CHANGE IN ANOMERIC FORM OF THE PRODUCT INDUCED BY THE AGLYCONE α-AMYLASE-CATALYZED HYDROLYSES OF PHENYL α-MALTOSIDES

Toshio SHIBAOKA, Nobuyuki SUETSUGU*, Keitaro HIROMI** and Sôzaburo ONO

The Laboratory of Biophysical Chemistry, College of Agriculture, University of Osaka Prefecture, Sakai, Japan

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1. Introduction

Amylases have been classified into α- and β-types according to the anomeric form of the sugar produced [1, 2]. This is generally inferred rather qualitatively from the direction of mutarotation of the enzymatic hydrolyzate. We established a method for determining the anomeric form of the product distinctly and quantitatively [3–6]. The method involves simultaneous measurements of reducing value and the optical rotation in the initial phase of reaction with simple substrates. Thus we found that the glucose produced from maltose, isomaltose and phenyl α-maltoside by glucoamylase from *Rhizopus delemar* is the β-anomer [3, 4], and that α-maltose is exclusively produced in the hydrolysis of phenyl α-maltoside catalyzed by Taka-amylase A [5] and the α-amylase of *Bacillus subtilis* [6]. These results have confirmed the nature of the anomeric forms as predicted qualitatively from the direction of mutarotation of hydrolyzates of the natural substrates. The configuration of the products produced by an enzyme has been believed to be inherent in the enzyme itself and independent of substrate. To our knowledge, no enzyme reactions showing substrate-dependent alteration of the configuration of the products are yet known.

In the present communication, we present evidence that the anomeric form of maltose produced from phenyl α-maltoside by *B. subtilis* α-amylase is changed from the α-form to the β-form by substitution of the aglycone by an electron-withdrawing or electron-repelling group, i.e. *p*-nitro- or *p-tert.*-butyl, respectively. The result, though it looks very striking from the conventional view of specificity of enzyme action, unequivocally indicates an alteration of reaction mechanism caused by substituents, which is also suggested from the catenary dependence of maximal velocity on the inductive constant of substituent observed in our separated study [7].

2. Materials and methods

Materials: Crystalline *B. subtilis* α-amylase was purchased from Nihon Seikagaku Kogyo Co., Ltd.

p-Nitrophenyl α-maltoside and *p-tert.*-butylphenyl α-maltoside were prepared by the method of Helferich and Peterson [8]. The detailed procedure and the properties of the substrates are described elsewhere [7, 8].

Methods: The enzyme reactions were carried out at 24.8°C and pH 5.40 with 0.02 M acetate buffer. The concentrations of substrate and enzyme were 0.436 mM and 3.69×10^{-7} M respectively, throughout the experiments. At this low substrate concentration, the products were only maltose and phenols. Therefore, the molar concentration of phenol produced is equal to that of total maltose (sum of α- and β-maltose). The production of phenols and

* Present address: The Laboratory of Biopolymers, Department of Agriculture, Kyoto Prefectural University, Kyoto, Japan.

** Present address: The Laboratory of Enzyme Chemistry, Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto, Japan.

the change in optical rotation were followed continuously at appropriate wavelengths by the use of a spectrophotometer (Hitachi-Perkin-Elmer 139) and a spectropolarimeter (Yanagimoto ORD-185), respectively [6]. The wavelengths used for spectrophotometric and optical rotatory measurements were 286 nm and 320 nm, respectively, with *p-tert.*-butylphenyl α-maltoside, and 400 nm for both measurements with *p*-nitrophenyl α-maltoside. The molar concentrations of phenols produced were calculated from the absorbance change by using the molar absorptivity differences between the phenols and the substrates, which were determined at the wavelengths employed (590 cm^{-1} at 286 nm for *p-tert.*-butylphenyl α-maltoside, and 456 cm^{-1} at 400 nm for *p*-nitrophenyl α-maltoside). At least three runs for both measurements were made under strictly the same conditions.

3. Results and discussion

Anomeric forms of maltose produced from the hydrolyses of phenyl α-maltosides by *B. subtilis* α-amylase can be determined quantitatively by the following equations [3–6].

$$C_{\alpha M} = \frac{10\alpha + ([M_{\phi M}] - [M_{\beta M}])\, C_M - [M_{\phi M}]\, C^0_{\phi M}}{[M_{\alpha M}] - [M_{\beta M}]} \quad (1)$$

$$C_{\beta M} = \frac{[M_{\phi M}]\, C^0_{\phi M} - ([M_{\phi M}] - [M_{\alpha M}])\, C_M - 10\alpha}{[M_{\alpha M}] - [M_{\beta M}]} \quad (2)$$

where $C^0_{\phi M}$ is the initial molar concentration of the phenyl α-maltoside, and C_M, $C_{\alpha M}$ and $C_{\beta M}$ are the molar concentrations of total free maltose, α-maltose and β-maltose respectively, α is the optical rotation of the reaction mixture for 10 cm optical path and $[M_{\phi M}]$, $[M_{\alpha M}]$ and $[M_{\beta M}]$ are the molecular rotations of the phenyl α-maltoside, α-maltose and β-maltose, respectively.

The time course of C_ϕ ($= C_M$) and α during the hydrolysis of *p-tert.*-butylphenyl α-maltoside by *B. subtilis* α-amylase are shown in fig. 1. From these curves, $C_{\alpha M}$ and $C_{\beta M}$ were calculated by using eqs. 1 and 2 and the following values of molecular rotations; $[M_{\phi M}] = 3967°$ (for *p-tert.*-butylphenyl α-maltoside), $[M_{\alpha M}] = 2395°$ and $[M_{\beta M}] = 1728°$ at 320 nm and 24.8°C. The results are shown in fig. 2 together with the curve of C_M ($= C_\phi$). At the begin-

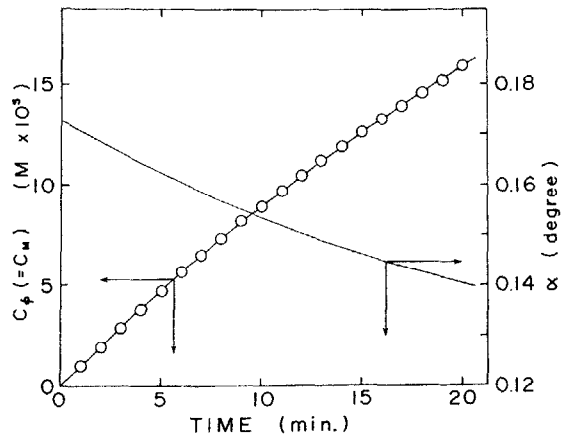

Fig. 1. Changes in optical rotation (——) and concentration of *p-tert.*-butylphenol (○) during the hydrolysis of *p-tert.*-butylphenyl α-maltoside catalyzed by *B. subtilis* α-amylase at pH 5.40 and 24.8°C. Substrate concentration, 0.436 mM; enzyme concentration, 3.69×10^{-7} M. α = Optical rotation (degrees) of the reaction mixture for a 10 cm optical path at 320 nm, C_ϕ = concentration of *p-tert.*-butylphenol, which is equal to the total free maltose concentration C_M.

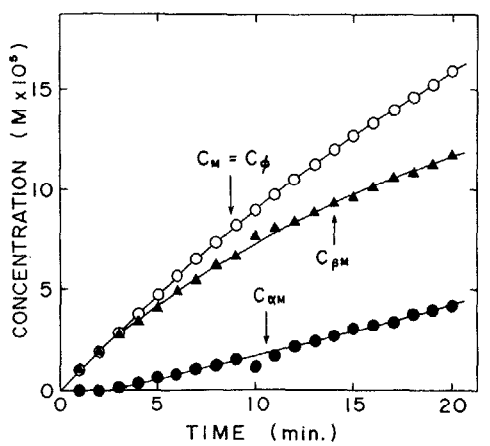

Fig. 2. Concentrations of total free maltose, α-maltose and β-maltose during the course of hydrolysis of *p-tert.*-butylphenyl α-maltoside catalyzed by *B. subtilis* α-amylase ○—○ : Total free maltose (C_M), ●—●: α-maltose ($C_{\alpha M}$), ▲—▲: β-maltose ($C_{\beta M}$). $C_{\alpha M}$ and $C_{\beta M}$ were obtained from the results of fig. 1 by using eqs. 1 and 2.

Table 1
Anomeric forms of maltose produced and breakdown rate constant of ES complex for *B. subtilis* α-amylase catalyzed hydrolyses of phenyl α-maltosides at pH 5.40 and 24.8°.

Substituent	Anomeric form of maltose produced	k_{+2} (sec^{-1}) [a]	Inductive constant [b] (σ^0)
p-$C(CH_3)_3$	β	8.1	−0.174
H	α	1.2	0
p-NO_2	β	5.6	0.82

[a] Breakdown rate constant of ES into product, obtained in a separate study [7].
[b] From [10, 11].

ning of the reaction, the rate of β-maltose formation ($C_{\beta M}$) is equal to that of total maltose formation (C_M). It was clearly demonstrated, therefore, that maltose released from *p-tert.*-butylphenyl α-maltoside is entirely in the β-form. The slow appearence of α-maltose is obviously due to the spontaneous mutarotation of β-maltose. By the same procedure, it was found that the maltose released from *p*-nitrophenyl α-maltoside by the same enzyme is entirely the β-anomer.

These results are in remarkable contrast to the previous finding that α-maltose is exclusively produced from unsubstituted phenyl α-maltoside by the same enzyme [6]. The anomeric form of maltose produced from phenyl α-maltoside was reinvestigated in the present series of experiments and the previous result was confirmed. The anomeric forms of maltose produced by the α-amylase-catalyzed hydrolyses of the three phenyl α-maltosides are summarized in table 1, which includes the breakdown rate constant of the ES complex, k_{+2}, [7] and the inductive constant σ^0 of the substituents [10, 11].

The fact that the anomeric form of the maltose changes from α to β according to the nature of the aglycone seems peculiar in view of the conventional concept of enzyme specificity. In organic reactions, however, it is often observed that the reaction mechanism is altered by the nature of a substituent, which may lead to a change in configuration of product. Thus the substituent-induced change of product configuration may not necessarily be unexpected even in enzyme reactions. In fact, we have found for the α-amylase-catalyzed hydrolyses of a series of *p*-substituted phenyl α-maltosides that the breakdown rate constant k_{+2} changes with σ^0 in a catenary shape [7] (cf. table 1), which strongly indicates that the reaction mechanism changes with the nature of the aglycone [12]. The observed dependence of k_{+2} on σ^0 suggests acid catalysis for *p-tert.*-butyl- and unsubstituted phenyl α-maltoside and an S_N mechanism for *p*-nitrophenyl α-maltoside [7]. Moreover, it is generally accepted that inversion of configuration is expected from an A-2* or single S_N2 mechanism, while retention of configuration is possible either in a double S_N2 mechanism or in an A-1* mechanism followed by stereospecific hydration [13, 14]. Therefore, a reasonable interpretation of our results, which are consistent with both the change in anomeric form of maltose and the dependence of k_{+2} on σ^0, may be that the reaction mechanism changes for A-2 through A-1 (followed by stereospecific hydration) to single S_N2 with increasing electron-withdrawing tendency of substituent (p-$C(CH_3)_3 < H < p$-NO_2). Further studies with other substituents are now being made.

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* A-1 represents unimolecular acid catalysis and A-2 biomolecular acid catalysis according to Ingold's nomenclature [13].

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