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

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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

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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

reduced and labeled by reaction with NaB^3H_4.[13,16] The oxidized Me-α-Gn (500–750 mg) was dissolved in 10 mL of water, and the pH was adjusted to 8.5 with 3.0 mL pyridine and 0.1 M KOH, and then pre-incubated for 20 min at 70 °C. NaB^3H_4 solution (59 μCi, 152 μL) in 0.1 M NaOH was added to the carbohydrate solution and allowed to react for 30 min at 70 °C. $NaBH_4$ (3 mmol, 113.5 mg in 1.5 mL of water at pH 9.25) was added to the carbohydrate solution and incubated at 70 °C for 1.5 h. The reaction was terminated by adjusting the pH to 4.5 with glacial HOAc, and after 10 min it was neutralized with 1 M NaOH. Methyl α-6-[^3H]-maltooligosaccharide glycosides were purified by BioGel P-2 column chromatography, and the radioactive fractions were pooled and lyophilized. The carbohydrate composition was analyzed by TLC (two ascents of 18 cm on 20 × 20 cm Whatman K5 plates), using 85/25/55/50 volume proportions of MeCN–EtOAc–PrOH-1–H_2O, with the carbohydrates visualized by dipping the dried plate vertically into an MeOH solution containing 0.3% (w/v) *N*-(1-naphthyl)ethylenediamine and 5% (v/v) H_2SO_4, followed by drying and heating at 120 °C for 10 min. The compounds were quantitatively determined by scanning densitometry.[15] The radioactivity was determined by liquid scintillation spectrometry,[13] and ^{13}C NMR spectra were obtained, showing the starting material, the oxidized material, and the reduced labeled material, in which the latter showed that the reduced labeled materials did not have any free aldehyde groups (see Fig. 1).

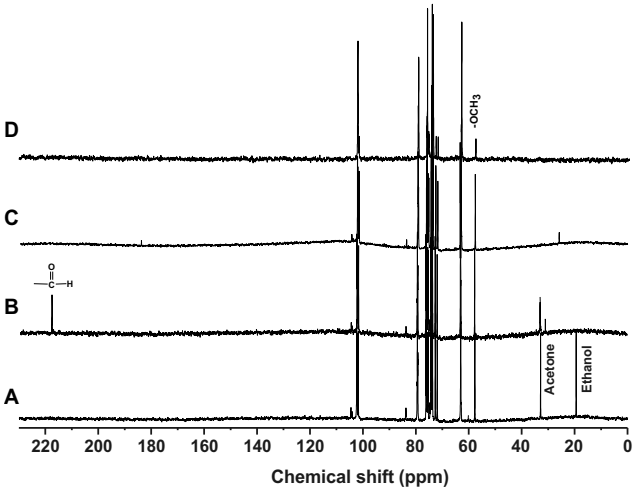

Figure 1. ^{13}C NMR spectrum of the methyl α-6-[^3H]-maltooligosaccharide glycoside substrates used to react with α-amylases. A, spectrum of the starting material, methyl α-maltooligosaccharide glycosides; B, spectrum of the pyridinium dichromate oxidized methyl α-maltooligosaccharide glycosides with C-6 carbons as aldehydes; and C, spectrum of the NaB^3H_4 reduced methyl α-maltooligosaccharide glycosides (dp = 3–7); and D, spectrum of the NaB^3H_4 reduced methyl α-maltooligosaccharide glycosides (dp = 8–16).

2.6. Trapping of *B. amyloliquefaciens* α-amylase–carbohydrate covalent intermediate

Methyl α-[^3H]-maltooligosaccharide glycosides (dp 8–16) 7.34 mg were dissolved in 350 μL of 1 °C water. *B. amyloliquefaciens* α-amylase (0.05 μmol) in 350 μL of 1 °C water was added to the Me-α-[^3H]-G8-G16 solution and allowed to react for 15 s at 1 °C. The reaction was stopped by adding 10 volumes (7.0 mL) of 21 °C acetone to stop the reaction and to denature and precipitate the enzyme. The precipitate was centrifuged for 30 min at 15,000*g* at 20 °C. The precipitated enzyme was suspended in 90% (v/v) acetone–H_2O and centrifuged again. Excess acetone was removed from the precipitate by a stream of air, and the precipitate was dissolved in 350 μL of 12 M urea and then diluted to 700 μL with water. The solution was added to a BioGel P-2 (fine) column (1.5 × 45 cm) and eluted with water at 21 °C at a rate of 0.2 mL/min, and 1.0 mL fractions were collected. Protein was determined for each fraction by the Bradford method,[18] and carbohydrate was assayed by the micro phenol–sulfuric acid method.[19] The carbohydrate composition of each fraction was analyzed by TLC as described in the previous section. The radioactivity of each fraction was determined by adding 500 μL of sample to 2 × 23 cm Whatman 3 MM paper and putting the rolled-up papers in 15 mL of liquid scintillation counting cocktail containing 5.0 g POP and 0.1 g PoPoP in 1.0 L of toluene and counting the radioactivity by heterogeneous liquid scintillation spectrometry.

2.7. Trapping of porcine pancreatic α-amylase–carbohydrate covalent intermediate

Radioactive methyl α-6-[^3H]-maltooligosaccharide glycosides (dp 3–7) 5.78 mg were used as substrates for PPA. The preparation, reaction, and analytical conditions were the same as those used in trapping the BAA covalent enzyme intermediate complex.

2.8. ^1H NMR analysis of the α-amylase–carbohydrate covalent intermediates

Non-labeled enzyme–carbohydrate intermediates were prepared by reacting 350 μL (0.02 μmol) BAA with 350 μL (20 mg) of methyl α-maltooligosaccharide glycosides (dp 8–16) and 350 μL of PPA (0.1 μmol) with 350 μL (5.78 mg) of methyl α-maltooligosaccharide glycosides (dp 3–7) each for 5 s at 1 °C. The reactions were stopped, and the enzymes were precipitated and denatured by adding 7.0 mL of acetone. The precipitated enzymes were centrifuged at 15,000*g* for 30 min. The remaining acetone was removed by a continuous vacuum, and the precipitated protein was dissolved in 350 μL of 12 M urea that was diluted to 700 μL. The dissolved precipitate was chromatographed on a BioGel

P-10 column (1.5 × 45 cm) with elution by 0.5 M urea and 0.05 M NaCl. The eluted covalent intermediate was dialyzed three times against 4 L of deionized water, and then lyophilized. The lyophilized intermediate was dissolved in 4 mL of D_2O and lyophilized. This was repeated three times, and then BAA and PPA protein–carbohydrate intermediates were dissolved in 500 μL D_2O (Aldrich Chemical Co.) to give 0.2 mM protein.

^1H NMR spectra of the intermediates were obtained on a Bruker DRX 500 spectrometer, operating at ^1H frequency of 499.867 MHz at 25 °C. The spectrometer was equipped with a 5-mm $^1H/^{13}C/^{15}N$ gradient probe. Chemical shifts were internally referenced to the methyl frequency of 3-(trimethylsilyl)-1-propanesulfonic acid. All spectra were recorded at 298 K. One-dimensional ^1H (2048 scans, with a sweep width of 8012 Hz) were acquired using standard experimental protocols. The data were processed and analyzed using Bruker Xwinnmr 2.6 software.

^1H NMR saturation-transfer experiments[20] were performed to provide definitive evidence that the carbohydrate proton resonances observed in the ^1H NMR spectra of PPA and BAA were due to the covalent attachment of the carbohydrate D-glucopyranose unit to the protein and not due to the α-(1→4) covalent linkages between D-glucopyranose units in a maltooligosaccharide chain. For each sample, saturation and reference spectra were obtained. The saturation-transfer experiment consisted of 1 s relaxation delay, followed by a 1 s selective radio frequency pulse (800 Hz power, with the frequency shifted to 0.8 ppm), followed by free induction decay collection. The selective pulse was located within the protein methyl region at ~2000 Hz upfield from the carbohydrate resonances at 4 ppm. The reference spectrum was obtained identically except that the selective radio frequency pulse was shifted to −10 ppm.

3. Results

3.1. Synthesis of methyl α-6-[^3H]-maltooligosaccharide glycosides

Methyl α-maltooligosaccharide glycosides were prepared as previously described.[15] The hydroxymethyl groups on the glucose residues were oxidized by pyridinium dichromate to aldehyde groups, which were labeled by reduction with $NaB^3H_4/NaBH_4$. Figure 1 is a ^{13}C NMR spectrum showing the complete reduction of the oxidized substrates to labeled primary alcohol groups. The substrates used for PPA had dp values of 3–7 and a specific activity of 178,136 cpm/mg and are shown in Figure 2, lane 2. The substrates used for BAA had dp values of 8–16 and a specific activity of 58,525 cpm/mg and are shown in Figure 2, lane 3.

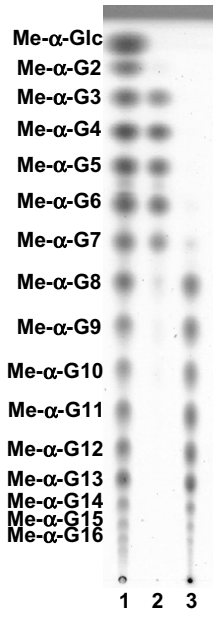

Figure 2. TLC of the substrates used in the reactions of *Bacillus amyloliquefaciens* α-amylase (BAA) and porcine pancreatic α-amylase (PPA). Lane 1, methyl α-maltooligosaccharide glycoside standards; lane 2 (dp 3–7), substrates for PAA; lane 3, methyl α-maltooligosaccharide glycosides (dp 8–16) substrates for BAA.

Two different sizes of substrates were used for the two α-amylases. PPA has five D-glucopyranose binding subsites and substrates having 3–7 D-glucopyranose residues were used in the reactions. BAA has nine D-glucopyranose binding subsites, and substrates having 8–16 D-glucopyranose residues were used in the reactions.

3.2. Trapping of *B. amyloliquefaciens* α-amylase carbohydrate intermediate

Precipitated and denatured BAA mixed with methyl α-6-[^3H]-maltooligosaccharide glycosides (dp 8–16) were chromatographed on BioGel P-2 column. Two peaks were obtained, a protein peak from fractions 61–65, and a ^3H-labeled carbohydrate peaks from fractions 70–100 (Fig. 3). The protein peak was completely devoid of ^3H-labeled carbohydrate.

The precipitated and denatured BAA reaction digests with methyl α-6-[^3H]-maltooligosaccharide glycosides (dp 8–16) was chromatographed on BioGel P-2. A protein peak containing ^3H-labeled carbohydrate appeared between fractions 31 and 40, and a broad ^3H-labeled carbohydrate peak appeared between fractions 45 and 70 (Fig. 4). Two ^3H-labeled peaks occur from BAA catalysis and are different from the control chromatogram of Figure 3 where no catalysis occurred, as BAA was denatured when it was mixed with the substrates. The first ^3H-labeled carbohydrate peak that occurs with the protein peak in Figure 4 is tightly associated with the pro-

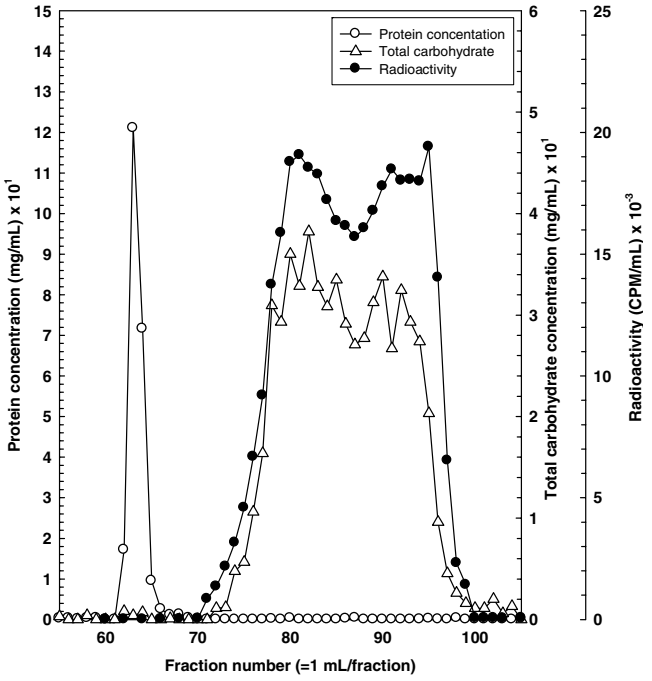

Figure 3. BioGel P-2 column (1.5 × 110 cm) chromatography of the denatured *B. amyloliquefaciens* α-amylase and ^{3}H-labeled methyl α-6-[^{3}H]-maltooligosaccharide glycosides (dp 8–16).

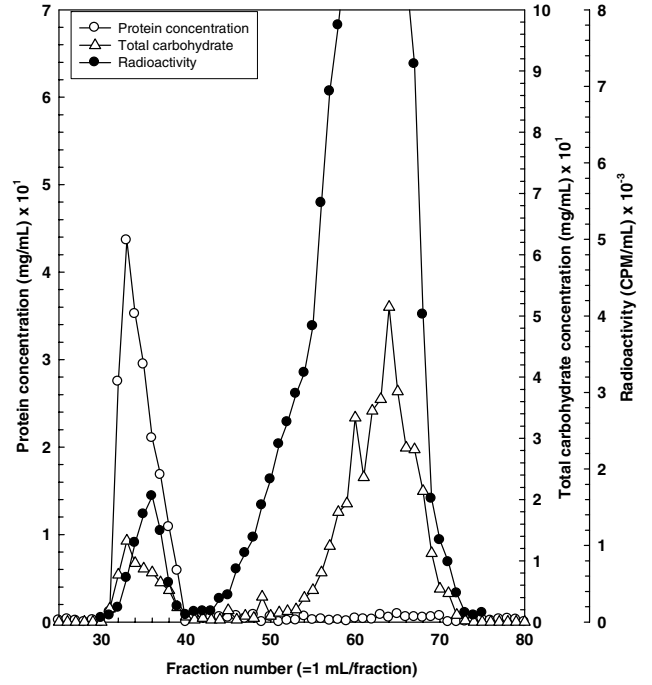

Figure 4. BioGel P-2 column (1.5 × 45 cm) chromatography of 10 volumes of acetone precipitated and denatured *B. amyloliquefaciens* α-amylase reaction digest with methyl α-6-[^{3}H]-maltooligosaccharide glycosides (dp 8–16).

tein, as it does not appear in the protein peak of the control of Figure 3. This is, therefore, the trapped enzyme–carbohydrate covalent intermediate. The second peak in Figure 4 (fractions 58, 60, 64, and 67) was analyzed by TLC and shown to be reaction products of BAA (data not shown). Fraction 58 contained Me-α-G3, Me-α-G4, G5, and G6. Fraction 60 contained a trace of Me-α-G2, Me-α-G3, G4, and G5. Fraction 64 contained G3 and fraction 64 contained G2. These were all of the products expected for the hydrolysis of methyl α-6-[^{3}H]-maltooligosaccharide glycosides (dp 8–16) by BAA.[2] BAA thus effectively hydrolyzed the substrates, and the denaturation of BAA that occurred during the reaction trapped the covalently linked BAA–carbohydrate intermediates.

3.3. Trapping of the porcine pancreatic α-amylase carbohydrate intermediate

Precipitated and denatured PPA mixed with methyl α-6-[^{3}H]-maltooligosaccharide glycosides (dp 3–7) were chromatographed on a BioGel P-2 column. Two peaks were obtained, a protein peak from fractions 30–40 and a ^{3}H-labeled carbohydrate peak from fractions 55–70 (Fig. 5). The protein peak was completely devoid of ^{3}H-labeled carbohydrate.

The precipitated and denatured PPA reaction digests with methyl α-6-[^{3}H]-maltooligosaccharide glycosides (dp 3–7) was chromatographed on BioGel P-2. A protein peak containing ^{3}H-labeled carbohydrate appeared between fractions 30–40, and two ^{3}H-labeled carbohydrate peaks appeared in fractions 55–65 and 65–75 (Fig. 6). The three ^{3}H-labeled peaks occur from PPA

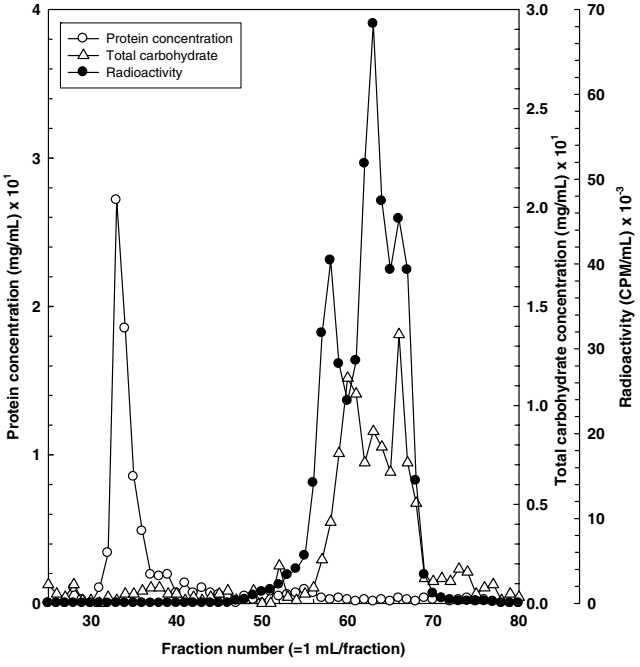

Figure 5. BioGel P-2 column (1.5 × 45 cm) chromatography of the acetone-denatured porcine pancreatic α-amylase and methyl α-6-[^{3}H]-maltooligosaccharide glycosides (dp 3–7).

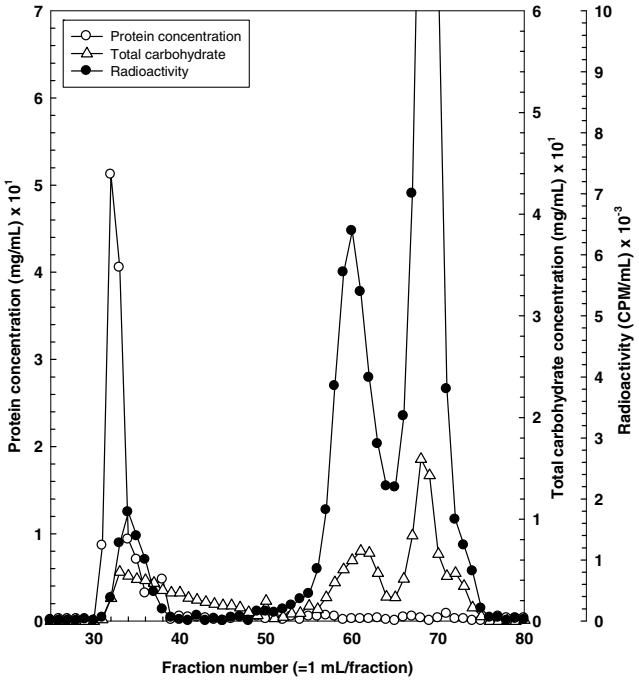

Figure 6. BioGel P-2 column (1.5 × 45 cm) chromatography of the acetone-denatured porcine pancreatic α-amylase reaction digest with α-6-[^3H]-maltooligosaccharide glycosides (dp 3–7).

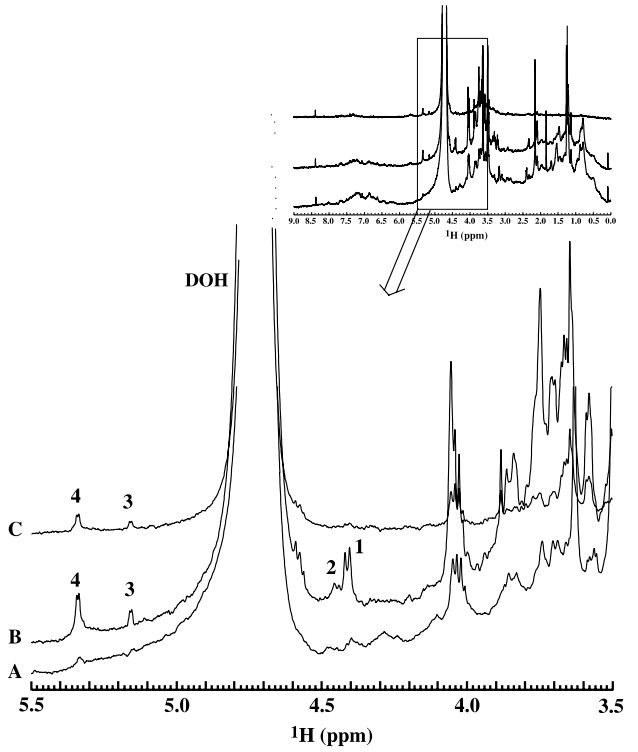

Figure 7. Proton NMR spectra of (A) the acetone-denatured porcine pancreatic α-amylase (PPA); (B) denatured PPA covalent intermediate; and (C) protein–proton saturation transfer experiment of (B) obtained at 500 MHz and 25 °C.

catalysis and do not appear in the control chromatogram of Figure 5 where no catalysis occurred, as PPA was denatured when it was mixed with the substrates. The first ^{3}H-labeled carbohydrate peak that occurs with the protein peak must be tightly associated with the protein. This is the trapped enzyme–carbohydrate intermediate, similar to what was found for the BAA reaction (Fig. 4). The second and third ^{1}H-labeled peaks in Figure 6 were analyzed by TLC. The second peak was Me-α-[^{3}H]G2 and the third peak was ^{3}H-labeled maltose and ^{3}H-labeled D-glucose as determined by TLC (data not shown); these were the products expected for the hydrolysis of methyl α-6-[^3H]-maltooligosaccharide glycosides (dp 3–7) by PPA.[3] PPA thus effectively hydrolyzed the substrates, and the denaturation of the PPA during the reaction trapped the PPA–carbohydrate covalent intermediates.

3.4. ^{1}H NMR analysis of the denatured enzyme reaction with methyl α-maltooligosaccharide glycosides

When denatured PPA protein was analyzed by ^{1}H NMR spectroscopy, no peaks were found between the chemical shift range of 4.4 and 5.5 ppm expected for carbohydrate anomeric protons[20] (Fig. 7A). The ^{1}H NMR spectrum obtained from the denatured PPA reaction with methyl α-maltooligosaccharide glycosides, however, gave four distinct anomeric proton peaks that were characteristically split into doublets due to scalar coupling (Fig. 7B). Peak 1 had a chemical shift, $\delta_H = 4.41$ and a coupling constant, $J_{1,2} = 8.3$ Hz and peak 2 had $\delta_H = 4.44$ and $J_{1,2} = 8.1$ Hz that are characteristic of carbohydrate anomeric protons on carbons with the β-configuration; peak 3 had $\delta_H = 5.51$ and $J_{1,2} = 3.4$ Hz and peak 4 had $\delta_H = 5.34$ and $J_{1,2} = 3.2$ Hz that are characteristic of carbohydrate anomeric protons on carbons with the α-configuration.[21–23]

In the ^{1}H NMR saturation-transfer experiment, the methyl protons of the protein are saturated by a selective radio frequency pulse. This results in all of the ^{1}H resonances of the protein to also become rapidly saturated due to spin diffusion, and they are attenuated and essentially disappear from the spectrum.[24] Further, any molecule that is covalently attached to the protein will also have the protons on the carbon directly attached to the protein saturated, and their resonances will also disappear from the spectrum. The proton saturation-transfer experiment (Fig. 7C) shows that peaks 1 and 2 disappeared, while peaks 3 and 4 remained relatively unaltered. Other anomeric protons, for example, those on carbons involved in α-(1→4) glycosidic linkages between D-glucose units in a relatively flexible saccharide chain that is covalently attached to the protein will not be subject to an efficient spin diffusion and hence will not be attenuated and will not disappear in the saturation-transfer spectrum.[24] The disappearance

of peaks 1 and 2 thus definitively shows that the trapped carbohydrate is covalently attached to the protein by a β-configuration. Furthermore, peaks 3 and 4, which do not disappear, are the anomeric protons for the α-(1→4) glycosidic linkages in the carbohydrate chain that is covalently attached—but not *directly* attached—to the protein, as shown in Figure 7C.

The occurrence of two β-peaks indicates that there are two kinds of chemical environments and that two distinct β-covalent linkages were formed, with peak 1 being 2.5 times more prevalent than the other peak 2, as determined from the integration of the peaks. For further details on the nature of these two β-linked intermediates, see the Discussion section. The two peaks for the α-anomeric linkages also indicate that there are two distinct α-linkages, with peak 3 being the anomeric proton on the carbon attached to the first glucopyranose unit and peak 4 being the anomeric protons on the carbons attached to the other D-glucopyranose units in the covalently linked carbohydrate chain, indicating that at least some of the carbohydrate chains are longer than maltose.[22,23]

Similar ^{1}H NMR spectra were obtained for BAA (data not shown) as were obtained for PPA. Denatured BAA did not have any anomeric proton peaks. The spectrum of the denatured BAA digest of the methyl α-maltooligosaccharide glycosides (dp 8–16) had four proton anomeric peaks, two β-anomeric peaks ($\delta_H = 4.41$, $J_{1,2} = 8.3$ Hz and $\delta_H = 4.45$, $J_{1,2} = 8.1$ Hz) and two α-anomeric peaks ($\delta_H = 5.14$, $J_{1,2} = 3.1$ Hz and $\delta_H = 5.33$, $J_{1,2} = 3.1$ Hz), with chemical shifts and coupling constants very close to those obtained for PPA. As with PPA, the β-anomeric peaks are due to the linkage of the covalent intermediate to the protein, and the α-anomeric peaks are due to the α-glycosidic linkages in the maltooligosaccharide chains attached to the enzyme.

4. Discussion

α-Amylases hydrolyze α-(1→4) glycosidic linkages in starch and related materials to give products that have the α-configuration at the reducing-end. Thoma et al.[25] showed that porcine pancreatic α-amylase gives 100% retention of the α-configuration during catalysis. Fischer and Stein first postulated that the mechanism for α-amylase glycosidic bond hydrolysis was a double-displacement process involving a covalent carbohydrate–enzyme intermediate that gives the required retention of configuration at the reducing-end of the products.[26] In this mechanism, it was proposed that a carboxylate anion at the active site of the α-amylases acts as a nucleophile and attacks the C-1-carbon of an α-(1→4)-linked glucopyranosyl unit of starch and related substrates that are properly aligned at the active site by the binding-site subsites to give cleavage and the formation of a covalent high-energy, β-carboxyl acetal ester. This high-energy group is then readily hydrolyzed at C-1 of the carbohydrate in a second reaction to give a product that has the α-configuration at C-1 of the reducing-end moiety.[1,27]

The first isolation of a covalent carbohydrate–enzyme intermediate was reported in 1970 by Voet and Abeles for the enzyme sucrose phosphorylase.[28] They showed that D-glucopyranose was covalently attached to the denatured enzyme and had the β-configuration. They concluded that the β-glucopyranosyl enzyme intermediate reacts with inorganic phosphate, to give inversion of the β-configuration at the C-1 atom of the D-glucopyranosyl moiety of the intermediate enzyme complex, forming α-Glc-1-phosphate. It was also concluded that the trapping of the intermediate provided support for the hypothesis that sucrose phosphorylase proceeds through a double-displacement mechanism with the formation of a β-linked glucose–enzyme intermediate. Covalent glucosyl enzyme intermediates have also been isolated for α-glucosidase[29] and β-glucosidase[30–32] and for *Streptococcus sorbrinus* glucosyltransferase (dextransucrase).[33]

The reactions of *Bacillus* sp. cyclomaltooligosaccharide glucanyltransferases (CGTases) also have been found to form covalent carbohydrate intermediates and proceed by a double-displacement mechanism. Mosi et al.[34] showed that *B. circulans* CGTase formed a maltotriosyl intermediate by using an inactive mutant enzyme in which Glu 257 was replaced by Gln and α-(4-deoxymaltotriosyl) fluoride was the substrate. Lee and Robyt[13] trapped the covalent carbohydrate–enzyme intermediate by denaturation during the reaction of 6-[^{3}H]-cyclomaltohexaose with *B. macerans* CGTase. The CGTases have some similarities to α-amylases. Like α-amylases, they are known to have the TIM-barrel structure, comprising the active site,[35,36] but they are also distinct in that they are transferases rather than hydrolases and are exo-acting enzymes that catalyze a transfer reaction, using the C-4-hydroxyl group at the non-reducing ends of starch chains to form non-reducing α-(1→4)–linked cyclomaltooligosaccharides, primarily having six, seven, and eight D-glucopyranose residues, whereas α-amylases are endo-acting enzymes, catalyzing the hydrolysis of α-(1→4) glycosidic linkages from the interior parts of starch chains to give reducing maltooligosaccharide products having the α-anomeric configuration. *B. macerans* CGTase also catalyzes transfer reactions between cyclomaltohexaose and acceptors that have hydroxyl groups on carbohydrates or non-carbohydrates.[15,37]

Tao et al.[38] found that the action of porcine pancreatic α-amylase formed a covalent carbohydrate–enzyme intermediate by using 1-[^{13}C]-maltotetraose as substrate and following the reaction by ^{13}C NMR spectroscopy at −20 °C in aqueous 40% (v/v) Me_2SO. Spectral summation and difference techniques revealed a broad resonance peak whose chemical shift, relative signal

intensity, and time-course of formation corresponded to a carboxyl-β-carbohydrate acetal ester.

In the present study, three experiments have been performed that show that α-amylases form covalent intermediates during catalysis: (1) rapid denaturation of the enzyme during reaction with methyl α-6-[^{3}H]-maltooligosaccharide glycosides gave ^{3}H labeled material associated with the denatured enzyme that was not associated with denatured enzyme before reaction; (2) ^{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; and (3) ^{1}H NMR saturation-transfer analysis showed that the carbohydrate was attached to the denatured enzyme by a β-configuration.

Arguments might be made that the ^{3}H-labeled carbohydrate associated with the denatured enzyme in Figures 4 and 6 were formed as an oxocarbonium ion paired with an anion on the enzyme, rather than a covalent intermediate. The ion-pair would not be particularly stable, and it would be quite unlikely to survive the denaturation unfolding of the enzyme. The oxocarbonium ion would readily dissociate from the enzyme and would not subsequently form a covalent intermediate, and hence it would not be chromatographed with the denatured protein. Another spurious argument might be that the formation of the covalent intermediate only takes place during the denaturation process, after the dissociation of the glycosidic bond and the formation of the oxocarbonium ion. If the oxocarbonium ion was forming first by a dissociation reaction and the denaturing process was unfolding the enzyme, the subsequent formation of a covalent linkage between the carbohydrate and the enzyme would be expected to be a random process in which both α- and β-linkages would be formed. The ^{1}H NMR saturation-transfer experiments, however, showed that the covalent carbohydrate intermediate was exclusively linked by a β-linkage, the linkage expected for a covalent carbohydrate intermediate, if it was being formed by a nucleophilic displacement reaction by α-amylase rather than a dissociation reaction.

The three-dimensional structural analysis of α-amylases by X-ray crystallography has shown that three amino acids, Asp197, Glu233, and Asp300 (PPA numbering) are strictly conserved in the active sites of all known α-amylases.[5–11,39–42] These three amino acids are located at the upper middle of the active-site cleft or groove of the $(\beta/\alpha)_8$-TIM barrel located in domain A. Recently, the roles of the three conserved amino acids were more specifically studied by point mutation of each amino acid and kinetic analysis of human pancreatic α-amylase.[10,43] The results suggest that Asp197 is the nucleophile that attacks C-1 of the substrate glycosidic bond, and Glu233 is the general acid that gives a proton to the leaving oxygen of the glycosidic linkage, and Asp300 abstracts the hydrogen of water or polarizes the water to facilitate the attack of water on C-1 of the β-carboxyl acetal ester to give inversion of the configuration and the formation of the α-anomeric product. The conservation of the three catalytic amino acids and the presence of the TIM barrel at the active sites of the α-amylases indicate that the S_N2 mechanism, with the formation of the carboxyl acetal β-carbohydrate intermediate, is universal for all of the known α-amylases.

The ^{1}H NMR analysis of the β-glucopyranosyl covalent intermediate in this study showed that there were two distinct β-linkages. One peak was 72% more prevalent (from the integration of peaks 1 and 2 in Fig. 7) than the other peak. Asp197 is the preferred carboxylate to make the attack, but Asp300 might also make the attack and form the β-linkage to the glucopyranosyl unit (peak 2 in Fig. 7B), probably during the denaturation process.

Thus the mechanism for the α-amylase-catalyzed hydrolysis of α-(1→4)-glycosidic bonds appears from this study to be the attack of an enzyme nucleophile (Asp197 carboxylate group) onto C-1 of an α-(1→4)-linked glucopyranosyl residue of starch and related substrates to give covalent β-linked enzyme–maltooligosaccharide intermediates that are then hydrolyzed to give products that have the α-configuration at the reducing end (see Fig. 8 for the mechanism).

A carbonium ion mechanism for α-amylase catalysis has been proposed, involving an S_N1 reaction with the formation of a carbonium ion at C-1 of the cleaved glucopyranose residue.[44] This carbonium ion has one resonance form, the oxocarbonium ion. However, there has never been any experimental evidence showing the formation of a carbonium ion for the α-amylase cleavage of the α-(1→4) glycosidic bond. From a consideration of the mechanism for the formation of a carbonium ion, there is no good driving force for cleaving the glycosidic linkage to form the carbonium ion at C-1. In the usual S_N1 reactions, proceeding through dissociation, the dissociation step is more rapid than the subsequent attack onto the carbonium ion[45] that in the cleavage of the α-(1→4) bond would be the hydrolytic step, which for α-amylase catalysis is very fast. Further, S_N1 reactions lead to a combination of inversion and retention of the substrate configuration; that is, the product has a configuration that has much less optical purity than an S_N2 reaction, which has 100% retention of the configuration of the substrate.[46] Furthermore, base-catalyzed reactions, as would be the case for the nucleophilic attack by the carboxylate anion onto C-1 of the α-(1→4) glycosidic bond, are $\sim 4–5 \times 10^{3}$ times faster than acid-catalyzed reactions, which is the required reaction for the S_N1 cleavage of the α-(1→4) glycosidic bond in which there would be the transfer of a proton from Glu233 to the C-4 oxygen leaving group as the first step in the catalytic reaction.

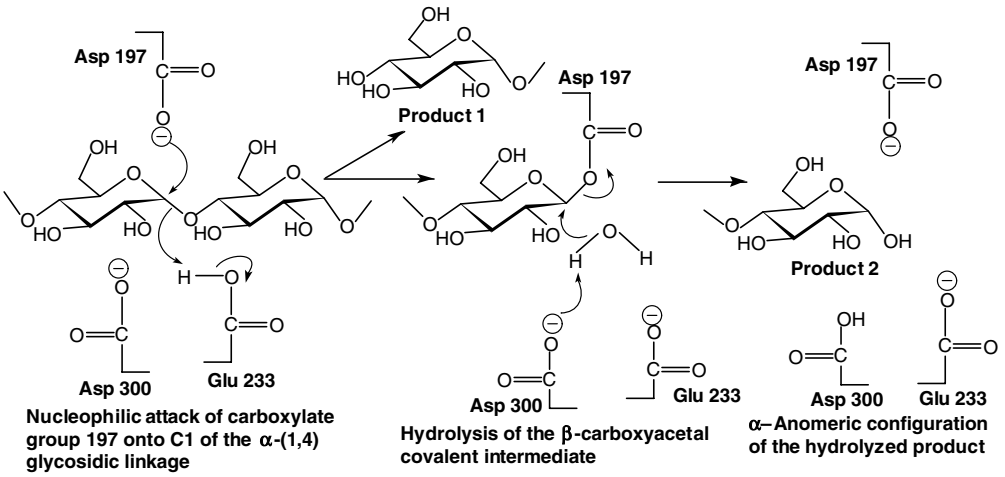

Figure 8. S_N2 reaction mechanism for the formation of the β-linked, carbohydrate carboxyl acetal, and its hydrolysis to give an α-anomeric product during the reaction of α-amylases with α-(1→4)-linked substrates. The numbering system for the catalytic amino acids is that of porcine pancreatic α-amylase. Other α-amylases have the same three amino acids as catalytic groups at their active-sites, but with different sequence numbers.

Spector[47] has stated that there are two main reasons for thinking that enzymes catalyze their reactions by forming covalent intermediates: (1) they are that the other two kinds of chemical catalysis, non-enzymatic catalysis in solution and heterogeneous non-enzymatic catalysis on a solid surface, both proceed by forming covalent intermediates, and (2) there is not a single authenticated experiment reported showing that an enzyme-catalyzed reaction proceeds by a single displacement, S_N1, carbonium ion mechanism.

There is a 66-year-old Pauling paradigm[48,49] that enzymes have such an awesome power as catalysts because of non-covalent molecular recognition by the active site of enzymes for their substrates, which is essentially the Fischer 'Lock and Key hypothesis' that explains the phenomenon of enzyme specificity. The proficiencies, $[(k_{cat}/K_M) \div k_{uncat}]$, of enzyme-catalyzed reactions range from 10^8 to $10^{27}\,M^{-1}$ for enzymes studied to date.[50] Houk et al.[51] have found, however, that there are limits to the binding of substrates to enzymes by non-covalent interactions and posit that it is the formation of covalent intermediates that explains why enzymes are such proficient catalysts.[50,51]

The formation of covalent carbohydrate–enzyme intermediates that have been shown in this study gives experimental evidence for the S_N2 double-displacement mechanism for catalysis by α-amylases, and now replaces and refutes the alternate S_N1 carbonium-ion hypothesis[44] and supports the hypothesis that covalent intermediates are indeed the driving force in the catalytic power of enzyme catalysis.

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