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CARBODIIMIDE-REACTIVE CARBOXYL GROUPS AT THE ACTIVE SITE OF AN INSECT MIDGUT TREHALASE

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Summary

Carbodiimide modification of the *Rhynchosciara americana* midgut trehalase (α,α-trehalose glucohydrolase, EC 3.2.1.28) at different pH values revealed the existence of two essential groups (pK_a 5.28 and pK_a 7.74) for the trehalose activity. Those groups must be carboxyl groups since the alternative possibilities (sulfhydryl and phenol groups) have been discarded by selective modification and attempts to reactivate the modified enzyme with hydroxylamine. Furthermore, the increase of the pK_a values of carbodiimide-reactive groups in the presence of dioxane supports further evidence that they are carboxyls. The results suggest the pK_a 5.28 carboxyl is in the active site, while the pK_a 7.74 carboxyl is in its neighborhood buried in the enzyme molecule. The possible role for the carbodiimide-reactive carboxyl groups in catalysis is discussed.

Introduction

Insect midgut trehalases (α,α-trehalose glucohydrolase, EC 3.2.1.28) are soluble and highly specific for trehalose [1]. They are found in *Rhynchosciara americana* larvae mainly in the fluid between the midgut epithelium and the peritrophic membrane [2]. Their function is certainly digestive, although other functions have also been proposed [3].

Despite the fact that trehalases occur in the majority of living organisms [3] few papers have dealt with the nature of their active sites [4—6].

Abbreviations: EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; TEMED, *N,N,N'N'*-tetramethylethylenediamine.

Recently *R. americana* midgut trehalase has been partially purified and thermodynamic data have been provided supporting the proposition that both the acidic and alkaline group in its active site are carboxyl groups [7]. In the present paper we confirmed our previous findings by selectively modifying carboxyl groups with EDC, a water soluble carbodiimide.

Materials and Methods

Materials. EDC, glycine ethyl ester, TEMED, tetranitromethane, and hydroxylamine were purchased from Sigma. Bio-Gel P-30 was from Bio-Rad Laboratories. All other reagents were analytical grade reagents from E. Merck (Darmstadt, F.R.G.).

Trehalase purification and assay. R. americana midgut trehalase was prepared, purified and assayed as previously described [7].

Modification studies with carbodiimide. The reagents and reaction conditions are described for each experiment in the legends of the figures. The pH values of the TEMED/HCl buffers, as well as the glycine ethyl ester solutions, were adjusted by titration with 1 M HCl at the temperature of the assay (30°C). The pH of solutions in dioxane was adjusted with the precautions described previously [7]. The reaction between trehalase and carbodiimide was stopped by a 100-fold dilution with 0.05 M citrate/phosphate buffer (pH 6.0) in most cases. When trehalose was present in the reaction medium, reaction was stopped by running aliquots of the incubation mixture on Bio-Gel P-30 columns (0.6 × 9 cm) in 0.05 M citrate/phosphate buffer (pH 6.0). Elution was achieved with 1.2 ml buffer and the enzyme activity was determined in 0.4 ml of the eluate. The enzyme was found to be stable in the pH of all the reaction mixtures used.

pK_a values of EDC-reactive groups. The solid lines in the inactivation-pH profiles have been calculated for Eqn. 1 with K'_{obs}, K''_{obs} and pK_a estimated from the data by an iterative process with the aid of a programmable pocket calculator (Texas Instruments TI 58).

$$K_{obs} = \frac{K'_{obs}}{1 + K_a/(H^+)} + \frac{K''_{obs}}{1 + (H^+)/K_a} \quad (1)$$

where K_{obs} is the observed second-order rate constant; K'_{obs} the apparent second-order rate constant for the modification of the protonated species, and K''_{obs} the one for the deprotonated species.

Modification with tetranitromethane. The incubation mixture with tetranitromethane (0.2 ml) contained trehalase (50 mU), 4.2 mM tetranitromethane, 0.5% ethanol and 75 mM phosphate buffer (pH 7.0). The reaction mixture was maintained at room temperature (22°C) and samples were collected at different time (up to 100 min) and run on Bio-Gel P-30 columns as described for carbodiimides.

Results

Kinetics of inactivation of trehalase by EDC plus glycine ethyl ester

The inactivation of trehalase by EDC plus glycine ethyl ester at different pH

values is shown in Fig. 1. Similar results were obtained using only EDC. Exhaustive dialysis of the inactivated enzyme gave no reactivation. Carbodiimides are known to react with carboxyl, sulfhydryl and phenol groups [8]. Sulfhydryl groups are absent from the trehalase [7]. Reaction with tyrosine is improbable, since less than 10% inactivation of the enzyme was found with tetranitromethane in conditions in which it is a fairly specific reagent for phenol groups in enzymes [9]. Furthermore, if part of the enzyme inactivation had been caused by reaction of phenol groups with carbodiimide, at any of the pH values tested, treatment with 0.5 M hydroxylamine at pH 7.0 and 25°C for 5 h would have released the carbodiimide from the tyrosine residue [8], and a partial reactivation of the enzyme should have been observed. Since this was not the case (data not shown), the data suggest that only carboxyl groups have been modified in all pH values tested.

The inactivation of trehalase by EDC plus glycine ethyl ester at pH 6.8—8.5 (6.9 is shown in Fig. 1) follows pseudo first-order kinetics. At those pH values the enzyme is not protected against modification by 140 mM (100 k_i) sucrose, which is a simple intersecting linear competitive inhibitor of trehalase [7], or by 70 mM (100 k_m) trehalose.

At pH values from 6.0 to 6.6 (6.2 is shown in Fig. 1) the inactivation of trehalase by EDC plus glycine ethyl ester follows pseudo first-order kinetics. Sucrose partially protects the enzyme against modification at those pH values (pH 6.2 is shown in Fig. 1).

The k_m and pH optimum values of the partially inactivated trehalase at pH 7.1 and 6.2 did not differ from the values of the native enzyme. It seems likely that the measurable activity of the partially inactivated enzyme at both pH values represent residual unmodified enzyme rather than altered enzyme with distinct kinetic properties. It also seems likely that there is a carboxyl reacting at pH 6.2 which differs from the one reacting at pH values above 6.8, since only the modification at pH 6.2 is decreased in the presence of sucrose.

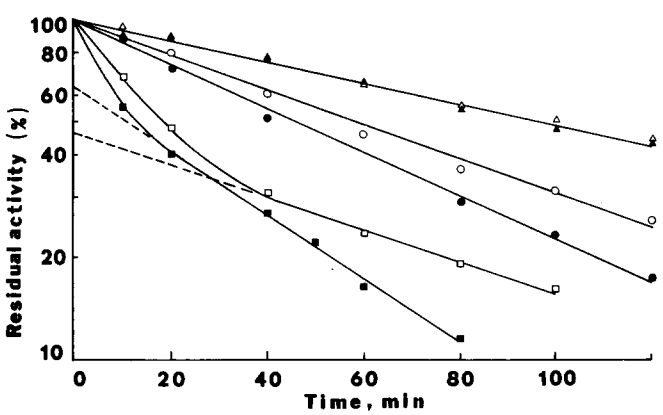

Fig. 1. Inactivation of trehalase by EDC plus glycine ethyl ester at 30°C. Reaction mixtures (0.2 ml) contained trehalase (50 mU), 6 mM EDC, 40 mM glycine ethyl ester and 60 mM TEMED/HCl buffer, pH 6.9 (▲, △), pH 6.2 (●, ○) or pH 5.6 (■, □) in the presence (△, ○, □) or in the absence (▲, ●, ■) of 140 mM sucrose. Ionic strength: 0.15 M. Samples (10 μl) were removed from the reaction mixture at the indicated times and diluted to 1 ml with 0.05 M citrate/phosphate buffer (pH 6.0). From these diluted samples 0.4 ml portions were used for assaying residual activity as described elsewhere [7].

Semi-logarithmic plots of trehalase activity as a function of time of inactivation by EDC and glycine ethyl ester at pH values from 5.4 to 6.0 (5.6 is shown in Fig. 1) is a curve at first and rectilinear afterwards. Such inactivation behavior should be a consequence of the modification of two types of carboxyl groups. One such group is completely modified in the first 30 min, rendering the enzyme partially active, while the modification of the other is paralleled by total inactivation of the enzyme according to pseudo first-order kinetics. The presence of sucrose in the reaction medium confers partial protection against modification of the late carboxyl group. Otherwise in its presence more carboxyl groups, whose modification partially inactivate the enzyme, react (compare extrapolations of the rectilinear segments of the plot at pH 5.6, in the presence and absence of sucrose, in Fig. 1).

The enzyme modified at pH 5.6 has altered kinetic properties, since its k_m has changed from 0.7 to 0.9 mM and its pH optimum from 6.2 to 6.0.

The inactivation of trehalase by EDC plus glycine ethyl ester at pH values lower than 5.4 follows kinetics that are too complex to be interpreted.

The reaction order with respect to the inhibitor was determined from a plot of log K_{obs} (K_{obs} is the observed first-order rate constant for enzyme inactivation) against log [EDC]. This type of plot should give a straight line with a slope equal to n, the apparent number of molecules of inhibitor reacting with each active site of the enzyme to give an inactive enzyme-inhibitor complex [10]. When the data were plotted in this manner, straight lines (correlations better than 0.99) were obtained from K_{obs} determined at pH 5.6, 6.2 and 7.1 with slopes respectively equal to 1.04, 1.03 and 1.05. This suggests that the reaction of one molecule EDC per active site of trehalase is necessary for inactivation. In other words the data support the assumption that the modification of any of the two carboxyl groups supposed to be reacting at pH 6.2 (see above) is sufficient to inactivate the enzyme.

pK_a and other properties of EDC-reactive groups

The pH profile of trehalase inactivation by EDC plus glycine ethyl ester in the pH range from 5 to 7 is shown in Fig. 2 and that from 7 to 9 in Fig. 3.

A profile of the observed rate constant (under conditions where the reagent concentration is low enough for the reaction to be first order in reagent) against pH can, in general, reflect free-reactant-state molecular pK_a values only if a quasi-equilibrium exists around the reactive protonic state of the adsorptive complex [11]. Although this has not been assessed directly for trehalase, the fact that the pH-independent K_{obs} values (K'_{obs}) are much less than $10^6\ M^{-1} \cdot s^{-1}$ makes the equilibrium assumption valid [11]. Thus our data indicate the existence of a carboxyl group ionizing at low pH ($pK_a = 5.28 \pm 0.08$) and another ionizing at high pH ($pK_{a_2} = 7.74 \pm 0.08$). A carboxyl group having pK_a 7.74 must be in a highly hydrophobic environment and hence one can expect it is buried in the enzyme molecule. The finding that the carboxyl with pK_a 5.28 ($K'_{obs} = 731\ M^{-1} \cdot s^{-1}$) is 10 times more reactive than the carboxyl with pK_a 7.74 ($K'_{obs} = 68.7\ M^{-1} \cdot s^{-1}$) supports this assumption.

The presence of dioxane at pH values below 6.4 results in inactivation of trehalase by EDC plus glycine ethyl ester according to kinetics too complex to be interpreted here. In spite of this problem, the data shown in Fig. 2 suggest

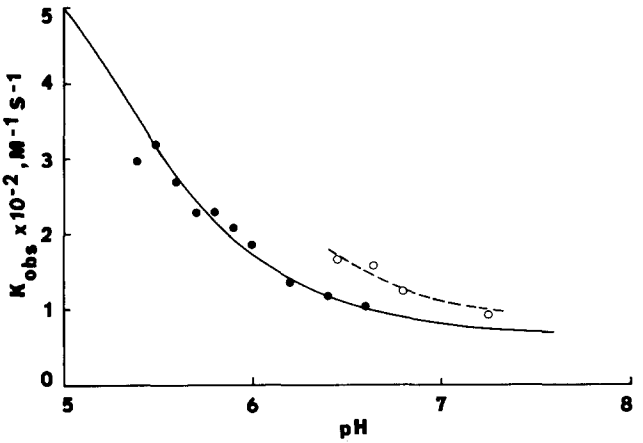

Fig. 2. Effect of pH (range from 5 to 7) on the inactivation of trehalase by EDC plus glycine ethyl ester in the presence or in the absence of dioxane at 30°C. Reaction mixtures (0.2 ml) contained trehalase (50 mU), 6 mM EDC, 40 mM glycine ethyl ester and 60 mM TEMED/HCl buffer in the indicated pH values in the presence (○) or in the absence (●) of 15% (w/v) dioxane. Ionic strength: 0.15 M. Sampling and assaying as in Fig. 1. Observed second-order rate constants for trehalase inactivation (K_{obs}) were calculated from first-order rate constants determined from slopes of plots similar to those in Fig. 1. Slopes were determined from straight lines calculated by simple regression and had correlations better than 0.99. The inactivation rate constant at pH values lower than 6.0 were calculated from the rectilinear portion of the plots. Points are averages of two independent determinations.

the pK_a of the low pH EDC-reactive group increases in the presence of dioxane. The same occurs with the pK_a of the high pH EDC-reactive group which is displaced from 7.74 ± 0.08 to 7.91 ± 0.07 in the presence of dioxane (Fig. 3). Dioxane increases the pK_a of the groups which ionize with separation

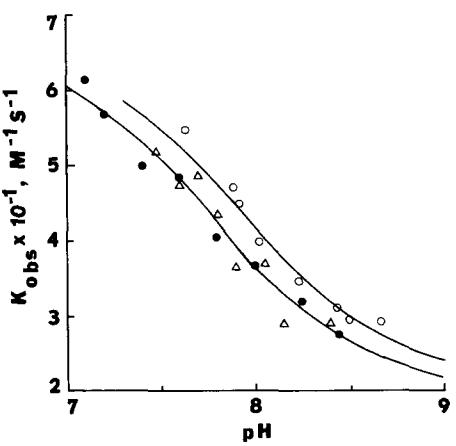

Fig. 3. Effect of pH (range from 7 to 9) on the inactivation of trehalase by EDC plus glycine ethyl ester at different conditions at 30°C. Reaction mixtures (0.2 ml) contained trehalase (50 mU), 6 mM EDC, 40 mM glycine ethyl ester and 60 mM TEMED/HCl buffer in the indicated pH values in the presence (○) or the absence (●) of 15% (w/v) dioxane or in the presence of 15% (w/v) dioxane plus 140 mM sucrose (△). Points are averages of two independent determinations. Other details as in Fig. 2.

of charges [12], thereby supporting the above state that both EDC-reactive groups are carboxyls.

Sucrose present at the trehalase-active site prevents binding of dioxane [7]. Thus the failure of dioxane to change the pK_a of the carboxyl with pK 7.74 in the presence of sucrose (Fig. 3) suggests the carboxyl is in the neighborhood of the active site.

Discussion

The EDC-reactive carboxyl groups might be involved in catalysis considering the following: (a) their pK_a values agree approximately with those of the trehalase essential groups calculated from the pH effects on kinetic data [7]; (b) their modification by EDC seems to result in a completely inactive enzyme. A catalytic role for the pK_a 5.28 carboxyl is further substantiated by the finding that extension of its modification by EDC is decreased in the presence of sucrose, a good competitive inhibitor of trehalase, suggesting that the carboxyl is in the active site. Nevertheless, some results must be considered before one can propose a direct catalytic involvement for the pK_a 7.74 carboxyl group. The presence of this carboxyl group in the active site is improbable, as judged from the failure of sucrose and trehalose to confer partial protection against modification by EDC, although the lack of change in its pK_a in the presence of dioxane plus sucrose suggests it is at least in the neighborhood of the active site. Otherwise the high pK_a and low reactivity of pK_a 7.74 carboxyl towards EDC suggest it is buried in the enzyme molecule. Thus if the pK_a 7.74 carboxyl is actually placed near but outside the active site (understood here as the substrate binding place) it makes it difficult to act as an acid to protonate the substrate glycosyl oxygen during catalysis as was proposed [7]. Nevertheless it could function in the protonation step through another amino acid residue, such as the proton transfer from a serine to a buried carboxyl group through histidine reported to occur in the chymotrypsin-active site [13]. This possibility, however, is under investigation at the present time.

Acknowledgements

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