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THE SPECIFICITY OF THE SYNTHETIC REACTION OF TWO YEAST α-GLUCOSIDASES*

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Summary

The specificity of the hydrolytic reaction has been compared to that of the synthetic reaction for maltase and isomaltase§ (α-methyl-D-glucosidase) from *Saccharomyces oviformis*. Maltase which hydrolyzes the α-1,4-disaccharide, maltose, and the α-1,6-disaccharide, isomaltose, catalyzes the formation of both maltose and isomaltose from free glucose. Isomaltase, which hydrolyzes isomaltose but not maltose, catalyzes the formation only of isomaltose from glucose. Both enzymes hydrolyze *p*-nitrophenyl-α-D-glucoside releasing the α-anomer of glucose. The enzymes utilize the α-anomer but not the β-anomer for the synthesis of the disaccharides. These results are consistent with the double displacement mechanism for glycosidases and with the proposal that the glucosyl-enzyme complex is an intermediate in the reaction. The competitive inhibition by D-glucose is independent of its anomeric form for both enzymes.

Introduction

A number of lines of evidence suggest that glycosidases may function via the formation of enzyme-glycosyl complex which subsequently releases glycose

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§ In the absence of unequivocal designation among the recommendations of the Commission for Biochemical Nomenclature to suit our enzymes, we are reluctant to assign an official EC number to them. We tentatively suggest that our "maltase" may be in the category described by the systematic name α-D-glucoside glucohydrolase (EC 3.2.1.20) although it has an appreciable specificity for both α-1,4 and α-1,6 linkages and it thus does not fit the accompanying comments and description of reaction. Our "isomaltase" (also known as α-methyl-D-glucosidase) does not correspond to any of the available categories.

to the acceptor molecule. When the glycosidase acts as a hydrolase, the acceptor is water. When it acts as a transferase, the acceptor can be a carbohydrate or an alcohol. On the principle of microscopic reversibility it would be expected that the specificity requirements for the reversal of hydrolysis, i.e. synthesis, would conform precisely to those governing the forward reaction and would proceed through the same intermediate enzyme-glycosyl complex. Indeed, an elegant demonstration of this point has been made by Hehre and his co-workers for α- and β-amylase and glucoamylase [2].

We have available in this laboratory two pure α-glucosidases [1] isolated from *Saccharomyces oviformis*, which have overlapping specificities [3]. One enzyme, maltase, is induced by growth on maltose. The second, isomaltase, is induced together with maltase, on α-methyl-D-glucoside [4]. Both enzymes are highly active on *p*-nitrophenyl α-D-glucoside. While maltase hydrolyzes both maltose and isomaltose, isomaltase acts only on isomaltose. It has been previously demonstrated that these enzymes are effective transferring enzymes (Fukui and Axelrod, unpublished). Dr C.P. Cepurneek, in this laboratory has examined the kinetics of transfer with several substrates and acceptors with these enzymes and obtained results that are consistent with the mechanism involving an enzyme-glycosyl intermediate, in which the release of the glucosyl moiety is the rate-limiting step [5].

It was of interest to see if the hydrolytic specificities of these enzymes were reflected in their synthetic behavior. We therefore examined the anomeric requirement of the reverse reactions catalyzed by these two glucosidases using pure anomers of glucose and we also identified the synthetic products in order to see if the anomeric configuration was retained in the product. To minimize the non-enzymatic mutarotation of glucose, even at pH 6.8, and to overcome the unfavorable ΔG of synthesis, we found it necessary to employ relatively high concentrations of glucose, large amounts of enzyme and short reaction times as described by Hehre et al. [2]. Examination of the reaction products showed that maltase synthesized both maltose and isomaltose from α-D-glucose while isomaltase formed only the latter. The results in the synthetic reactions were consistent with the expectations based on the hydrolytic specificities of the two enzymes.

Materials and methods

Chemicals

Pure α-D-glucose ($[\alpha]_D^{25} = +112°$) and pure β-D-glucose ($[\alpha]_D^{25} = +18°$) were prepared by Hudson's method [6]. Optical rotations were measured in a Bendix digital polarimeter. Maltose was obtained from Nutritional Biochemical Corporation and isomaltose from Pierce Chemical Co. The commercial sugars were purified by paper chromatography before using them as chromatographic standards.

Maltitol and isomaltitol were prepared by reducing the corresponding sugars with $NaBH_4$ [7]. $NaBH_4$, 100 mg, plus 10 ml of 5% (w/v) maltose or isomaltose was stirred for 2 h at room temperature. A few drops of acetic acid were then added to destroy the excess $NaBH_4$. The solution was evaporated to dryness under reduced pressure and boiled 1 h with 2 ml of 6% methanolic

hydrogen chloride. After the removal of sodium chloride by filtration and cooling, the corresponding alcohol was recovered from the filtrate.

Enzymes

Maltase and isomaltase were purified from freshly harvested cells of *S. oviformis* which had been induced on α-methyl-D-glucoside. We are much indebted to Dr Catherine Roberts of the Carlsberg Laboratorium, Copenhagen for the original cultures. The purification procedure which involves fractionations by ammonium sulfate and chromatography on DEAE-Sephadex and hydroxyapatite is described elsewhere [3,8,9]. The enzymes were homogeneous by disc gel electrophoresis. The preparations which were stored as ammonium sulfate precipitates, were dialyzed against pH 6.8 phosphate buffer, 0.067 M, before use. A unit of enzyme activity is that amount of enzyme which releases 1.0 nmol of *p*-nitrophenol per min at 30° from 3 ml of a reaction mixture containing 2.7 ml of 0.067 M phosphate buffer, pH 6.8, 0.1 ml of reduced glutathione (0.1 mg) and 0.1 ml *p*-nitrophenyl-α-D-glucoside (0.3 mg) plus 0.1 ml of enzyme.

Paper chromatography

Chromatography of the sugars was performed with butanol/pyridine/water (6/4/3, v/v/v). The sugars were visualized with the silver nitrate dip [10].

Electrophoresis

Electrophoretic identification of the reduced disaccharides was carried out on cellulose acetate strips using the Millipore Phoroslide apparatus. The electrolyte solution was prepared by dissolving 2.5 g of sodium molybdate dihydrate in 120 ml of water and adjusting the whole to pH 5.0 with concentrated sulfuric acid [11]. Electrophoresis was carried out at 100 V for 15 min. Compounds were detected with alkaline silver nitrate [10].

Results

Reversal of hydrolysis by maltase and isomaltase

The technique described by Hehre et al. [2] for the demonstration of enzymic reversions with various amylases was used with slight modifications in our studies. Mixtures (100 µl) containing 15 mg of α-D-glucose or 15 mg of β-D-glucose and purified enzyme (100 µl, $6.03 \cdot 10^4$ enzyme units of maltase or $1.01 \cdot 10^4$ enzyme units of isomaltase) were incubated for 2, 6 and 15 min at 30° C. After the addition of 100 µl dimethyl sulfoxide and heating at 100° C to inactivate the reaction mixture, 25 µl samples were chromatographed in the descending direction on Whatman No. 1 paper. Controls with enzyme inactivated by heating were included. Fig. 1 indicates the synthesis from α-D-glucose of maltose and isomaltose, by maltase, and isomaltose, by isomaltase. A limited and delayed synthesis of those saccharides occurred when starting with β-D-glucose, owing to the formation of α-D-glucose through mutarotation.

Identification of the synthetic products

A reaction mixture as described above and initially containing α-D-glucose

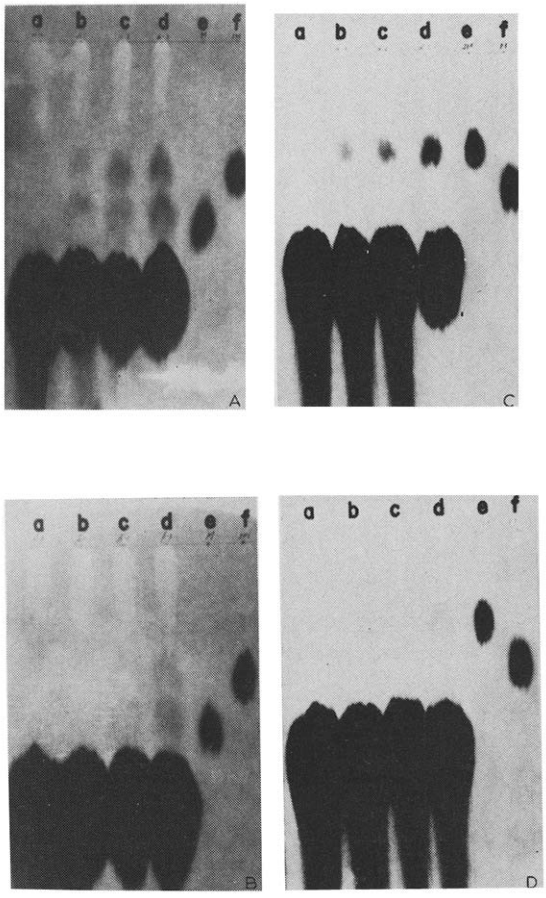

Fig. 1. Chromatograms of products obtained from incubation of maltase with (A) α-D-glucose or (B) β-D-glucose. Incubation was done at 30°C for (a) 0 time, (b) 2 min, (c) 6 min, (d) 15 min. Standards included (e) maltose, (f) isomaltose. (C) and (D) represent the corresponding experiments performed with isomaltase instead of maltase.

was incubated for 2 h and chromatographed on Whatman No. 3MM paper. Guide strips of 2 cm width were cut from the sides and stained with the silver nitrate dip. The saccharides in the corresponding sections of the unstained paper were eluted with distilled water, to give 1.0 ml volumes. As found previously (Fig. 1) maltase leads to two products, I, the slower one, and II, the faster. Isomaltase yielded a single product III having the same mobility as I. The identity of the products with maltose and isomaltose was further confirmed by the observation that following reduction with sodium borohydride they migrated as maltitol and isomaltitol, respectively (Table I). Electrophoresis in molybdate solution which discriminates between the disaccharides on the basis of the linkage [11] afforded an independent chromatographic procedure for identification (Table II).

Inhibition of maltase and isomaltase by α- and β-D-glucose

D-Glucose is known to be a competitive inhibitor of the two α-glucosi-

TABLE I

PAPER CHROMATOGRAPHY OF $NaBH_4$-REDUCED PRODUCTS FORMED FROM GLUCOSE BY MALTASE AND ISOMALTASE

Eluates (0.98 ml) were treated with 10 mg $NaBH_4$ for 2 h at 25°C. Any unreacted $NaBH_4$ was destroyed by acidification with acetic acid. The reaction mixture was evaporated to dryness in vacuum and the residue taken up in 0.1 ml H_2O. Aliquots of 10 µl were chromatographed on paper as described. For purposes of comparison the untreated eluates, as well as maltose, isomaltose, maltitol, isomaltitol, and glucose were also run.

	R_F
Product I	0.14
Product II	0.21
Product I after reduction	0.07
Product II after reduction	0.15
Product III after reduction	0.07
Maltose	0.21
Isomaltose	0.14
D-Glucose	0.30
D-Maltitol	0.15
D-Isomaltitol	0.07

dases used in this work, Fukui (unpublished results) and Cepurneek [5] found K_I values of 4 mM and 1.1 mM for maltase and isomaltase, respectively, employing a mutarotated equilibrium mixture of the glucose anomers. In view of the anomeric specificity exhibited in the hydrolytic and synthetic reactions, it was of interest to see if the inhibition was similarly specific. To minimize mutarotation the α- and β-glucose was added as the pure solid to a cuvette containing the substrate solution. The sugar dissolved rapidly (<5 s) and the enzyme was added to start the reaction. The equilibrium mutarotation mixture was taken from a previously prepared solution. The results show that inhibition by glucose is independent of the anomeric form, both for maltase and isomaltase (Fig. 2). Although the experiments described above which demonstrate anomeric specificity in glucoside synthesis make it unlikely that the enzyme has any mutarotase activity, a direct test showed that maltase has no appreciable mutarotase activity (Fig. 3). Isomaltase (curve not shown) was also inactive.

TABLE II

ELECTROPHORETIC IDENTIFICATION OF $NaBH_4$-REDUCED PRODUCTS FORMED FROM GLUCOSE BY MALTASE AND ISOMALTASE

Aliquots (0.3 µl) of the reduced products (see Table I) were subjected to electrophoresis as described in Materials and Methods. Mobilities are relative to the mobility of sorbose which is taken as 1.0.

	R_S
Product I after reduction	0.77
Product II after reduction	0.56
Product III after reduction	0.77
Sorbitol	1.0
Maltitol	0.56
Isomaltitol	0.78

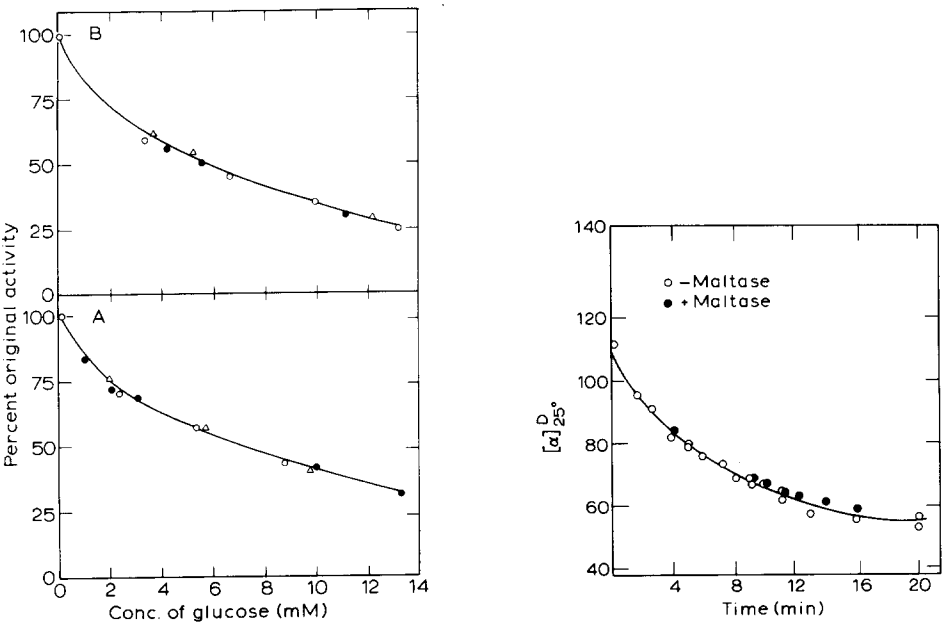

Fig. 2. Inhibition of maltase (a) and isomaltase (b) by glucose anomers. The reaction mixture is similar to that described for the assay employing *p*-nitrophenol α-D-glucoside as described under Materials and Methods. The reaction was monitored directly in the spectrophotometer. Mutarotation equilibrium mixture of glucose •; α-glucose ○; β-glucose, ▲.

Fig. 3. Effect of yeast maltase on the mutarotation of α-D-glucose. The polarimeter tube contained 5 ml of 4% α-D-glucose in 0.067 M phosphate buffer pH 6.8, ○——○; same plus maltase ($2 \cdot 10^4$ units) •——•; t = 25° C.

Anomeric configuration of D-glucose formed in glucohydrolase reactions

Although maltase and isomaltase are known to be specific for the hydrolysis of α-glucosides, it was of interest to know, as an aid in understanding their mechanism of action, which anomer of glucose is released on hydrolysis. *p*-

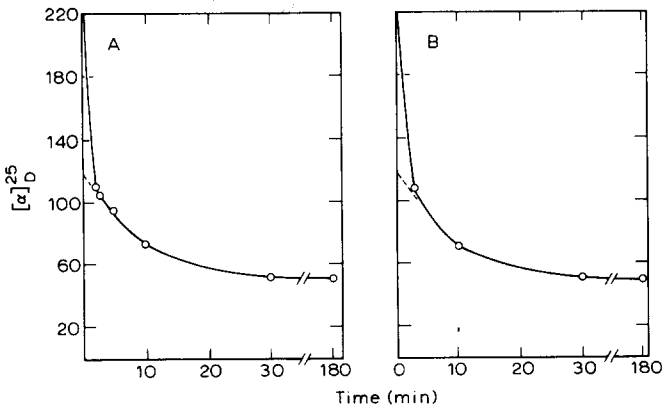

Fig. 4. Determination of anomeric configuration of D-glucose by (A) maltase and (B) isomaltase. To 5 ml of 0.01 M *p*-nitrophenyl α-D-glucoside, 1.0 ml of maltase (25 000 units) or isomaltase (40 000 units) were added and the optical rotation was read; t = 25° C.

Nitrophenyl-α-D-glucoside was used for this purpose since its aglycone, nitrophenol, is optically inactive. High concentrations of enzyme were employed to release easily measurable amounts of the product in a relatively short time and minimize the degree of non-enzymatic mutarotation. The optical rotation changed downward toward the equilibrium value showing that α-D-glucose is the anomer which is released by both maltase and isomaltase (Fig. 4). In addition, extrapolation of the equilibrium reaction curve gives an $[\alpha]_D$ of about +120°, the value for α-D-glucose.

Discussion

The disaccharidases, maltase and isomaltase, exhibit a correspondence between the anomeric specificities for the hydrolytic and synthetic reactions. In addition the correspondence extends to their respective specificities vis à vis the inter-glucose bond positions which undergo hydrolysis and synthesis. Such correspondence of anomeric specificity is in agreement with the findings of Hehre and co-workers obtained with several amylases. Moreover these workers have observed that glucoamylase, which hydrolyzes α-1,4 and α-1,6 glucosidic bonds in glucans, form both maltose and isomaltose on reversion. This enzyme which belongs to the class of glucosidases operating with inversion of configuration at the anomeric C-1 atom, was found to require β-glucose.

The determination of anomeric specificity for the synthetic reaction does not, of course, establish, as Hehre et al. [2] have noted, whether the specificity applies to the glucose as a donor or as an acceptor. These workers do suggest, however, that the anomeric configuration of the acceptor must be of little or no significance. In the case of maltase and isomaltase, work in this laboratory has shown a variety of primary alcohols to be suitable as acceptors in the transferase reaction when *p*-nitrophenol-α-D-glucoside is the donor [5], supporting the reasonable presumption that it is the anomeric configuration of the donor that is important.

The findings in this report are consistent with a mechanism involving a glycosyl-enzyme intermediate as are the following previous observations with these enzymes [5]. (a) The respective k_3 values for maltase and isomaltase are the same with a number of glucosidases. (b) When butanol and ethanol are present as competing acceptors in the transferase reaction the respective ratios of products formed by transfer are independent of the nature of the donor glucoside.

Koshland [12] has suggested a double displacement mechanism for those carbohydrase reactions which occur with retention of configuration of the glycone. He has proposed that an enzyme-substrate complex forms with inversion of configuration at the C-1 position which is followed by a second displacement to yield the free sugar in its original configuration. However, such a complex may be either a covalent combination or any reasonably stable non-covalent association. Strong support for the concept of a glycosyl-enzyme complex has come from Fink and Good [13] who succeeded in the resolution of the aglycone-releasing step from the glycosyl-releasing step, with the aid of low temperature kinetic studies. The foregoing results are consistent with a glucosyl-enzyme intermediate and indeed we have recently obtained a covalently bound glucose-isomaltase intermediate [14].

The observation that α- and β-glucose inhibit the hydrolysis equally well does raise a question about the specificity. However, there is no a priori reason why the binding of glucose by a specific region need be influenced by its anomeric condition. While the interaction of the glucose with the catalytic site is anomerically specific, such an interaction must be a relatively rare event compared to the binding site encounter which does not contribute appreciably to the overall binding.

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