Available online at www.sciencedirect.com
ScienceDirect
Carbohydrate Research 342 (2007) 55–64
Carbohydrate RESEARCH

Formation of covalent β-linked carbohydrate–enzyme intermediates during the reactions catalyzed by α-amylases

Seung-Heon Yoon,[a,b] D. Bruce Fulton[b] and John F. Robyt[a,b,*]

[a]*Laboratory of Carbohydrate Chemistry and Enzymology, Iowa State University, Ames, IA 50011, USA*
[b]*Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, IA 50011, USA*

Received 30 August 2006; received in revised form 26 October 2006; accepted 30 October 2006
Available online 6 November 2006

Abstract—Porcine pancreatic and *Bacillus amyloliquefaciens* α-amylases were examined for the formation of covalent carbohydrate intermediates during reaction. The enzymes were precipitated and denatured by adding 10 volumes of acetone. When these denatured enzymes were mixed with methyl α-6-[^3H]-maltooligosaccharide glycosides and chromatographed on BioGel P-2, no carbohydrate was found in the protein void volume peak. When the enzymes were added to the methyl α-6-[^3H]-maltooligosaccharide glycosides and allowed to react for 15 s at 1 °C and then precipitated and denatured with 10 volumes of acetone, ^{3}H-labeled carbohydrates were found in the BioGel P-2 protein void volume peak, indicating the formation of enzyme–carbohydrate covalent intermediates. ^{1}H NMR analysis of the denatured enzyme from the reaction with methyl α-maltooligosaccharide glycosides confirmed that carbohydrate was attached to the denatured enzyme. ^{1}H NMR saturation-transfer analysis further showed that the carbohydrate was attached to the denatured enzyme by a β-configuration. This configuration is what would be expected for an enzyme that catalyzes the hydrolysis of α-(1→4) glycosidic linkages by a two-step, S_N2 double-displacement reaction to give retention of the α-configuration of the substrates at the reducing-end of the products.

Keywords: Alpha-amylases; Mechanism; Covalent intermediates; S_N2 double-displacement reaction; Methyl 6-[^3H]-α-maltooligosaccharide glycosides; Proton NMR

1. Introduction

α-Amylases (EC 3.2.1.1) catalyze the hydrolysis of α-(1→4) glycosidic linkages of starch, amylose, amylopectin, glycogen, and various maltooligosaccharides.[1,2] α-Amylases are produced from a wide variety of biological sources: bacteria, fungi, plants, and animals. α-Amylases from the different biological sources produce maltooligosaccharides of different sizes and amounts from starch and related materials due to a different number of D-glucopyranose binding subsites.[1] From the study of the action patterns, Robyt and French postulated that *Bacillus amyloliquefaciens* α-amylase (BAA) had nine D-glucopyranose binding subsites, with the catalytic groups positioned between the third and fourth subsites from the reducing-end subsites,[2] and porcine pancreatic α-amylase (PPA) had five D-glucopyranose binding subsites, with the catalytic groups positioned between the second and third subsites.[3] These hypotheses were later confirmed by the mapping of the free energy of the subsites[4] and by X-ray crystallography.[5]

α-Amylases from different origins do exhibit similar three-dimensional structures despite differences in their primary structures.[6–8] They are characterized by the presence of three different domains: a central $(\beta/\alpha)_8$ TIM-barrel domain (domain A), which is interrupted by an irregular β-strand (domain B) inserted between the third β-strand and the third α-helix of the TIM barrel, and a third domain (domain C), which is a key motif located on the opposite side of the barrel.[6,7,9,10] The substrate-binding sites and the catalytic groups are located in a cleft or groove at the interface between domain A and domain B, comprising several β-strands of variable

* Corresponding author. Tel.: +1 515 294 1964; fax: +1 515 294 0453; e-mail: jrobyt@iastate.edu

0008-6215/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2006.10.028

length, depending on the biological origin of the amylase.[6,11] The catalytic residues of α-amylases are composed of two aspartic acid and one glutamic acid, and these are positioned at the C-terminal side of the central TIM-barrel.[6,8–10] The α-amylase catalytic active site residues are very strictly conserved in all of the known α-amylases: bacterial α-amylase (BAA), fungal α-amylase from *Aspergillus oryzae* (AOA), to PPA and human salivary and pancreatic α-amylases (HSA and HPA).[6–11]

In the present study, we have trapped the covalent enzyme intermediates of porcine pancreatic and *B. amyloliquefaciens* α-amylases by using methyl α-6-[^3H]-maltooligosaccharide glycosides as substrates. The intermediates were trapped by conducting the reaction at 1 °C and rapidly denaturing them by acetone precipitation. The reaction mixture of denatured α-amylases with α-6-[^3H]-maltooligosaccharide glycosides was chromatographed on a BioGel P-2 column in the presence of urea; ^{3}H-activity was found associated with the enzyme peak, indicating the trapping of a covalent intermediate. The covalent linkage of a non-labeled purified protein–carbohydrate intermediate was analyzed by ^{1}H NMR saturation-transfer experiments.

2. Experimental

2.1. Chemicals

Methyl α-D-glucopyranoside (Me-α-Glc) was purchased from Eastman Kodak Co. (Rochester, NY) and recrystallized two times in distilled water. Cyclomaltohexaose (CD6) was obtained from Ensuiko Sugar Co. (Yokohama, Japan) and was pure by TLC analysis. Pyridinium dichromate was purchased from Aldrich (Milwaukee, WI). Sodium borotritide (NaB^3H_4) was obtained from New England Nuclear (Boston, MA). Other chemicals were of reagent grade.

2.2. Enzymes

B. macerans cyclomaltooligosaccharide glucanyltransferase (CGTase) [EC 2.4.1.19] was obtained by growing *B. macerans* ATCC 8517 on a wheat bran medium and purifying it by a modification of the method of Kobayashi et al.,[12] as previously described.[13] *B. amyloliquefaciens* α-amylase (BAA) concentrate was obtained from Miles Laboratories (Elkhart, IN) and crystallized two times.[14] Crystalline porcine pancreatic α-amylase (PPA) was purchased from Sigma Chemical Co. (St. Louis, MO).

2.3. Synthesis of methyl α-maltooligosaccharide glycosides

Methyl α-maltooligosaccharide glycosides (degree of polymerization, dp = 3–16) were prepared as previously described,[15] using substrate solution A of 100 mM CD6 and 100 mM Me-α-Glc (molar ratio, $R = 1$) and substrate solution B of 50 mM CD6 and 200 mM Me-α-Glc (molar ratio, $R = 4$) both in 25 mM imidazolium HCl buffer (pH 6.5) and 1 mM $CaCl_2$. The solutions were pre-incubated for 10 min at 37 °C, and the reactions were started by adding 100 IU of CGTase to 100 mL of each of the substrate solutions. One CGTase unit (an International Unit, IU) is the number of micromoles of D-glucose, divided by 6, which are formed per minute in the reaction of cyclomaltohexaose with methyl α-glucopyranoside in the presence of excess glucoamylase. Substrate solution A was incubated for 1 h, and substrate solution B was incubated for 12 h. The reactions were stopped by placing them in a boiling water bath for 10 min and concentrating them to 25 mL by rotary vacuum evaporation. The methyl α-maltooligosaccharide glycosides were fractionated on BioGel P-2 (fine) column (2.5 × 195 cm) with a flow rate of 3.0 mL/min and the collection of 5.0 mL fractions. The fractions containing Me-α-G8 to Me-α-G16 from substrate solution A were used for BAA reactions, and the fractions containing Me-α-G5 to Me-α-G7 from substrate solution A and Me-α-G3 to Me-α-G5 from substrate solution B were used for PPA reactions. The fractions (30–50 mL) were pooled, concentrated to 5 mL by rotary vacuum evaporation, and the maltooligosaccharide glycosides were precipitated by adding 10 volumes of acetone, centrifuged and treated five times with acetone and once with EtOH, and dried in a vacuum oven at 40 °C for 15 h.

2.4. Oxidation of methyl α-maltooligosaccharide glycosides

The primary alcohol groups of Me-α-Gn, where n is some number of glucose residues, were oxidized to aldehydes at C-6 by pyridinium dichromate (PDC).[13,16,17] The isolated fractions of Me-α-Gn, were each dissolved in 4.0 mL of water. PDC (1.2 g, 3.2 mmol) was dissolved in 4.0 mL of 0.1 M H_2SO_4, and the oxidation was initiated by adding the PDC solution to the carbohydrate solution. The solution was incubated for 18 h at 21 °C with stirring. The reaction was stopped by adding 4.0 g of $BaCO_3$ with vigorous stirring. The suspension was filtered, and the filtrate was treated three times with 4 g of BioRad AG11A8 ion-exchange resin to remove salts and acid. The oxidized methyl α-maltooligosaccharide glycosides were recovered by acetone precipitation and dried as described in the previous section.

2.5. Reduction of the oxidized methyl α-maltooligosaccharide glycosides with sodium borotritide/sodium borohydride

The aldehyde groups on the glucose residues of the oxidized methyl α-maltooligosaccharide glycosides were