (Birmingham, AL, U.S.A.). Octyl glucoside was purchased from Calbiochem-Behring (Los Angeles, CA,

Abbreviation used: EDAC, 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide [1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide] hydrochloride.

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U.S.A.). Iodo[^{14}C]acetate was obtained from Amersham Corp. (Arlington Heights, IL, U.S.A.). EDAC was purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

Enzyme assay

Trehalase activity was assayed by measuring the rate of glucose production after adding trehalose (20 mM) to the membrane preparations. A 100 μl portion was removed, placed into thin-walled glass tubes and the reaction stopped by placing the tubes in boiling water for 1 min. The tubes were cooled, and another 500 μl of glucose assay solution (Flozyme; Worthington Diagnostic Systems, Freehold, NJ, U.S.A.) was added to determine the glucose production. The trehalase specific activity was determined by dividing the glucose production rate by the protein concentration of the enzyme preparation. For kinetic studies, enzyme assays were performed at different time points and the slopes used to calculate the glucose production rate. Protein was assayed by the method of Schaffner & Weissmann (1973). One unit of enzyme activity was defined as the amount of the enzyme producing 1 μmol of glucose/min at 37 °C.

Membrane preparation

Membranes were prepared by rapid hypo-osmotic lysis of intestinal cells present on everted sacs of rat small intestine. Male Sprague-Dawley rats (100–150 g) (Charles River Laboratories, Wilmington, MA, U.S.A.) were killed by transcervical dislocation, and the small intestine was removed, cut into three segments of equal length and extensively flushed with phosphate-buffered saline (135 mM-NaCl/2.7 mM-KCl/1.5 mM- KH_2PO_4 /6.5 mM- Na_2HPO_4 , adjusted to pH 7.4). The intestinal loops were then everted (mucosal side out) and ends ligated. These everted loops were then rinsed again in 50 ml of phosphate-buffered saline. Cells were lysed by dipping the loops into chilled distilled water and keeping them for 15 min on ice. Ruptured cells were gently rinsed from the loops by mild stirring. The medium containing the ruptured membranes was saved and the process of rinsing the loops was repeated once more. The media were pooled and centrifuged at low speed (500 g) for 10 min to remove the intact cells and nuclei. Membranes were collected by centrifuging the supernatant at 100 000 g for 1 h at 4 °C. The pellet was resuspended in 100 mM-Hepes/KOH buffer, pH 7.0, yielding a protein concentration in the range 10–16 mg/ml and stored at –70 °C. The above procedure enriched the sucrase, lactase, trehalase and alkaline phosphatase specific activities about 20-fold.

Membrane solubilization and reconstitution of trehalase activity

Trehalase can be extracted in a functional state by using detergents such as Triton X-100, sodium cholate or octyl glucoside (Newman & Wilson, 1980). In the present experiments, enzyme solubilization and purification was performed in 100 mM-Hepes/KOH buffer, pH 7.0, containing 1.0% octyl glucoside. With this detergent, the solubilized enzyme was stable for more than 1 week at 0 °C and indefinitely at –70 °C. Octyl glucoside did not interfere with the glucose assay, and the K_m measured in

1.0% octyl glucoside was the same as that of the membrane-bound form. The solubilized enzyme could be reconstituted into liposomes by first adding sonicated bovine brain phospholipid and then rapidly diluting the lipid/protein/detergent mixture 20–25-fold into 100 mM-Hepes/KOH or 100 mM-potassium phosphate buffer without octyl glucoside (Newman & Wilson, 1980). Proteoliposomes with typical double-leaflet plasma-membrane structure, 100 nm in diameter, were seen by electron microscopy. The K_m for trehalase in the reconstituted form was 5 mM in 100 mM-Hepes/KOH buffer, pH 7.0, and 8 mM in 100 mM-potassium phosphate buffer, pH 7.0.

Glucose transport by proteoliposomes

For measuring the uptake of Na^+ -dependent D-glucose transport, proteoliposomes were obtained by diluting the lipid/protein/detergent mixture into 100 mM-potassium phosphate buffer, pH 7.0. The proteoliposomes were centrifuged and resuspended in 100 mM-potassium phosphate buffer containing 0.5 μM -valinomycin. Proteoliposomes were diluted 100-fold in 100 mM-sodium phosphate or -potassium phosphate buffer, pH 7.0 (the latter as control), containing D-[^{14}C]glucose (1 $\mu\text{Ci}/\text{ml}$). To terminate transport, proteoliposomes were quickly filtered on Millipore filters (0.22 μm pore size) with suction (Yamada *et al.*, 1983), and washed immediately with 100 mM-sodium phosphate buffer containing 1 mM- HgCl_2 . The ratio of [^{14}C]glucose uptake in sodium phosphate compared with potassium phosphate buffer was then determined.

Trehalase purification

Trehalase was purified by four major procedures. Initially, intestinal cell membranes were solubilized by incubating them in 100 mM-Hepes/KOH buffer, pH 7.0, containing 1% octyl glucoside for 30 min and then centrifuged at 80 000 g for 30 min at 4 °C. Some 77% of the enzyme from this membrane preparation was solubilized and recovered in this procedure. The supernatant was then subjected to DEAE-cellulose chromatography on a 1 cm $\times$ 12 cm column of Whatman DE 52 microgranular anion-exchange DEAE-cellulose that had been pre-equilibrated with 100 mM-Hepes/KOH buffer, pH 7.0, containing 1% octyl glucoside. The solubilized supernatant was then added to the Hepes/octyl glucoside buffer followed by the buffer containing first 37.5 mM-NaCl and then 75 mM-NaCl. Most of the trehalase activity was eluted with the buffer containing 75 mM-NaCl. As shown in Fig. 1, the trehalase activity was eluted following the fractions containing alkaline phosphatase activity. Further elution with increasing NaCl concentrations (up to 150 mM) yielded no additional trehalase activity. The trehalase-containing fractions were then pooled and concentrated under high-salt conditions. In these experiments the enzyme preparation was adjusted to yield a 2.5 M- $(\text{NH}_4)_2\text{SO}_4$ concentration. Proteins that were co-precipitated with octyl glucoside were found as a film floating on the high-salt solution after a 1 min spin with the micro-centrifuge. The surface layer was recovered by aspirating the solution below and then redissolved in 100 mM-Hepes/octyl glucoside buffer. Further protein purification (and desalting) was performed by gel filtration. A 0.6 cm $\times$ 90

Fig. 1. Chromatography of solubilized intestinal trehalase on DE 52 DEAE-cellulose

Membranes were solubilized and the supernatant was collected as described in the Materials and methods section. In a typical experiment, 1 or 1.5 ml of the supernatant was loaded on a 1 cm × 12 cm column of Whatman DE 52 DEAE-cellulose pre-equilibrated with the solubilizing buffer. Proteins were eluted at a flow rate of 2 ml/min into separate tubes (0.8 ml/tube). Bound proteins were eluted first with the same buffer containing 37.5 mM-NaCl followed by buffer containing 75 mM-NaCl. Enzyme (●) and protein (○) assays were performed on samples of each fraction as described in the Materials and methods section.

cm Sephadex G-100 column was prepared with 100 mM-Hepes/octyl glucoside buffer, and the concentrated protein solution was loaded on to the column and eluted at a flow rate of 0.5 ml/min. Protein in the eluate was monitored at 280 nm and collected in 2 ml fractions. Trehalase activity was assayed as described above.

Labelling procedure and gel electrophoresis

Membranes, freshly prepared as described above, were incubated in 100 mM-Hepes/KOH buffer, pH 8.0, containing 20 mM non-radioactive iodoacetate with or without 40 mM-trehalose at 20 °C for 1 h (final protein concentration 4 mg/ml). The tubes containing the membrane preparation were wrapped with aluminium foil to prevent the degradation of the light-sensitive iodoacetate. After incubation, membranes were centrifuged at 100 000 g, the supernatant was discarded and the membranes were then resuspended in 100 mM-Hepes/KOH buffer, pH 8.0. The membranes were centrifuged once more and finally resuspended in the 100 mM-Hepes/KOH buffer, pH 8.0, containing iodo[¹⁴C]acetate (5 μCi/ml) and incubated at 20 °C for 2 h. Thereafter the membranes were centrifuged and washed twice with Hepes buffer. The polyacrylamide gels (10 %) and buffer were prepared as described by Laemmli (1970). Electrophoresis was performed at room temperature with the treated membranes applied at protein concentration of 100 μg/ml. The gels were stained with Coomassie

Blue, photographed, and then sliced for measurement of radioactivity in each fraction.

RESULTS

Enzyme purification

After intestinal mucosal cells were exposed to cold distilled water for rapid hypo-osmotic lysis, the crude membrane preparation was found to contain not only trehalase but also other brush-border enzymes, such as sucrase, maltase, lactase, aminopeptidase and alkaline phosphatase. In most experiments this initial lysis resulted in about a 20-fold increase in the specific activities of these hydrolases. The supernatant from the octyl glucoside extraction was loaded on a short column of Whatman DE 52 DEAE-cellulose. In the initial studies, proteins were eluted with a continuous 0–200 mM-NaCl gradient, and trehalase was identified in the 60–80 mM-NaCl fractions. However, to recover more of the bound trehalase, a three-step gradient (0, 37.5 mM- and 75 mM-NaCl) was used (as shown in Fig. 1). Trehalase appeared predominantly in 75 mM-NaCl fractions.

The major six or seven fractions of trehalase were pooled, and (NH₄)₂SO₄ was added to give a final concentration of 2.5 M. A thin layer of octyl glucoside and membrane proteins (containing most of the trehalase) could be easily separated and removed after a 1 min spin in a micro-centrifuge. This procedure effectively concentrated the enzyme-containing solution (from 4–5 ml to 50 μl) in less than 2 min and gave a recovery of nearly 67% of the enzyme from DE 52 DEAE-cellulose chromatography (Table 1). The surface film containing the enzyme was redissolved and run through a 0.6 cm × 90 cm Sephadex G-100 column (Fig. 2). Trehalase appeared after the void volume and a 3-fold increase in specific activity was obtained (Table 1). The four major trehalase-containing fractions were pooled, and sonicated phospholipid was added for reconstitution. The reconstituted proteoliposomes gave a 1.3-fold increase of the specific activity. SDS/polyacrylamide-gel electrophoresis showed a single band of molecular mass 67 kDa (Fig. 3).

The purification steps, yield and increases in specific activity are summarized in Table 1. From the initial

Table 1. Purification of trehalase from rat small intestine

For details see the Materials and methods section.

Step	Protein (mg)	Trehalase activity	
		Total (units)	Specific (units/mg of protein)
Intestinal cells	1500	17	0.011
Membrane fraction	65	13	0.2
Detergent supernatant	26	10	0.38
DE 52 DEAE-cellulose chromatography	0.8	3	3.76
(NH ₄) ₂ SO ₄ flotation	0.4	2	5
Gel filtration	0.1	1.5	15
Reconstitution	0.05	1.0	20

Fig. 2. Chromatography of solubilized trehalase on Sephadex G-100

The major trehalase-containing fractions (nos. 84–90) from the DE 52 DEAE-cellulose column were pooled, concentrated and redissolved as described in the Materials and methods section for the gel-filtration chromatography. Proteins were fractionated on a 0.6 cm × 90 cm Sephadex G-100 column at room temperature at a flow rate of 0.5 ml/min and collected in 2 ml fractions. Both protein concentration (○) and enzyme activity (●) of each fraction are shown.

Fig. 3. SDS/polyacrylamide-gel electrophoresis of purified trehalase after gel filtration (see Table 2)

Protein obtained after filtration on Sephadex G-100 was subjected to SDS/10%-polyacrylamide-gel electrophoresis. The gel was stained with Coomassie Blue. Molecular-mass markers are shown on the left. The arrow points to the single band of molecular mass 67 kDa.

harvesting of intestinal cells to the final formation of reconstituted proteoliposomes more than 99% of the total protein was removed, 5.9% of the total enzyme was recovered and a greater than 1800-fold increase in specific activity of trehalase was obtained.

Fig. 4. Trehalase activities at different pH values

(a) Enzyme activities at different pH were measured in presence of 20 mM-trehalose as described in the Materials and methods section. ●, Phosphate buffer; ○, citrate buffer. (b) and (c) $V_{\max(\text{app.})}$ and $K_{m(\text{app.})}$ were measured and plotted at different pH. The continuous lines in (a), (b) and (c) were calculated from the following equations:

$$\frac{v}{V_{\max.}} = \frac{[S]}{K_s \left(1 + \frac{[H^+]}{K_{e1}} + \frac{K_{e2}}{[H^+]} \right) + [S] \left(1 + \frac{K_{es2}}{[H^+]} \right)} \quad (\text{for } a)$$

$$\frac{V_{\max(\text{app.})}}{V_{\max.}} = \frac{1}{1 + \frac{K_{es2}}{[H^+]}} \quad (\text{for } b)$$

$$K_{s(\text{app.})} = K_s \frac{1 + \frac{[H^+]}{K_{e1}} + \frac{K_{e2}}{[H^+]}}{1 + \frac{K_{es2}}{[H^+]}} \quad (\text{for } c)$$

These equations were derived from the diprotic model of the enzyme (three forms of trehalase exist as E, EH and EH_2 from alkaline to acidic; E and EH effectively bind substrate S to form ES and EHS; only EHS yields the product). K_{e1} , K_{e2} , K_s and K_{es2} are dissociation constants for EH_2 , EH, EHS (to $\text{EH} + \text{S}$) and EHS (to $\text{ES} + \text{H}$); $V_{\max.}$ is the maximal enzyme activity at optimal pH.

pH-activity profile

The optimal pH for purified trehalase was pH 6 at a trehalose concentration of 10 mM (Fig. 4a). The apparent enzyme activity decreased to 50% at pH 4.5 and 7.2, and

Table 2. Structure-inhibition analysis of Tris-like compounds on trehalase activity

Trehalase was purified through gel-filtration step as described in the Materials and methods section and Table 1. For each compound tested, the enzyme assay was performed in 100 mM-Hepes buffer, pH 7.0, containing 1.0 % octyl glucoside and 20 mM-trehalose. Inhibition was calculated as the difference between enzyme activities in the absence and in the presence of tested compound at 5 mM.

Compound tested	Structure	Inhibition (%)
Tris	$\begin{array}{c} \text{CH}_2\text{OH} \\ \\ \text{HOCH}_2-\text{C}-\text{NH}_3^+ \\ \\ \text{CH}_2\text{OH} \end{array}$	69
2-Amino-2-methylpropane-1,3-diol	$\begin{array}{c} \text{CH}_2\text{OH} \\ \\ \text{CH}_3-\text{C}-\text{NH}_3^+ \\ \\ \text{CH}_2\text{OH} \end{array}$	70
2-Amino-2-methylpropan-1-ol	$\begin{array}{c} \text{CH}_2\text{OH} \\ \\ \text{CH}_3-\text{C}-\text{NH}_3^+ \\ \\ \text{CH}_3 \end{array}$	66
Ethanolamine	$\begin{array}{c} \text{CH}_2\text{OH} \\ \\ \text{H}-\text{C}-\text{NH}_3^+ \\ \\ \text{H} \end{array}$	45
Isobutylamine	$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_3-\text{C}-\text{NH}_3^+ \\ \\ \text{CH}_3 \end{array}$	30
<i>NN</i> -Dimethylethylenediamine	$\begin{array}{c} \text{CH}_2-\text{N} \begin{array}{l} \text{CH}_3 \\ \text{CH}_3 \end{array} \\ \\ \text{H}-\text{C}-\text{HN}_3^+ \\ \\ \text{H} \end{array}$	26
Methylamine	$\begin{array}{c} \text{H} \\ \\ \text{H}-\text{C}-\text{NH}_3^+ \\ \\ \text{H} \end{array}$	24
5-Aminopentan-1-ol	$\begin{array}{c} \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH} \\ \\ \text{H}-\text{C}-\text{NH}_3^+ \\ \\ \text{H} \end{array}$	23
Choline	$\begin{array}{c} \text{CH}_2\text{OH} \\ \\ \text{H}-\text{C}-\text{N}^+ \begin{array}{l} \text{CH}_3 \\ \text{CH}_3 \\ \text{CH}_3 \end{array} \\ \\ \text{H} \end{array}$	0
Serine	$\begin{array}{c} \text{CH}_2\text{OH} \\ \\ \text{H}-\text{C}-\text{NH}_3^+ \\ \\ \text{CO}_2^- \end{array}$	0
Tricine	$\begin{array}{c} \text{CH}_2\text{OH} \\ \\ \text{HOCH}_2-\text{C}-\text{NH}-\text{CH}_2\text{CO}_2^- \\ \\ \text{CH}_2\text{OH} \end{array}$	0
δ -Hydroxylysine	$\begin{array}{c} \text{OH} \\ \\ \text{H}-\text{C}-\text{CH}_2\text{CH}_2-\text{CH} \begin{array}{l} \text{CO}_2^- \\ \text{NH}_3^+ \end{array} \\ \\ \text{H}-\text{C}-\text{NH}_3^+ \\ \\ \text{H} \end{array}$	0

Table 3. Effect of EDAC on trehalase activity

Proteoliposomes of purified trehalase were incubated in 100 mM-Hepes buffer, pH 7, containing 20 mM-EDAC plus the additions shown below. After 2 h incubation at 20 °C, the proteoliposomes were centrifuged and washed twice with 100 mM-Hepes buffer. Trehalase activity was then assayed as described in the Materials and methods section.

Addition	Concn. of EDAC (mM)	Enzyme activity [units/mg (%)]
None	0	18 (100)
None	20	8 (44)
Phosphate (50 mM)	20	8.5 (47)
Tris (20 mM)	20	17 (94)
Trehalose (20 mM)	20	15 (83)

to 20% at pH 4 and 8. Further studies of changes in K_m and V_{max} between pH 4 and pH 8 showed that the decrease of enzyme activity from pH 6 to pH 8 was due to a decrease in V_{max} , whereas the decrease from pH 6 to pH 4 was due to an increase in K_m . It is possible that the active form of the enzyme involves two functional groups, one with a pK_a of approx. 7.2 and the other with a pK_a of 4.8 (Figs. 4b and 4c).

Enzyme stability was also tested and trehalase was found to retain its full activity for more than 2 h between pH 4.5 to 8. A 20% decrease in activity occurred after incubation at pH 4 for 1 h (results not shown).

Tris inhibition

Tris, a known competitive inhibitor of trehalase and many other brush-border hydrolases, was tested for its

inhibiting effects on purified trehalase. The K_i appeared to be 4.0 mM, consistent with a K_i of 3.7 mM observed by others (White *et al.*, 1985). Several other compounds with Tris-like structure were also tested. The results shown in Table 2 indicate that the structure yielding the best inhibition was $RR'(\text{HOCH}_2)\text{C}-\text{NH}_3^+$. Since choline gave no inhibition at all, it was evident that a protonated unsubstituted ammonium group was essential for the inhibitory effect. Since neither D-serine nor L-serine inhibited trehalase, it appears that a negative charge on either R or R' prevents the binding of the inhibitor to the active site on the enzyme.

Tris protected trehalase from inactivation by EDAC. As shown in Table 3, the purified enzyme activity decreased to 44% after 2 h of incubation with EDAC, a carboxy-group-specific agent. This inhibition could be prevented by 20 mM-Tris and high trehalose concentrations but not by phosphate ions (see below). Studies at various pH values showed that Tris inhibited the enzyme by 50% or more only at pH 6.0 or above; at pH 5 or less, Tris inhibition was much diminished or absent (results not shown). From these observations it would appear that a carboxy group on the active site is essential both for Tris binding and Tris inhibition. Although Tris binding is favoured by a low- pK_a group, it is still not clear whether this carboxy group is the same (pK_a 4.8) as that involved in the change in K_m (Fig. 4c).

Inhibition by iodoacetate

Although it is well known that several thiol-blocking agents, such as *p*-chloromercuribenzoate and HgCl_2 , irreversibly inhibit trehalase activity (Counter & Veiga, 1985), we used iodoacetate to probe the active site for several reasons. First, its action is more gentle and specific than that of HgCl_2 . In Figs. 5 and 7(a) the time course for iodoacetate inactivation indicated that after

Fig. 5. Effect of substrate and inhibitors of trehalase in protecting against iodoacetate inactivation

Trehalase, purified and reconstituted as described in the Materials and methods section, was used to study its active site, which is sensitive to iodoacetate inhibition. Reconstituted enzyme was first incubated in 100 mM-Hepes buffer, pH 8.0, containing 20 mM-iodoacetate with indicated concentrations of either trehalose or inhibitor (Tris or phlorizin) at 20 °C for 1 h (●) or 2 h (○). Proteoliposomes were then centrifuged at 100 000 *g* to remove the reagents present in the supernatant. The pellet obtained after 1 h centrifugation was resuspended in 100 mM-Hepes buffer, pH 7.0, and re-centrifuged. Trehalase activity of the proteoliposomes was then assayed after two washings as described in the text. The control value of 100% was the activity of proteoliposomes not treated with iodoacetate. (a) Effect of Tris; (b) effect of phlorizin; (c) effect of trehalose.

Fig. 6. Evidence that trehalose can protect the protein binding of iodo[^{14}C]acetate

Freshly prepared crude brush-border membranes were incubated at room temperature for 1 h in 100 mM-Hepes buffer, pH 8.0, containing 20 mM-iodoacetate with (—) or without (-----) 40 mM-trehalose. Both trehalose and iodoacetate were then removed from the membranes by centrifugation. Iodo[^{14}C]acetate was then added to label the membranes as described in the Materials and methods section. Membranes were then washed to remove unbound iodoacetate. Radioactive labelling of proteins was measured after staining and slicing the SDS/polyacrylamide gel.

1 h incubation 50% of the activity was lost and after 2 h only 30% activity remained. Complete substrate protection was found at a trehalose concentration of 20 mM or greater and half protection with 10–15 mM-trehalose, a concentration greater than the K_m of the enzyme (Fig. 5b). The absence of substrate protection at low trehalose concentrations and the discrepancy between the 50% protection concentration and K_m value may be due to the hydrolysis of the substrate during the incubation (glucose was detected at the end of incubation). Protection against inactivation by iodoacetate was also seen with the inhibitors phlorizin and sucrose. The concentrations of these compounds resulting in 50% protection were close to their K_i values (e.g. 1.1 mM for phlorizin, as indicated in Fig. 5b).

We carried out experiments to examine the protective effect of trehalose on the inactivating effects of iodoacetate. Thus brush-border membranes were incubated with iodoacetate either in the presence or in the absence

Table 4. Tris inhibition of trehalase activity in Hepes or phosphate buffer

Enzyme was purified and reconstituted as described in the Materials and methods section. Tris inhibition was examined at 20 mM-trehalose concentration in 100 mM-Hepes buffer, pH 7, or in 100 mM-potassium phosphate buffer, pH 7. The enzyme activity measured in the absence of Tris was defined as 100% activity.

Concn. of Tris (mM)	Inhibition of enzyme activity (%)	
	In 100 mM-Hepes, pH 7	In 100 mM-phosphate, pH 7
0	0	0
2.5	20	22
5	60	60
10	85	82
20	93	95

of trehalose. After incubations, the membranes were washed and then exposed to iodo[^{14}C]acetate. As shown in Fig. 6, there was a single protein band labelled on SDS/polyacrylamide-gel electrophoresis with a molecular mass 67 kDa only when the membranes had been exposed to trehalose plus iodoacetate. This finding indicated that there was substrate protection against iodoacetate binding and inactivation of a specific protein component of the brush-border membrane fraction. This finding was also consistent with the results obtained during enzyme purification. Tris, a fully competitive inhibitor, showed no protection whatsoever at concentrations far above its K_i (Fig. 5a).

Another reason for using iodoacetate is its interaction with phosphate ions. Thus inhibition of trehalase by iodoacetate was completely prevented by either 50 mM-potassium phosphate or -sodium phosphate buffer. Iodoacetate inactivation was also prevented by acetate ions (Fig. 7b). However, phosphate did not prevent the inactivation produced by HgCl_2 (Fig. 7a).

In Fig. 7(b) are shown the results obtained with various phosphate concentrations. The results suggest that phosphate protected against iodoacetate in a complicated positive co-operative pattern. This, plus the observations that phosphate by itself does not interfere with normal enzyme function and that iodoacetamide does not inhibit the enzyme (whereas iodoacetate does), suggest that there must be separate sites for phosphate and acetate binding, which are very close to but different from the functional thiol group. There appeared to be no interaction between Tris and phosphate ions. This is supported by the findings that Tris inhibition in phosphate buffer was the same as that in Hepes buffer (Table 4) and that phosphate did not protect the carboxy group in the Tris-binding site (Table 3).

Active-site model

As a result of the above findings, it is apparent that there are at least three regions in the active site of the trehalase, as shown in an active-site model depicted in Fig. 8, namely, A, a region for Tris binding, B, a region with a functional thiol group that was attacked by

Fig. 7. Evidence that phosphate and acetate prevent inactivation of trehalase by iodoacetate

(a) Trehalase was purified and reconstituted as described in the Materials and methods section, and proteoliposome pellets were resuspended in either 100 mM-potassium phosphate buffer, pH 8.0 ($\blacktriangle$ and $\triangle$), or 100 mM-Hepes buffer, pH 8.0 ($\bullet$ and $\circ$), containing 20 mM-iodoacetate ($\bullet$ and $\blacktriangle$) or 1 mM- HgCl_2 ($\circ$ and $\triangle$). After incubation at room temperature for various times, the reconstituted enzyme was washed by high-speed centrifugation as described in the Materials and methods section and assayed in 100 mM-Hepes buffer, pH 7, containing 20 mM-trehalose. (b) Reconstituted proteoliposomes of purified trehalase were incubated for 2.5 h at room temperature in 100 mM-Hepes buffer, pH 8, containing 20 mM-iodoacetate and indicated concentrations of acetate ($\circ$) or phosphate ($\bullet$). Proteoliposomes were then washed and assayed in 100 mM-Hepes buffer, pH 7, containing 20 mM-trehalose. The enzyme activity of the untreated trehalase was taken as 100 %.

HgCl_2 and iodoacetate (iodide moiety), and C, adjacent to B, which binds phosphate and iodoacetate (acetate moiety).

From the data in Fig. 5(a) and the findings in the kinetics analysis at different pH values (Fig. 4), it is reasonable to believe that the A region (Tris-binding site) must be far from the B region (thiol-containing group). Furthermore, on the basis of the findings shown in Fig. 7, the C region (phosphate-binding site) appears to be very close to the B region. The reasons for placing the

C region at a distance from the A region (instead of placing the C region between A and B) were the (1) phosphate itself does not affect the enzyme activity, (2) Tris inhibition of trehalase in phosphate buffer is identical with that in Hepes buffer (results not shown), and (3) phosphate provides no protection of the EDAC-inactivated carboxy group at the Tris-binding site (Table 3).

DISCUSSION

The purification of trehalase from mammalian intestine has been reported previously with the use of various methods, including butanol extraction (Saseyima *et al.*, 1975). The molecular mass has been reported to range from 30 to 240 kDa. In the present paper we have described a method of purification without the use of proteolytic enzymes or organic solvents. The active enzyme was found to have a molecular mass of 67 kDa and could be functionally reconstituted into liposomes. Our labelling experiments further confirmed that a protein of molecular mass 67 kDa was the predominant form of the enzyme in our brush-border membrane preparation. The discrepancy in the molecular-mass data between the present findings and prior observations (Saseyima *et al.*, 1975; Nakano *et al.*, 1977; Galand, 1984) may be due to the fact that others used different detergents having different hydrophilic/hydrophobic indices, the drastic denaturation involved in the long purification steps and the application of digestive enzymes.

A symmetric bell-shaped pH-activity profile for trehalase was found with the use of buffers ranging from pH 4.5 to 8. The plot of $V_{\text{max. (app.)}}/K_{\text{s (app.)}}$ versus pH indicated that two ionizable groups were involved in the

Fig. 8. Suggested distribution of three regions (A, B and C) of the active site of trehalase

For explanation see the text.

active site. Data from our experiments suggest a diprotic model for three ionic forms, EH_2 , EH and E , with pK_{e_1} at pH 4.8 and pK_{e_2} at pH 7.2 (E represents enzyme and H represents proton). Substrate binds equally well with both EH and E forms of the enzyme (but poorly with the EH_2 form), but only EH -substrate and EH_2 -substrate complexes can efficiently give the hydrolytic product, glucose. In other words, the active site of trehalase may functionally consist of two separate regions: a binding region (with a functional group of pK_{a} 4.8) and a catalytic region (with another functional group, of pK_{a} 7.2).

Tris, a well-known reversible competitive inhibitor of various intestinal disaccharidases (sucrase, lactase, isomaltase and trehalase; Semenza, 1968), was examined for its interaction with the active site of trehalase. In our study, the basic structural requirement for the Tris inhibition was elucidated. Protonated ammonium ions and one hydroxymethyl group were absolutely required. The presence of a bulky group or negatively charged group decreased the inhibition. This suggests that the binding site for Tris may be very small and that a negatively charged group may be present in that region. Tris inhibition was much more efficient at high pH (6–8) than at low pH (4.5–5.5). Since in this pH range (4.5–8) Tris is always protonated, its decreased inhibition at an acid pH must be due to the change of some functional group at this Tris-binding area. This observation, together with the finding that Tris fully protects against EDAC inactivation, suggests that a carboxy group is related to this Tris-binding site. The pK_{a} of this group strongly suggests that Tris may bind directly to this domain (defined as the acidic pK_{e_1} group of the enzyme in our kinetic study; Fig. 8, A).

The inhibition by iodoacetate demonstrated another domain of the active site (Fig. 8, C). The inhibition was less pronounced than the Tris inhibition and was irreversible. The inhibition could be fully protected by either trehalose or phlorizin but not by Tris. This indicated that a site separate from the Tris-binding site accounted for the iodoacetate binding. The fact that in our labelling experiments both Hg^{2+} and *N*-ethylmaleimide blocked the binding strongly suggests the presence of a thiol group in that area. However, it is not yet clear whether this thiol group is the catalytic pK_{e_2} group. The interaction between phosphate and trehalase was unexpected, since the enzyme activity is not affected by phosphate ions. The iodoacetate inhibition could be fully prevented by the presence of 20 mM-phosphate. This observation, together with the findings that iodoacetate inhibition could be prevented by acetate but not by acetamide (results not shown), indicates that the binding of iodoacetate on trehalase is stereospecific and that a region adjacent to the thiol site may provide the microenvironment for specific acetate or phosphate binding. The finding that Tris inhibition was not affected at all by phosphate ions may be explained in our model whereby the Tris-binding site (Fig. 8, A) is far from the iodoacetate-binding site (Fig. 8, C).

It should be noted that many membrane-associated enzymes of the intestinal brush border are inhibited by similar biochemical agents. As mentioned above, Tris inhibits not only trehalase but also some other disaccharidases. Phlorizin inhibits both trehalase and the Na^+ /glucose transport system. Phlorizin was used as a labelling agent by Klip *et al.* (1979) for partial purification

of Na^+ /glucose transporter of intestinal microvilli; its polymer was also used by Lin *et al.* (1981) for the affinity-chromatography purification of the Na^+ /glucose transporter. A peptide of molecular mass 60–70 kDa was reported as a component of the possible polymeric subunits of the Na^+ -dependent D-glucose co-transport system (Klip *et al.*, 1979; Lin *et al.*, 1981). The similarity of the molecular mass of our purified trehalase and its phlorizin-binding property to a presumed subunit of the Na^+ /glucose transporter led us to examine the glucose-uptake activity of our reconstituted trehalase proteoliposome. However, neither facilitated nor Na^+ -dependent glucose-transport activities were detected in our purified trehalase preparation [although the reconstitution with the crude intestinal membrane preparation gave a weak but significant (5-fold) Na^+ /glucose-uptake activity (in versus out)]. Conceivably, the lack of glucose-transport activity may have been due to the selective denaturation process, which affected the transporter function more than the enzyme activity. However, the facts that Tris has not been found to be an inhibitor of the Na^+ -dependent transporter and that Na^+ does not stimulate trehalase activity indicate that there is a fundamental difference between their active sites. Further studies of the active sites of other brush-border membrane proteins may provide a better understanding of the divergence of these proteins. In addition, once peptide and gene sequences of purified trehalase have been obtained, they may also provide an easier means of identifying other brush-border proteins that are very difficult to isolate and purify.

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