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Time-dependent inhibition of porcine kidney trehalase by aminosugars

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The inhibitory effects of various nitrogen-containing sugars on porcine kidney trehalase were studied. Validamycin A, validoxylamine A and MDL 25637 were found to be potent, time-dependent inhibitors of the enzyme in vitro. The validoxylamine A-inhibited enzyme showed slow but reversible reactivation over time ($t_{1/2} = 1.2$ h). To our knowledge, this is the first report of time-dependent inhibition exhibited by either these particular aminosugars or a trehalase.

Trehalase; Amino sugar; Enzyme inhibition; (Porcine kidney)

1. INTRODUCTION

Trehalase (α,α-trehalose glucohydrolase, EC 3.2.1.28) is the enzyme involved in the catabolism of the nonreducing disaccharide α,α-trehalose into glucose. Trehalose is a key storage carbohydrate in certain insects, fungi and bacteria [1]. The enzyme is widespread in nature, even in organisms presently known not store the sugar, such as mammals. There is currently great interest in the development of inhibitors of glycohydrolytic enzymes as biological probes of enzyme mechanism and physiology as well as potential medicinal agents [2–5]. Several compounds have recently been reported to inhibit trehalases from various sources [6–8]. The current investigation concerns the previously unreported time-dependent inhibition of porcine kidney trehalase by these N-containing compounds which are structural analogues of the natural substrate, trehalose (fig.1).

2. MATERIALS AND METHODS

2.1. *Materials*

Porcine kidney trehalase (3.4 units/mg), glucose oxidase (type II), horseradish peroxidase (type II), trehalose dihydrate, *o*-dianisidine dihydrochloride, castanospermine and 2[*N*-morpholino]ethanesulfonic acid (Mes) were purchased from Sigma Chemical (St. Louis, MO). Validamycin A and MDL 25 637 were kind gifts from Takeda Chemical Industries (Tokyo, Japan) and Merrell Dow Research Institute (Ohio, USA), respectively. Validoxylamine A was obtained from the hydrolysis of validamycin A using a modified procedure of Kameda and colleagues [9] employing Dowex 50W-X2 as catalyst. All other chemicals were from local suppliers and were of analytical grade.

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Dedicated to the memory of Dr Bernard Belleau

2.2. *Methods*

Trehalase activity was assayed at 30°C in a total vol. of 1.0 ml containing 10.0 mM trehalose, porcine kidney trehalase (2 mU) and 50 mM Mes buffer (pH 5.6). After 30 min of incubation, the reaction was interrupted by the addition of 2.0 ml glucose oxidase-peroxidase (GOP) reagent [10] which was used for the determination of the liberated glucose. After further incubation at 30°C for 30 min, 4.0 ml of 5 N HCl was added, and measurement at 525 nm was performed, IC_{50} values are concentrations of inhibitor necessary to inhibit trehalase by 50% at a trehalase concentration of 10.0 mM (K_M = 3.7 mM at pH 5.6). Determination of K_i values was estimated by Dixon plots of reciprocal activity vs inhibitor concentration. In preincubation experiments, inhibitor and trehalase were incubated with shaking for the time stated, and the reaction was started by addition of trehalose. Otherwise, addition of the enzyme indicates the start of the reaction. Enzyme activity was proportional to protein concentration and time of incubation. For kinetic analysis, the ratio of inhibitor to enzyme concentration was never lower than 40:1. None of the inhibitors at the highest concentration used interfered with the assay of glucose by the GOP reagent. One unit of enzyme activity is defined as the quantity of enzyme which produces 1.0 μmol glucose/min. All experiments were conducted in replicate.

For reaction progress curves, reactions with or without preincubation with an inhibitor were started by the addition of 210 μl of 100 μmol/ml trehalose in a final vol. of 2.21 ml in 50 mM Mes, pH 5.6. At various times, aliquots (300 μl) were analyzed by GOP reagent (1.0 ml). The reaction was allowed to proceed for 30 min as described above before 2.0 ml of 5 N HCl was added.

3. RESULTS AND DISCUSSION

The incorporation of appropriately positioned amino functionalities into sugar analogues has led to the production of potent inhibitors of glycohydrolytic enzymes [3,6–9,11–13]. In some cases these effects are perhaps due to their purported resemblance, when protonated by essential glutamate or aspartate functionalities in the enzymes, to transition state or reactive intermediate structures. To further understand the connection between inhibitor structure and enzyme inhibition, pig kidney trehalase was investigated with various structural analogues of its natural substrate, trehalose. Pig

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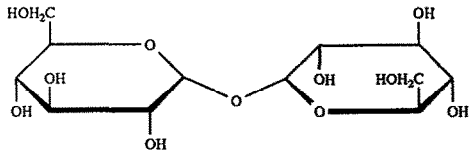
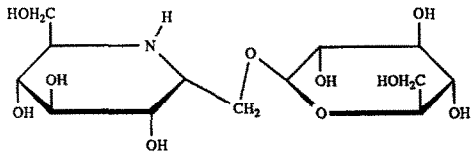
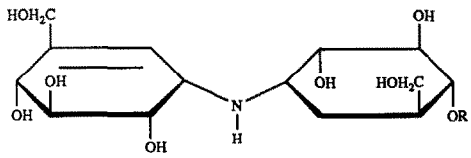


α,α-Trehalose

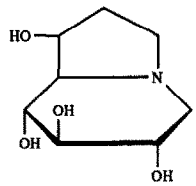


MDL 25,637



R = H Validoxylamine A

R = β-D-glucose Validamycin A



Castanospermine

Fig.1. Structures of trehalose and selected N-containing compounds that are inhibitors of trehalase.

kidney trehalase has not previously been studied with inhibitors of this type. In this investigation a time-dependent inhibition by 3 trehalose analogues, MDL 25 637, validamycin A and validoxylamine A has been observed. In addition, this is the first report of time-dependent inhibition exhibited by a trehalase or these compounds.

Castanospermine, an aminocyclitol having inhibitory activity against several glycohydrolases [14,15], has good inhibitory activity against the trehalase (IC_{50} = 5.5×10^{-6} M). The analogues that are structurally similar to the natural substrate were more potent having IC_{50} values of 4.5×10^{-9} M, 1.1×10^{-8} M, and 1.0×10^{-7} M for validoxylamine A, MDL 25 637 and

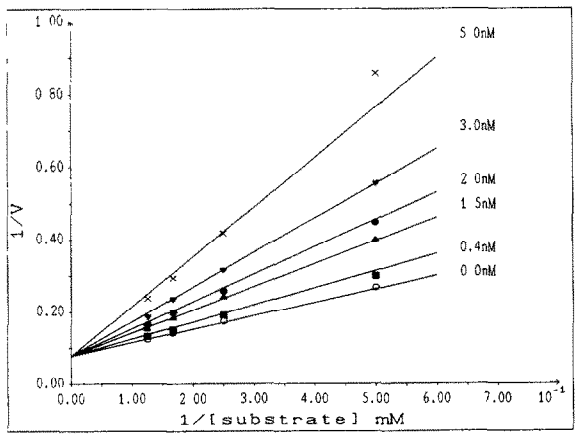


Fig.2. Lineweaver-Burk plot of enzyme activity in the presence of validoxylamine A.

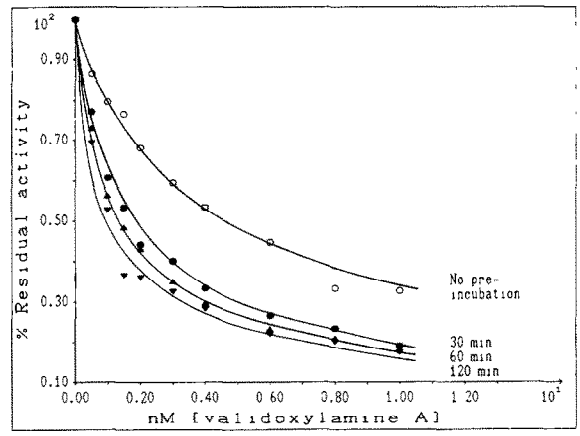


Fig.3. Inhibition of porcine kidney trehalase by validoxylamine A. Enzyme and inhibitor were preincubated for 0 (○), 30 (●), 60 (▲) and 120 (▼) min at room temperature before the addition of 100 μl of 100 μmol/ml trehalose in a final vol. of 1.0 ml. Values are presented as a percent of the enzyme activity. Activity was 4–7 nmol glucose/min.

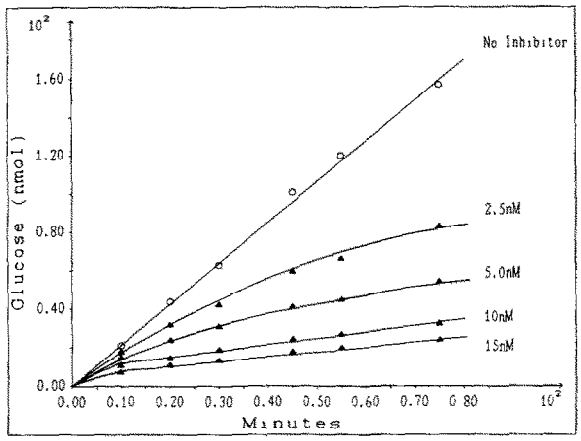


Fig.4. Reaction progress curve. Porcine kidney trehalase was incubated with different concentrations of validoxylamine A.

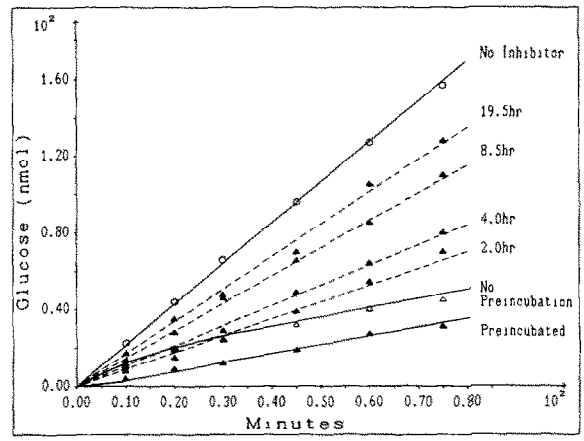


Fig.5. Reaction progress curve. No inhibitor (○), 12.5 nM validoxylamine A without preincubation (△), and 12.5 nM validoxylamine A preincubated for 60 min at room temperature (▲). Preincubated samples (2.0 ml) were dialyzed against 500 ml of 25 mM Mes buffer, pH 5.6. At appropriate times, the content of the dialysis bags was transferred into test tubes and reactions were started by the addition of 210 μl of 100 μmol/ml trehalose (dashed lines).

validamycin A, respectively. Without preincubation, validoxylamine A is a competitive inhibitor of pig trehalase (fig.2) with a K_i value of 2×10^{-9} M. MDL 25 637 and validamycin A are also competitive inhibitors of the enzyme (plots not shown) with K_i values of 3×10^{-9} M and 6×10^{-8} M, respectively.

However, in preincubation experiments, validoxylamine A showed noncompetitive inhibition upon simple steady-state kinetic analysis (data not shown). Fig.3 illustrates the relationship of preincubation time to enzyme activity for validoxylamine A. These observations, also observed for MDL 25 637 and validamycin A suggest that the interaction of these inhibitors and the enzyme is a slow binding process [16,17]. Analysis of the product vs time plot for the trehalose inhibitor analogues by the method of Baici and Gyger-Marazzi [18] resulted in presteady state K_i values for validoxylamine A (fig.4), MDL 25 637 and validamycin A of 8.5×10^{-10} M, 2.2×10^{-9} M and 2.3×10^{-8} M, respectively. The k_{on} values for validoxylamine A, MDL 25 637 and validamycin A were calculated to be 2.7×10^{5} $M^{-1}s^{-1}$, 1.8×10^{5} $M^{-1}s^{-1}$ and 1.4×10^{4} $M^{-1}s^{-1}$, respectively. The k_{off} values obtained by the above approach for validoxylamine A, MDL 25 637 and validamycin A were 2.3×10^{-4} s^{-1} ($t_{1/2}$ = 1.2 h), 3.8×10^{-4} s^{-1} and 3.3×10^{-4} s^{-1}, respectively. Plots of reaction progress with and without preincubation with validoxylamine A indicate linear first order kinetics upon preincubation (fig.5).

Investigations to determine the reversibility and the expected reactivation of the inhibited enzyme upon dilution were performed. Exhaustive dialysis of the enzyme preincubated with validoxylamine A resulted in time-dependent reactivation of the enzyme activity (fig.5). Interestingly, a recent report has shown that time-dependent inhibition of α-glucosidase with castanospermine is a nonreversible process even upon extensive dialysis [18]. This phenomenon is not observed with porcine kidney trehalase in the present studies. The interaction of MDL 25 637, validamycin A and validoxylamine A with porcine kidney trehalase appears to be in the category of slow-binding inhibition. Apparently, in the appropriate molecular context, an amino functionality can be positioned either in the ring structure or in the glycosidic bond to produce potent time-dependent interactions with the same enzyme.

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