Biochimica et Biophysica Acta, 524 (1978) 131—141

BBA 68422

PHYSICAL PROPERTIES AND TRIS INHIBITION OF AN INSECT TREHALASE AND A THERMODYNAMIC APPROACH TO THE NATURE OF ITS ACTIVE SITE

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(Received September 20th, 1977)

Summary

The midgut from *Rhynchosciara americana* larvae display a trehalase (α,α'-trehalose glucohydrolase, EC 3.2.1.28) which is soluble with a molecular weight of 122 000 and p*I* 4.6. The optimum pH of the enzyme is 6.0, its apparent K_m for trehalose is 0.67 mM and its energy of activation is 16.7 kcal/mol. Sulfhydryl reagents do not inhibit the trehalase. The results suggest the existence of two carboxyl groups in the active site, one of which has a very high (8.3) p*K*. The increase of the p*K* values of the essential groups of the free enzyme in the presence of increasing concentrations of dioxane supports the hypothesis that these groups are carboxyls. The purified enzyme hydrolyzes only α,α'-trehalose and it is competitively inhibited by several compounds.

Introduction

Trehalase (α,α'-trehalose 1-D-glucohydrolase, EC 3.2.1.28), which hydrolyzes α,α'-trehalose to two glucose molecules it is one of the most widespread carbohydrases in insects, occurring mainly in midgut, flight muscle, fat body and labial glands [1].

Some soluble trehalases have been highly purified from whole insects [2,3] and frequently they have been considered as being derived from the gut [1]. Nevertheless, since soluble trehalases also occurs in tissues other than the gut, such as muscle [4,5] and hemolymph [6] it is not possible to be sure the soluble trehalases purified from whole animals come from the gut and are not a mixture of trehalases. The trehalases, which unequivocally are soluble gut trehalases, have been poorly characterized. In the majority of cases only the molecular weight have been measured and the effect of pH, temperature and substrate concentration on the trehalase studied [7,8].

In this paper we describe some of the physical and kinetic properties of the

midgut trehalase of *Rhynchosciara americana* and try to identify the nature of their prototropic groups at the active site.

Materials and Methods

Materials. Glucose oxidase (Type II), peroxidase (Type II) were purchased from Sigma. Ampholine (pH 3—10) was supplied by LKB Corp. Pure proteins used as molecular weight markers were: egg albumin, catalase and ferritin purchased from Sigma and porcine pancreas α-amylase from Worthington. A universal buffer [9] was supplied by B.D.H. Chemicals Ltd. Iodoacetic acid (Sigma) was recrystallized and tested for the presence of free iodine before use [10].

Animals. R. americana (Diptera, Sciaridae) was reared in the laboratory [11] and we have used only females at the end of 2nd period of 4th instar [12].

Preparation of enzyme solution. It was performed as previously described [13] followed by centrifugation at 105 000 $\times g$ for 1 h at 4°C and dialysis of the supernatant for 24 h at 4°C against 100 vols. of distilled water. The enzyme solution could be stored for at least 6 months at —10°C without noticeable change in activity.

Standard trehalase assay. The standard assays were accomplished at 37°C in 0.05 M citrate/phosphate buffer (pH 6.0), 15 mM trehalose in a final volume of 0.5 ml. The reaction was stopped by placing the tubes in a boiling-water bath for 2 min. The glucose formed was determined with the Tris/glucose oxidase reagent [14]. In each determination, incubations have been carried out for 30, 60 and 120 min and the initial rates calculated. A unit of enzyme is defined as the amount that catalyzes the cleavage of 1 μmol of substrate per min.

Gel electrophoresis. Trehalase samples containing 25 munits were applied to acrylamide gel columns of different concentrations [15]. The electrophoretic separation was achieved with a current of 2.5 mA/column for 1 h at 4°C. After the runs the gel were fractionated as previously described [13]. To each fraction, buffered trehalose was added to become 15 mM trehalose, 0.05 M citrate/phosphate buffer (pH 6.0). The incubations were terminated after 2 h at 37°C by the addition of 2 ml 3,5-dinitrosalicylic acid reagent [16] followed by heating 5 min in a boiling-water bath and dilution with 2 ml water before reading the absorbance at 550 nm. The recovery of trehalase activity applied to the gels was 68—77%.

Molecular weight. The molecular weight of trehalase was determined by measuring its electrophoretical migration in acrylamide gels of different concentrations (4.0, 5.5, 7.0, 8.5 and 10.0%) [15] and comparing its behavior with those of proteins of known molecular weight [17]. The position of these proteins was marked by amido black staining and that of trehalase by fractionation of the gel followed by enzyme assay, as described above. The linear correlation coefficients found were higher than 0.99 for the molecular weight markers and than 0.97 for the treahalase.

Isoelectric focusing. Two columns of 7.5% polyacrylamide gels were used. Before polymerization was initiated, 12.5 munits trehalase and a volume of 40% Ampholine (pH 3—10, LKB Corp.) sufficient to the gel to become 1.5% Ampholine were added. Isoelectric focusing was carried out as previously

described [18]. One column was then fractionated and assayed as described for gel electrophoresis, while the other column was fractionated with deionized water replacing the buffer. The fractions in water were left overnight at 4°C and then the pH of each was measured with a glass electrode. The recovery of trehalase activity applied to the gels was approx. 67%.

Trehalase partial purification. Samples of trehalase were electrophoretically separated in several columns of 8.5% polyacrylamide gels. The regions of the columns containing the trehalase were cut with a razor blade and homogenized in 20 mM citrate/phosphate buffer (pH 6.0). After the pH of the homogenate was adjusted to 6.0 with 3 M HCl, it was left overnight at 4°C. Then, the homogenate was centrifuged and the supernatant dialyzed against 100 vols. of distilled water for 12 h at 4°C. The activity remaining in dialysates corresponded to 55—65% of that applied to the gel. The combination of ultracentrifugation followed by purification by gel electrophoresis results in a recovery of 53—63% of the activity found in the original homogenate and in a specific activity 26-fold higher (1.6 units/mg, at 37°C, pH 6.0). The purified trehalase was stable and no change in activity has been found after 2 months storage at −10°C in water at neutral pH.

Thermodynamic parameters of ionization. Trehalase assays were performed in universal buffer at more than 10 different pH values in which the enzyme is stable and apparent V (V_{app}) and apparent K_m ($K_{m,app}$) were determined at each pH (from Lineweaver-Burk plots). The buffer pH was adjusted by titration with 0.2 M NaOH at the temperature of the assay. The pK values of the ionizable groups in the active site were determined from linear plots of K_{mapp}/V_{app} versus $[H^+]$ and of $1/V_{app}$ versus $1/[H^+]$ [19]. The standard enthalpies of ionization (ΔH^0_{ion}) were calculated from the slope of a plot of pK against the reciprocal of the absolute temperature. The standard free energies of ionization (ΔG^0_{ion}) were calculated from the equation $\Delta G^0 = 2.303\,RT$ pK from pK values interpolated from the plot of pK versus $1/T$. The limits of error of the slopes of the straight lines [20] and of the interpolated pK values [21] were also calculated. The standard entropy of ionization (ΔS^0_{ion}) were calculated from the equation: $\Delta S^0_{ion} = (\Delta H^0_{ion} - \Delta G^0_{ion})/T$.

Enzyme pK values in dioxane. The pH of the universal buffer/dioxane solution was adjusted by titration at the temperature of the assay (25°C) as previously described [22]. Acetate buffers with dioxane were used to standardize the pH meter. The pK values of the acetic acid in the different concentrations of dioxane used were calculated from the data in the literature [23]. The assays and the determinations of enzyme pK values were accomplished as described above.

Multiple inhibition analysis. It was accomplished by plotting $1/v$ versus $[I]/K_i$ at several fixed $[j]/K_j$ [24]. When j was dioxane, only one fixed concentration was used to avoid displacements of the enzyme optimum pH. Theoretical straight lines were calculated from the equation below:

$$\frac{1}{v} = \frac{K_m}{[S]\,V}\left(1 + \frac{[j]}{\alpha K_j}\right)\frac{[I]}{K_i} + \frac{1}{V}\left(1 + \frac{K_m}{[S]} + \frac{K_m\,[j]}{[S]K_j}\right)$$

TABLE I

DISTRIBUTION OF TREHALASE IN SUBCELLULAR FRACTIONS OF *R. AMERICANA* MIDGUT

R. americana midguts after being rinsed thoroughly with saline were homogenized with neutralized deionized water and then passed through glass wool. The resulting filtrate were submitted to differential centrifugation. The sediment were dispersed in 0.1% Triton X-100/0.1 M NaCl before assay in the standard conditions as the supernatants. The data correspond to the average of two independent determinations.

Fraction	Activity	
	munits/midgut	Percent
Homogenate	50.1	100
Sediment, 10 000 X *g*, 10 min	0.05	0.1
Supernatant, 10 000 X *g*, 10 min	49.8	99.5
Sediment, 105 000 X *g*, 60 min	0.0	0.0
Supernatant, 105 000 X *g*, 60 min	48.4	97.0

Results

Subcellular distribution and physical properties

The trehalase activity found in *R. americana* midgut homogenates is completely soluble (Table I) and was not enhanced by repeated freezing and thawing. It corresponds to a protein with a molecular weight of 122 000, and it has a p*I* value of 4.6 (Fig. 1).

Stoichiometry of reaction and the effect of pH, temperature and substrate concentration

2 mol of glucose were formed per mol of trehalose by the action of the trehalase as determined by the methods of glucose oxidase or 3,5-dinitrosalicylic acid.

The effect of the substrate concentration in midgut trehalase is shown in

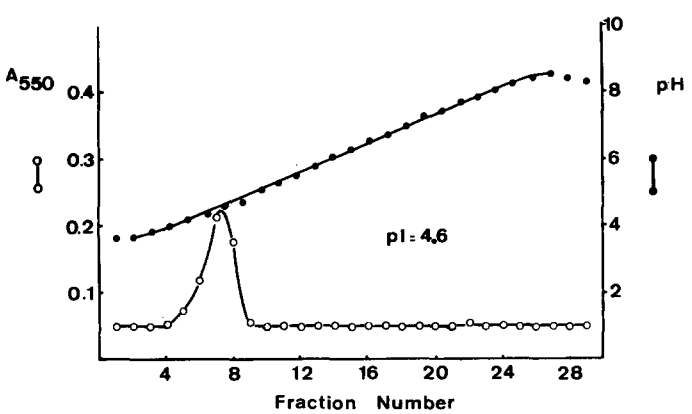

Fig. 1. Isoelectric focusing of *R. americana* midgut trehalase in polyacrylamide gel. Conditions are described in Materials and Methods.

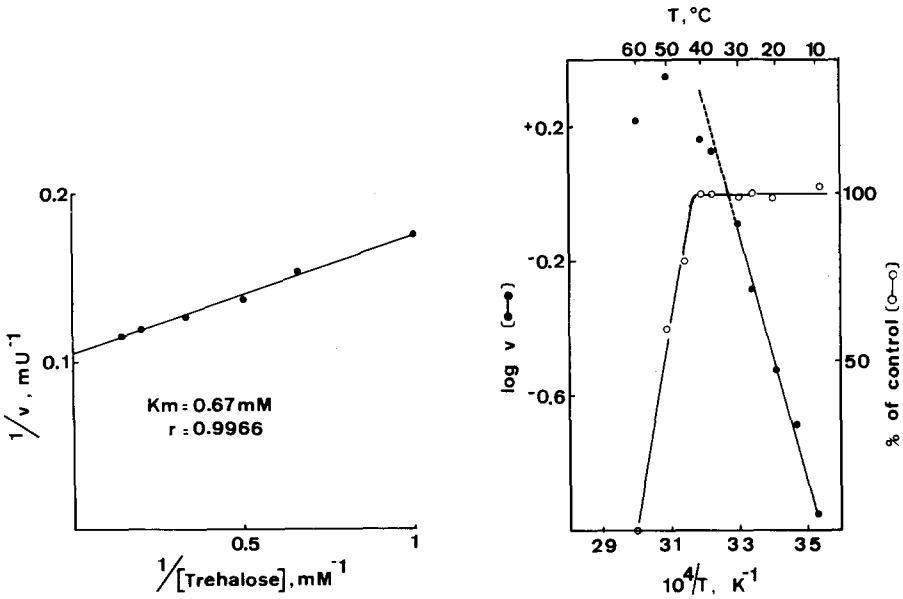

Fig. 2. Effect of substrate concentrations on the *R. americana* midgut trehalase activity. The assays were performed in the standard conditions. The straight line was adjusted to the points using the least-squares method. *r* is the correlation coefficient.

Fig. 3. Effect of temperature on the activity and stability of midgut trehalase. The assays for the Arrhenius plot were accomplished in the standard conditions at different temperatures. The straight line was adjusted to the points between 10 and 30°C ($r = 0.9978$). The heat stability of trehalase was determined by incubating enzyme samples in 0.2 M citrate/phosphate buffer (pH 6.0) at different temperatures for 2 h before assaying in the standard conditions.

Fig. 2 using a Lineweaver-Burk plot from which it was calculated an apparent K_m of 0.67 mM for trehalose. The energy of activation of the midgut trehalase determined in substrate-saturating conditions between 10 and 30°C is 16.7 kcal/mol (Fig. 3).

The effect of pH on the trehalase activity suggests the existence of a

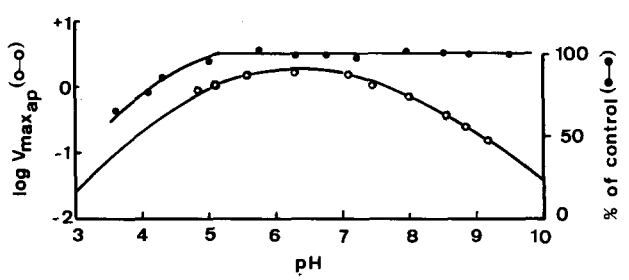

Fig. 4. Effect of pH on the activity and stability of trehalase. The enzyme samples were incubated in the different pH values at several trehalose concentrations and the apparent *V* were extrapolated from Lineweaver-Burk plots. The assays were performed in universal buffer [9] at 30°C. For the determination of the pH stability the enzyme was left for 2 h at 30°C in universal buffer at different pH values, before being diluted 10 times by the addition of 0.2 M citrate/phosphate buffer (pH 6.0) followed by assays in the standard conditions.

TABLE II

THERMODYNAMIC PARAMETERS OF IONIZATION OF THE PROTOTROPIC GROUPS OF THE MIDGUT TREHALASE-ACTIVE SITE AT 25°C

The figures are averages followed by the limits of error with 95% of confidence. The prototropic groups are numbered according to their pK values. E, ionizable groups in the free enzyme; ES, in the enzyme · substrate complex. See text for further details.

Prototropic group	ΔG^0_{ion} (kcal/mol)	ΔH^0_{ion} (kcal/mol)	ΔS^0_{ion} (cal/mol per degree)
E_1	6.70 ± 0.19	0.6 ± 0.9	−19.3 ± 3.8
ES_1	6.73 ± 0.31	1.0 ± 1.3	−19.2 ± 5.6
E_2	11.23 ± 0.40	1.8 ± 2.4	−31.5 ± 9.4
ES_2	11.32 ± 0.42	1.9 ± 2.5	−31.5 ± 9.8

deprotonated (pK_1 4.9) and a protonated (pK_2 8.3) group in the active site of the functional form of the enzyme which shows an apparent optimum pH at approx. pH 6.0 (Fig. 4).

A control experiment using the following buffers (0.05 M): citrate/phosphate (pH 3.6—7.0), phosphate (pH 7.5—8.0) and glycine/NaOH (pH 8.6—10.0), instead of the universal buffer, it was accomplished and the results found were essentially the same as shown in Fig. 4.

Nature of the prototropic groups of the active site

The pK values of the prototropic groups in the active site of trehalase were determined at different temperatures and the thermodynamic parameters calculated from them are displayed in Table II. Although the enthalpies determined show large deviations (Table II), the only known group whose enthalpy of ionization can be found inside the limits of error calculated for the enthalpies of the prototropic groups in the active site of trehalase is the carboxyl group [25]. This imply in the occurrence of two carboxyl groups in the active site of trehalase: one group with a pK 4.9 and another with pK 8.3. Carboxyl groups with pK 4.9 have been described before [25], while a carboxyl group having pK 8.3 must be in a highly hydrophobic environment. The hydrophobicity of the environment increases the pK of the groups, which ionize with separation of charges, almost entirely due to a more negative entropy of ionization [26]. An inspection in Table II shows us that the ΔS^0_{ion}

TABLE III

EFFECT OF DIOXANE IN THE IONIZATION OF THE TREHALASE-ACTIVE SITE PROTOTROPIC GROUPS AND ON pH-INDEPENDENT V AND K_m AT 25°C

pK values are numbered in the same way as in Table II. See text for further details.

Solvent	pK_{E_1}	pK_{ES_1}	pK_{E_2}	pK_{ES_2}	V (munits)	K_m (mM)
Water	4.97	4.83	8.30	8.26	2.56	0.48
7% dioxane	5.20	4.94	8.48	8.18	2.53	1.42
15% dioxane	5.61	4.74	8.86	8.15	2.78	2.32

of the group with larger pK is much more negative than the one of smaller pK, in accordance with the assumption it is in a more hydrophobic environment.

Table III shows that the pH-independent V and K_m values are, respectively, constant and increasing as the concentration of dioxane becomes higher. A plot of pH-independent K_m versus concentration of dioxane results in a straight line ($r = 0.9984$) showing that dioxane is a simple intersecting linear competitive inhibitor with a K_i 0.48 M. The pK values shown in Table III were calculated assuming that the dioxane K_i does not change with pH. There we see that the pK values of the free enzyme increase with increasing dioxane concentration, supporting the hypothesis the prototropic groups of the trehalase-active site are carboxyl groups. Otherwise, the pK values of the enzyme · substrate complex do not change in the same conditions, suggesting that dioxane competes with trehalose in binding the trehalase, in accordance with the findings commented above.

Substrate specificity and inhibitors of trehalase

The midgut trehalase hydrolyzes only α,α'-trehalose among several other disaccharides (Table IV). It is highly competitively inhibited by sucrose and *p*-nitrophenyl-β-D-glucoside, and in a less extent by other carbohydrates (Table IV). The sulfhydryl reagents *p*-mercuribenzoate (0.1 mM) and iodoacetate (50 mM) do not inhibit the midgut trehalase.

The midgut trehalase inhibition by Tris at constant ionic strength (500 mM) is a simple intersecting linear competitive inhibition at pH 6.0 (Fig. 5) and an intersecting linear non-competitive inhibition at pH 9.2 (Fig. 6). The results suggest that the protonated Tris binds more (K_i 74 mM) than the deprotonated Tris (K_i 182 mM) in the enzyme. Otherwise, the binding of the two forms of Tris must be different, since the protonated Tris present at the active site prevent the binding of trehalose, while the deprotonated Tris only make it difficult (α 4.7). In both cases, however, there is the binding of only one molecule of Tris per active site, basing on the observed rectilinear replots of slopes and intercepts (Figs. 5 and 6).

TABLE IV

SUBSTRATE SPECIFICITY AND INHIBITION OF THE PURIFIED *R. AMERICANA* MIDGUT TREHALASE

Purified *R. americana* midgut trehalase, was incubated with each of the listed carbohydrates (15 mM) with and without trehalose (5 mM) in the standard assay conditions. None of the carbohydrates have substrate properties, although they affected in varied degrees the action of the enzyme on trehalose. The K_i values were determined from replots of slopes of Lineweaver-Burk plots against inhibitor concentration. Four different concentrations of the inhibitors were used in each case and the results showed that all of them are simple intersecting linear competitive inhibitors.

Carbohydrate	Inhibition (%)	K_i (mM)	Carbohydrate	Inhibition (%)
Sucrose	61.6	1.4	lactose	8.3
P-Nitrophenyl-β-D-glucoside	44.2	2.5	cellobiose	7.2
Turanose	23.1	5.9	melibiose	6.4
Phenyl-β-D-glucoside	18.3	6.5	maltose	2.0
P-Nitrophenyl-α-D-glucoside	25.2	7.1		

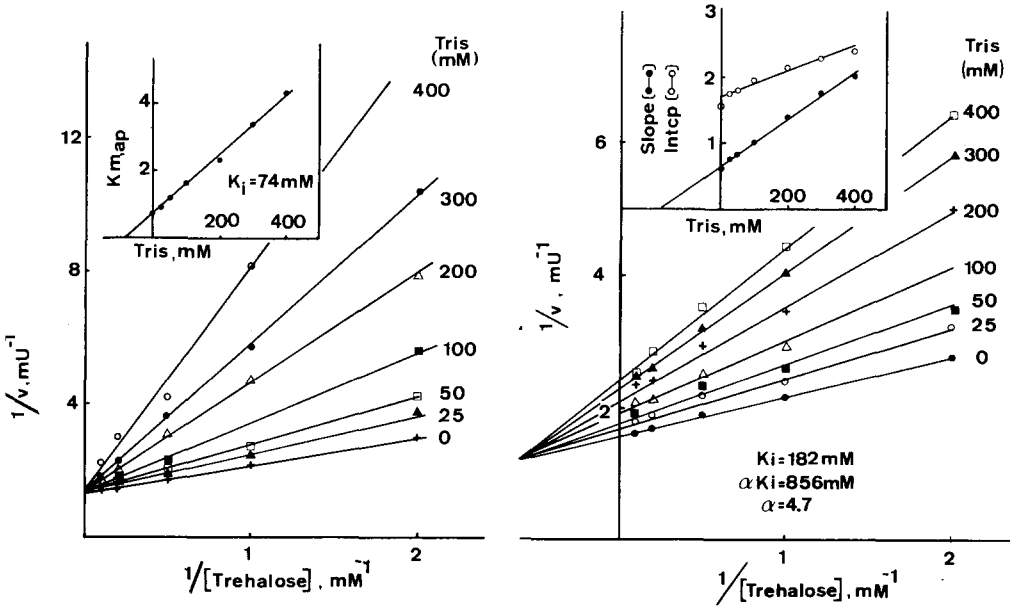

Fig. 5. Inhibition of the purified midgut trehalase by Tris at pH 6.0. Lineweaver-Burk plots for different concentrations of Tris; inset, replot of $K_{m,app}$ calculated from the Lineweaver-Burk plots against the concentration of Tris. The ionic strength (I) in assay tubes was maintained constant (500 mM) by the addition of suitable amounts of NaCl.

Fig. 6. Inhibition of the purified midgut trehalase by Tris at pH 9.2. Lineweaver-Burk plots for different concentrations of Tris; inset, replot of slopes and intercepts from Lineweaver-Burk plots against the concentration of Tris. Ionic strength = 500 mM.

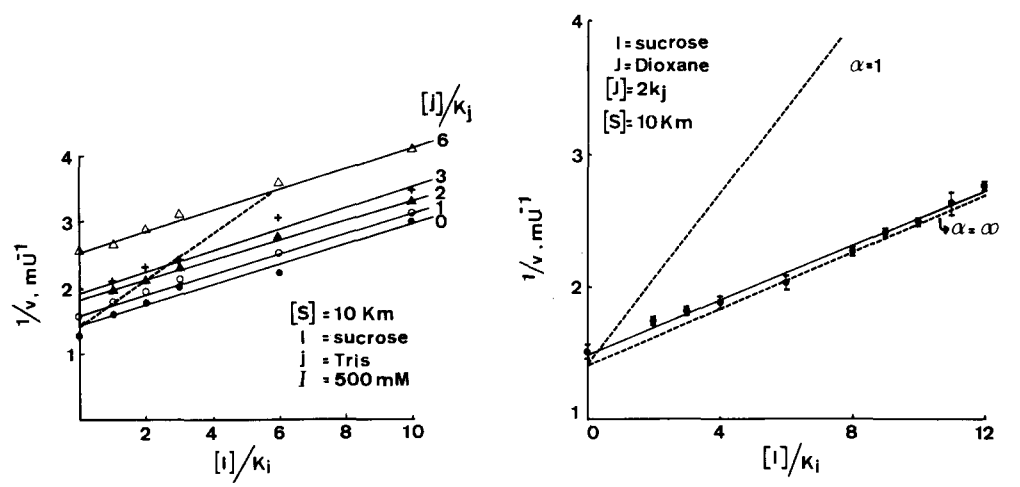

Fig. 7. Multiple inhibition of the purified midgut trehalase by Tris and sucrose at pH 6.0; Yonetani-Theorell plot [24].

Fig. 8. Multiple inhibition of purified midgut trehalase by dioxane and sucrose at pH 6.0. The vertical bars are standard deviations of means. The solid line is the straight line which fits better to the experimental points by using the least-squares method. The interrupted lines are theoretical. α is the interaction factor between the two inhibitors in the enzyme · inhibitor complex.

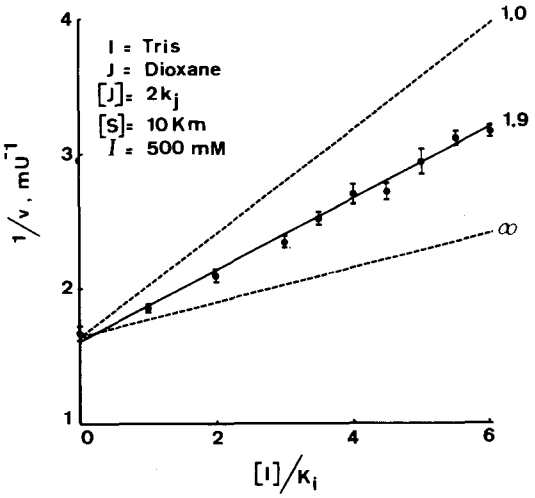

Fig. 9. Multiple inhibition of purified midgut trehalase by Tris and dioxane at pH 6.0. The vertical bars are standard deviations of means. α is the interaction factor between the two inhibitors in the enzyme · inhibitor complex. The lines for $\alpha = 1$ and $\alpha = \infty$ are theoretical, while that for $\alpha = 1.9$ corresponds to the straight line which fits better to the experimental points by using the least-squares method.

Multiple inhibition of trehalase

The results shown in Fig. 7 indicate that Tris and sucrose bind at the same site, since the interaction factor (α) of Tris and sucrose in the enzyme · inhibitor complex must be ∞ for the plot result in a family of parallel straight lines and the Yagi-Ozawa plot (interrupted line in Fig. 7) be linear [24]. The interaction factor (α) of dioxane and sucrose in the enzyme · inhibitor complex is, within the experimental error, ∞ (Fig. 8).

This supports the previous statement that dioxane binds at the active site of the trehalase.

The interaction factor (α) between dioxane and Tris in the enzyme · inhibitor complex is 1.9 (Fig. 9). This means that Tris and dioxane binds at different although a little interpenetrated sites in the trehalase active center.

Discussion

The specificity and inhibition of trehalase

The *R. americana* midgut trehalase is highly specific for trehalose as previously described for trehalases from several other sources [27]. This specificity seems to be a consequence of the symmetry of its active site, since asymmetric epimers of trehalose are bad substrates or are competitive inhibitors of trehalase [28].

Early evidence suggested that at least two hydroxyl groups and the amino group of Tris are necessary to inhibit the trehalase [3]. Those findings have been confirmed in this paper, which also showed that the binding of Tris to the trehalase-active site leave sufficient room there for the binding of a dioxane molecule.

The active site of trehalase

Sulfhydryl groups seem to be absent from the active site of trehalases from several sources [3,29]. Amino, carboxyl and imidazole groups have been proposed to occur in the active site of trehalases [8,29,30]. Nevertheless, the reports are based on pH studies carried out at only one temperature [8,30] or on chemical modification studies in conditions which do not exclude the involvement of other groups [29].

Thermodynamic data presented in this paper suggest that both the acid and basic groups in the active site of *R. americana* midgut trehalase are carboxyl groups. The occurrence of two carboxyl groups in the active site of the glycosidases lysozyme [31], β-glucosidase [32] and intestinal sucrase [33] it is very well documented. It is proposed for those enzymes that both carboxyl groups are involved in catalysis, one acting as an acid to protonate the glycosyl oxygen while the other functions as a base to stabilize a developing carbonium ion [31,34,35]. It is possible that the same mechanism occurs in other glycosidases including the trehalases.

Acknowledgements

We are much indebted to Professor F.J.S. Lara for encouragement and laboratory facilities and to Dr. F. Galembeck for helpful discussions. This work was supported by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (F.A.P.E.S.P.). C.F. is a graduate fellow of F.A.P.E.S.P.

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