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THE EFFECT OF SUBSTRATE MODIFICATION ON BINDING OF PORCINE PANCREATIC ALPHA AMYLASE: HYDROLYSIS OF MODIFIED AMYLOSE CONTAINING D-ALLOSE RESIDUES*

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ABSTRACT

A modified amylose containing 10% of tritiated D-allose residues has been hydrolyzed by porcine pancreatic alpha amylase (PPA). This reaction produced a number of radioactive oligosaccharides of low molecular weight, including modified mono-, di-, and tri-saccharides, as well as larger products. Analysis of these products by chemical and enzymic methods identified D-allose, two isomers of modified maltose, and isomers of modified maltotriose. These results may be interpreted in terms of current PPA models to indicate that D-allose residues may be productively bound at all five subsites of the active site of the enzyme. The distribution of modified residues in these products, however, further suggests that productive binding of D-allose at the subsite where catalytic attack occurs (subsite 3) is less favorable than binding of D-glucose. These results are compared with results of a series of PPA substrates having modifications at C-3 and at other positions. Trends observed in enzyme hydrolysis of these modified substrates reflect factors that contribute to PPA catalysis, with respect to steric, electronic, and hydrogen-bonding interactions between enzyme and substrate.

INTRODUCTION

Studies in this laboratory have determined the ability of porcine pancreatic alpha amylase (PPA) to hydrolyze α-(1→6)-branched substrates[1,2], hydroxyethylated amylose[3], oxidized–reduced amylose[4], and substrates containing substituents at C-2 and C-6 of small steric bulk (unpublished results). Other authors have also described the action of PPA on modified substrates[5–8], particularly in studies using PPA for structural analysis of polysaccharides. Previous experiments using substrates modified at C-3 have involved substrates containing bulky[3,6] and

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charged[8] groups. In this report, we describe the hydrolysis of amylose containing 10% of D-allose residues. As this modification entails inversion of configuration at C-3, these experiments may provide information about the effect of removal of the equatorial C-3 hydroxyl group and may also identify important enzyme–substrate interactions disrupted by the introduction of an axial hydroxyl group at C-3. The distribution of modified residues within amylolysis products reflects the ability of these residues to bind productively at the five subsites of the active site of PPA[9]. By comparing results from various substrate modifications, the relative effects of charged substituents, sterically large groups, and small groups having altered hydrogen-bonding properties may be assessed. These results should provide insight into factors contributing to PPA catalysis.

EXPERIMENTAL

Materials. — Amylose (Superlose) was obtained from Stein–Hall. Porcine pancreatic alpha amylase (E.C. 3.2.1.1, Boehringer–Mannheim, 10,000 U/mL) and *Rhizopus niveus* glucoamylase (E.C. 3.2.1.3, Seikagaku Fine Chemicals, 35 U/mL) were commercial preparations and were used without further purification. PPA contained no detectable α-D-glucosidase activity when assayed with *p*-nitrophenyl α-D-glucopyranoside. Tritiated sodium borohydride (40 mCi/mL, 252 mCi/mmol) was obtained from NEN–DuPont.

Methods. — Paper chromatography was performed on Whatman 3MM paper with 7:3 1-propanol–water (solvent *A*) or 8:1:1:1 nitromethane–acetic acid–ethanol–saturated aqueous boric acid (solvent *B*). Separations were performed at 37°. Saccharides were hydrolyzed with 3M trifluoroacetic acid for 2 h at 100° in sealed ampules. Reduction of oligosaccharides by sodium borohydride, for analysis, was performed by using the method of Sloneker[10]. Tritiated samples were detected on paper chromatograms by autoradiography using En^{3}Hance spray (NEN–DuPont). Labeled products were cut from chromatograms and placed in vials containing a toluene cocktail. Radioactivity was determined by heterogeneous liquid-scintillation spectrometry.

Modified amylose containing D-allose residues was prepared by partial oxidation of 2-*O*-benzoyl-6-*O*-tritylamylose with dimethyl sulfoxide and phosphorus pentaoxide, followed by reduction with sodium borohydride, according to the procedure of Kondo and Takeo[11]. A sample of this product was hydrolyzed in 3M trifluoroacetic acid, and analysis of the hydrolyzate by high-pressure liquid chromatography showed that 10% of the sugar residues had been converted into D-allose. Radioactive modified amylose was prepared by reduction of the partially oxidized amylose (150 mg) with sodium [^{3}H] borohydride (125 mg, 2 mCi). Acid hydrolysis of the labeled product, followed by paper chromatography (20 h descent, solvent *B*) and autoradiography, showed two products having the paper-chromatographic mobilities of D-allose and D-glucose, as well as a small amount of unhydrolyzed material. Results of liquid-scintillation counting indicated that reduc-

tion of the oxidized amylose gave products having ~80% in the D-*allo* configuration and 20% in the D-*gluco* configuration.

Hydrolysis by PPA of modified amylose containing D-*allose residues.* — Tritiated modified amylose (6 mg) was dissolved in 60 μL of 9:1 dimethyl sulfoxide–water. This was diluted with 600 μL of water, and 80 μL of pH 6.9 buffer containing 200mM sodium glycerophosphate, 100mM sodium chloride, and 0.2% sodium azide was added. A sample (25 μL) was removed and spotted onto a paper chromatogram. An enzyme solution was prepared by adding 40 μL of PPA to 80 μL of buffer, pH 6.9, containing 20mM sodium glycerophosphate, 10mM calcium chloride, and 0.02% sodium azide. A portion of this enzyme solution (100 μL) was added to the substrate solution (715 μL) and the mixture was incubated at 37°. Aliquots (25 μL) were removed at various times (1–96 h) and spotted onto the paper chromatogram. At the end of the reaction time, 50 μL was removed and reduced with sodium borohydride. The remainder of the solution was streaked onto a second chromatogram. Ascending chromatography was performed (2 ascents, solvent *A*), and autoradiograms were prepared. The molecular size of D-allose-containing products was determined by comparison of their paper-chromatographic mobilities with those of malto-oligosaccharide standards. Mobilities of the modified and unmodified oligosaccharides were nearly identical when two ascents with solvent *A* were used.

RESULTS

Paper chromatography of products obtained by PPA hydrolysis of the modified amylose showed formation of radioactive monosaccharides, disaccharides, trisaccharides, and larger products. Labeled mono-, di-, and trisaccharides were purified by paper chromatography (2 ascents, solvent *A*). Approximately 35% of the total radioactivity in the enzymolysis mixture consisted of labeled products that were relatively resistant to enzyme hydrolysis. A PPA digest of 6-deoxyamylose (d.s. 0.15) performed under identical conditions, except that half as much enzyme was used, contained less than one-third as much resistant material, which consisted of heptasaccharides and larger products (data not shown). We consider, but have not confirmed, that these resistant products contain regions of multiple D-allose residues, caused by nonrandom clustering of modified residues in the modified amylose.

Purified radioactive oligosaccharides were analyzed by using descending paper chromatography (solvent *B*, 18–20 h). A sample of the original PPA digest, which had been reduced with sodium borohydride, was subjected to total acid hydrolysis, followed by paper chromatography (solvent *B*) and autoradiography. Visual inspection of the autoradiogram indicated that D-allose, with a chromatographic mobility relative to D-glucose (R_{Glc}) of 1.4, was the major radioactive product obtained from this hydrolysis, with smaller amounts of D-glucose (R_{Glc} 1.0) and D-glucitol (R_{Glc} 2.1), and still smaller amounts of D-allitol (R_{Glc} 1.9). Rechromatography of the D-allose and D-allitol fractions confirmed the identities

of these fractions. Isolation of D-allitol in this hydrolyzate indicates that modified products containing D-allose in the reducing end are formed during PPA enzymolysis.

Paper chromatography (solvent *B*) of the monosaccharide fraction obtained from PPA hydrolysis of the modified amylose showed radioactive D-glucose (85% of the total labeled monosaccharide) with small amounts of radioactive D-allose (15%). Chromatography of the labeled disaccharide fraction indicated that this fraction consisted of maltose (R_{Glc} 0.19, 25% of the total radioactive disaccharide), plus a modified component with greater chromatographic mobility (R_{Glc} 0.31, 75% of the labeled disaccharide). Reduction of the disaccharide fraction with sodium borohydride produced maltitol (R_{Glc} 0.35) and a modified product (R_{Glc} 0.57). Total acid hydrolysis of the reduced, modified disaccharide, followed by paper chromatography and autoradiography, produced D-allose as the major labeled product (55%), with smaller amounts of D-glucitol (21%), D-glucose (18%), and D-allitol (6%). This result indicated that both possible modified maltoses [D-allosyl-α-(1→4)-D-glucose, and D-glucosyl-α-(1→4)-D-allose] were originally produced by PPA hydrolysis. The ratio of D-allose:D-allitol released during acid hydrolysis of the reduced disaccharide was determined to be 9:1, indicating that D-allose residues were located principally in the nonreducing end of the disaccharide. Thus, as seen for other experiments in which two isomers of modified disaccharide were produced, PPA hydrolysis favors formation of the disaccharide modified in the nonreducing residue (unpublished results).

The modified trisaccharide fraction obtained from PPA hydrolysis of modified amylose was examined by reduction, followed by treatment with *R. niveus* glucoamylase under conditions that gave complete hydrolysis of maltose but no hydrolysis of maltitol. As a result, only the nonreducing residue is hydrolyzed from the reduced trisaccharide, producing labeled monosaccharide and labeled reduced disaccharide products. The monosaccharide fraction obtained from glucoamylase hydrolysis of the reduced trisaccharide contained labeled D-allose (29% of the total radioactivity in the original trisaccharide) and a small amount of labeled D-glucose (8%). This result indicated that the trisaccharide mixture contained a maltotriose analog having D-allose in the nonreducing end. The reduced disaccharide obtained from glucoamylase hydrolysis of modified trisaccharides was purified, and hydrolyzed with acid, producing D-allose (35% of the total radioactivity in the original trisaccharide), D-glucose (16%), D-glucitol (8%), and D-allitol (4%). These results indicate that the original trisaccharide fraction obtained from PPA hydrolysis of the modified amylose included maltotrioses containing D-allose primarily in the middle and nonreducing residues, with a small proportion in the reducing end.

DISCUSSION

The active site of porcine pancreatic alpha amylase has been shown to consist

of five subsites which bind a series of five glucose residues, with hydrolysis occurring between the second and third residues from the reducing end[9]. When modified substrates are hydrolyzed by PPA, the structure of modified oligosaccharide products will reflect whether productive binding of the modified residues is allowed at some or all of the enzyme subsites. It has been observed, for example, that subsite 3 (the subsite where hydrolysis occurs) is particularly sensitive to bulky modifying groups[1-3,5-7]. As a result, modified oligosaccharides containing these substituents at the reducing-end residues are not formed during PPA hydrolysis.

The action of PPA on a modified amylose containing 10% of D-allose residues leads to formation of D-allose and two isomeric, modified disaccharides. This indicates that productive binding of D-allose residues is allowed at subsites 1 through 4 of the active site of the enzyme. The disaccharide modified in the nonreducing-end residue is formed in ~9 times greater amount then the disaccharide modified in the reducing-end residue. This is consistent with results for amylose analogs containing 2-deoxy-D-glucose, 6-deoxy-D-glucose, and 6-deoxy-6-fluoro-D-glucose residues (unpublished results). We have also obtained evidence for formation of a trisaccharide that contains D-allose in the nonreducing residue, which would require productive binding of D-allose residues at subsite 5. These results may be compared with results obtained for several unusual amylase substrates modified at C-3, as shown in Table I. Residues containing bulky modifying groups [3-*O*-(hydroxyethyl) or 3-*O*-D-glucosyl] are not productively bound at subsite 3, in contrast to D-allose. Introduction of the sterically large, negatively charged phosphate group seriously affects enzyme–substrate interactions, and this modification is productively bound only at subsite 4.

The results obtained from PPA substrates modified at C-3 are consistent with trends observed for modifications at C-2 and C-6. These trends are summarized as follows:

TABLE I

PRODUCTIVE BINDING OF SUBSTRATES MODIFIED AT C-3 TO THE SUBSITES OF PORCINE PANCREATIC ALPHA AMYLASE

C-3 Modification	*Subsite of porcine pancreatic alpha amylase*[a]					*Ref.*
	1	*2*	*3*	*4*	*5*	
D-Allose	+	+	+[b]	+	+	This study
3-*O*-(Hydroxyethyl)	+	+	−	+	*	3
3-*O*-α-D-Glucosyl	+	+	−	+	n.d.	6
3-Phosphate	−	−	−	+	−	8

[a]Subsites numbered from reducing end as in ref. 9. Plus signs indicate that productive binding is allowed. Minus signs indicate that productive binding is not allowed. Asterisks indicate that productive binding probably occurs, but has not been definitely demonstrated. N.d. indicates that binding has not been determined. [b]Productive binding is allowed at subsite 3, but does not seem to be as favorable as binding of D-glucose.

1. The effect of substrate modifications is greatest at subsite 3. Enzyme–substrate interactions at other subsites are relatively less specific.

2. Sterically large or charged modifications are not productively bound at subsite 3, nor at other specific subsites.

3. Productive binding of sterically small modifications is allowed at subsite 3 and at other subsites. Productive binding of these modified substrates, however, seems to be less favorable than binding of D-glucose.

The sensitivity of subsite 3 to bulky and charged modifications is not surprising, since the site of catalytic attack might be expected to require a highly specific orientation. Results from bulky modifiers are very consistent[1–3,5–7], and indicate that subsite 3 is sensitive to substituents at C-2, C-3, and C-6. In addition, subsite 2 is sensitive to bulky modifications at C-2, and subsite 4 is sensitive to bulky groups at C-6. This pattern of binding specificity may reflect the conformation of the substrate in the enzyme–substrate complex. Comparison of these results with results obtained from substrates modified with sterically small groups suggests that the effect of bulky substituents originates from steric, rather than electronic, disruption.

The active site of PPA has been shown by low-resolution X-ray diffraction to be a long crevice running down one side of the enzyme[12,13]. The conformation of bound substrate has not been determined. The results of this study indicate that the action of PPA is not critically affected by the presence of D-allose residues in the substrate. This may be consistent with binding of PPA substrates in a helical conformation in which the axial hydroxyl group at C-3 of D-allose residues protrudes into the interior of the helix, a region that may be of limited accessibility to the enzyme. Binding of PPA substrates in a conformation having helical character is consistent with studies that have proposed a helical conformation for amylose in neutral solution[14–18] with the observation that cyclomaltoheptaose is a competitive inhibitor of PPA[19], as well as being a poor substrate for the enzyme[20], and with the observation that maltoheptaose binds to the glycogen-storage site of glycogen phosphorylase in a left-handed helical conformation having 6.5 D-glucose residues per turn[21].

The active site of PPA may resemble the hydrophobic cleft found for many enzymes, including lysozyme[22]. The sensitivity of PPA to bulky or charged modifications at subsite 3 suggests that a highly organized ensemble of steric and electronic interactions is required at this subsite. Several authors, including Warshel and Levitt[22] and Wolfenden[23], have indicated that electronic interactions may be especially crucial for enzyme action in a hydrophobic enzyme-environment. Interactions between PPA substrates and subsites other than subsite 3 are considerably less sensitive to modification. Although binding requirements at subsites 1, 2, 4, and 5 are less stringent, their binding makes a major contribution to catalysis[24]. Elucidation of the role of the multi-subsite PPA binding site remains central to understanding the catalytic efficiency of this enzyme.

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