BBA 65633

INHIBITION OF BARLEY MALT α-GLUCOSIDASE BY TRIS(HYDROXYMETHYL)AMINOMETHANE AND ERYTHRITOL

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(Received April 17th, 1967)

SUMMARY

1. The competitive inhibition of barley malt α-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20) with Tris and erythritol has been studied in the pH interval 3–7 with maltose as substrate.

2. At pH 4.6, Tris and erythritol compete with each other and with maltose for the enzyme.

3. The variation of the inhibitor constant for Tris and erythritol and of the Michaelis constant for maltose with pH, shows that Tris and maltose react with different groups in the enzyme, which makes it unlikely that Tris is a substrate analogue.

4. Erythritol requires 2 groups in the enzyme and competes with maltose for one of the groups.

INTRODUCTION

It is known that several glycoside hydrolases are inhibited by Tris[1–4] and polyols such as erythritol[5]. Barley malt α-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20) is competitively inhibited by Tris and erythritol. To throw some light on the mechanism of this reaction, the inhibition of barley malt α-glucosidase by the two inhibitors was examined. The variation with pH of the Michaelis constant K_m, of the maximum velocity V, and of the competitive inhibitor constants K_i, has been studied with maltose as substrate.

MATERIALS AND METHODS

Substrate and inhibitors

Maltose (Kerfoot) was purified by charcoal adsorption. Tris (2-amino-2-hydroxymethylpropane-1,3-diol) was obtained from Sigma and erythritol from Merck.

Enzyme

The enzyme was extracted from barley malt and fractionated as described in a previous paper[6].

Enzyme activity determination

The initial velocity was measured as follows: In a test tube containing 400 μl reaction mixture (see below) 100 μl enzyme was added at time zero, while 100 μl enzyme was likewise added to another test tube containing 400 μl reaction mixture without substrate (blank). After incubation for 60 min at 37° the reaction was stopped, and the glucose released was determined by adding 3 ml Tris–glucose oxidase reagent[6,7]. Tris of the concentration used in this reagent inhibits the malt α-glucosidase activity. After incubation for 60 min at 37° the absorbance at 420 mμ was measured. Appropriate standards containing 0, 2, 5, 20, and 50 μg of glucose per 0.5 ml were run simultaneously. The determinations were run in duplicate, and it was found that when the hydrolysis of the maltose did not exceed 5% the degree of hydrolysis is apparently proportional to the enzyme concentration.

Determination of K_m, V, and K_i

K_m, V, and K_i were determined at different pH values by the Lineweaver–Burk method, plotting reciprocals of initial velocities *versus* reciprocals of substrate concentrations at each of the following pH values: 3.15, 3.25, 3.55, 4.00, 4.50, 5.10, 5.60, 6.15 and 6.85. The maltose concentrations 0, 1.0, 2.0, 4.0 and 8.0 mM were used with 2.0 mM Tris or with 10.0 mM erythritol or without any inhibitor added. The Tris solutions were brought to the desired pH by titration with HCl. The desired pH values in the final reaction mixtures were obtained by adding phosphate–acetate buffers (0.08 M acetate, 0.03 M phosphate).

In experiments in which the competition between Tris and erythritol was examined the maltose concentration was 0, 1.0, 2.0, 4.0 or 8.0 mM, the Tris concentration was 0 or 2.0 mM, and the erythritol concentration was 0, 10, 20, 30 or 50 mM, while the pH was 4.6.

RESULTS

Combined inhibition of Tris and erythritol

It was found in inhibition experiments that malt α-glucosidase was competitively inhibited by Tris and erythritol. Therefore it was of interest to examine if Tris and erythritol competed with each other when both inhibitors were used together. If the inhibitions by Tris and by erythritol are without influence on each other (*i.e.* they compete with maltose at different groups in the enzyme), then the initial velocity v can be calculated using the equation $v = V/(1 + K_p/s)$ where

$$K_p = K_m \left(1 + \frac{i_T}{K_{iT}}\right)\left(1 + \frac{i_E}{K_{iE}}\right) \qquad (a)$$

v is the initial velocity, V the maximum velocity, K_p the 'apparent Michaelis constant', s the initial maltose concentration, K_m the Michaelis constant for maltose, i_T the Tris concentration, i_E the erythritol concentration, and K_{iT} and K_{iE} the competitive inhibitor constants for Tris and erythritol, respectively. K_p is determined experimentally as K_m by the Lineweaver–Burk method.

If the two inhibitors compete with maltose at the same groups in the enzyme or give rise to steric hindrance, they may be expected to compete with each other. In this case the value of K_p is given by

TABLE I

COMBINED INHIBITION OF MALT α-GLUCOSIDASE BY Tris AND ERYTHRITOL

Maltose concentration 0–8 mM, pH 4.6, K_{iE} 10.9 mM erythritol, K_{iT} 3.0 mM Tris, and K_m 2.4 mM maltose.

i_E erythritol (mM)	i_T Tris (mM)	K_p	K_p*	K_p**
50	2	15.4	22.4	15.0
30	2	10.0	15.0	10.6
20	2	8.3	11.4	8.4
10	2	6.3	7.7	6.2
0	2	4.0	4.0	4.0
10	0	4.6	(4.6)	(4.6)
0	0	2.4	(2.4)	(2.4)

* Calc. from Eqn. a.
** Calc. from Eqn. b.

$$K_p = K_m \left(1 + \frac{i_T}{K_{iT}} + \frac{i_E}{K_{iE}}\right) \quad \text{(b)}$$

Table I shows the results of such an experiment. The values of K_p calculated from Eqn. b are in close agreement with the experimental K_p values. This shows that Tris and erythritol at pH 4.6 compete with each other and with maltose for the enzyme.

Inhibition by Tris

Table II shows the variation of K_m, V, and the competitive inhibitor constant K_{iT} with pH. The inhibitor constant is calculated on the basis of the concentration of total Tris or of Tris cation or of Tris base, using the ionization constant $5.8 \cdot 10^{-9}$.

Plotting pK_m (−log K_m), log V, and pK_{iT} (−log K_{iT}) against pH according

TABLE II

THE VARIATION WITH pH OF THE MICHAELIS CONSTANT K_m, THE MAXIMUM VELOCITY V, AND THE COMPETITIVE INHIBITOR CONSTANTS FOR Tris, Tris CATION, AND Tris BASE (K_{iT}, K_{iTc}, AND K_{iTb}) FOR THE MALT α-GLUCOSIDASE-CATALYZED HYDROLYSIS OF MALTOSE

V is expressed in μg glucose per 0.5 ml per h.

pH	K_m maltose (mM)	V	K_{iT} Tris total (mM)	K_{iTc} Tris cation (mM)	$10^4 \times K_{iTb}$ Tris base (mM)	K_{iE} erythritol (mM)
3.15	9.1	45.5				45.0
3.25	7.1	47.6	37.6	37.6	3.9	36.6
3.55	4.6	52.6	32.1	32.1	6.2	19.4
4.00	2.6	47.6	11.4	11.4	6.7	10.0
4.50	2.5	45.5	3.3	3.3	6.1	12.2
5.10	2.0	35.7	0.87	0.87	6.2	11.8
5.60	1.8	25.0	0.45	0.45	10	12.7
6.15	1.8	14.3	0.33	0.33	25	23.3
6.85	1.8	5.3	0.33	0.32	130	85.5

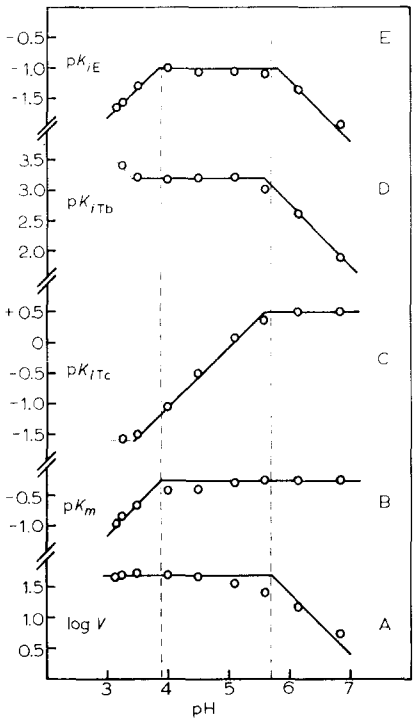

Fig. 1. Effect of pH on pK_m, pK_{iTc}, pK_{iTb}, pK_{iE}, and log V for the hydrolysis of maltose by an α-glucosidase from barley malt. K_m is Michaelis constant. K_{iTc}, K_{iTb}, and K_{iE} are competitive inhibitor constants for Tris cation, Tris base, and erythritol, respectively. V is maximum velocity. The straight-line sections are drawn with the slopes +1, 0, and −1.

to DIXON[8] and LAIDLER[9] (Fig. 1) biphasic curves are found. The curves for pK_m and log V suggest that 2 ionizable groups (pK_a 3.9 and pK_a 5.7) in the enzyme are involved in the hydrolysis of the non-ionized substrate. The group with pK_a 3.9 is involved in the complexing of the substrate and the enzyme. This is in accordance with the previous result[10], *viz.*, that the enzyme exists in three ionized forms $XHYH^+$, X^-YH^+, and X^-Y and that the substrate has the greatest affinity for X^-YH^+ and X^-Y or, in other words, for the negatively charged group in the enzyme.

The pK_{iT} *versus* pH curves can be interpreted in different ways. If it is assumed that the Tris cation is the inhibiting substance, Curve C (Fig. 1) is found, and it is seen that the Tris cation is bound with the greatest affinity to X^-Y, and the slope shows that in a reaction between Tris and $XHYH^+$ or X^-YH^+ a proton is liberated. If it is assumed that the Tris base is the inhibiting substance, Curve D (Fig. 1) is found, and it is seen that the Tris base is bound with the greatest affinity to $XHYH^+$ and X^-YH^+, and the slope shows that by a reaction between Tris base and X^-Y the complex takes on a proton. It is not possible from these experiments to decide whether the Tris cation or the Tris base is the inhibiting substance. The value of the inhibitor constant calculated on the basis of Tris base inhibition is very low, however, and this suggests a great affinity between enzyme and Tris base, which perhaps makes the Tris base inhibition unlikely. As Tris, in the reaction mixture,

mainly exists as Tris cation, it is not possible to distinguish between the inhibition by Tris cation and by total Tris (see Table II).

In view of the difficulties involved in obtaining accurate K_i values at low pH it is uncertain whether or not there is an inflexion point at pH 3.4. It is possible that the Tris–enzyme complex, but not the free enzyme, takes on a proton below pH 3.4.

Inhibition by erythritol

Table II shows the results of a similar experiment with erythritol as competitive inhibitor. Plotting pK_{iE} against pH, Curve E (Fig. 1) is obtained. The pK_{iE} curve shows 2 inflexion points at about pH 3.9 and pH 5.7; the same inflexion points are found in the pK_m and log V curves, respectively. This result shows that erythritol has the greatest affinity for the isoelectric enzyme form X^-YH^+.

DISCUSSION

On the assumption that malt α-glucosidase exists in three ionized forms, $XHYH^+$, X^-YH^+ and X^-Y, the substrate will react with X^-YH^+ and X^-Y, *i.e.* with a negatively charged group in the enzyme (pK_a 3.9). Tris is a competitive inhibitor; if Tris base is the inhibitor, it reacts with $XHYH^+$ and X^-YH^+, *i.e.* with the positive group YH^+; if Tris cation is the inhibitor, it reacts with X^-Y, *i.e.* with the uncharged group Y. Tris reacts in both cases with the group in the enzyme having pK_a 5.7. This means that Tris is not a substrate analogue of maltose. Comparison between the variation with pH of pK_m and of pK_{iE} shows that erythritol is not a true substrate analogue in the whole pH range. MYRBÄCK[11] has recently shown that the inhibition of yeast β-fructosidase by Tris at pH 5–7 can be interpreted in a similar manner as the inhibition of malt α-glucosidase by Tris.

The competitive Tris inhibition of maltose hydrolysis can be explained by the assumption that Tris, by complexing with the enzyme, causes a steric hindrance for maltose or that the complexing with Tris changes the conformation or the charge distribution of the enzyme. The competitive erythritol inhibition of maltose hydrolysis can be explained as a competition for the isoelectric enzyme form X^-YH^+, in which erythritol requires both groups, *i.e.* the same group as maltose and the same group as Tris. It is then to be expected that Tris and erythritol compete with each other, and this is confirmed by the experiments.

ACKNOWLEDGEMENTS

Financial support from Statens almindelige Videnskabsfond is gratefully acknowledged. Our thanks are due to Miss B. NIELSEN for skilful technical assistance.

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