Optimum pH Control Mechanism for Porcine Pancreatic α-Amylase

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We studied the substrate-dependence of pH activity of porcine pancreatic α-amylase by using a series of p-nitrophenyl maltoligosaccharides. The mechanism controlling the optimum pH of mammalian α-amylase involved the reception and recognition of a substrate component at some other substrate binding sites, in addition to those at subsite 5 that were reported previously [K. Ishikawa et al., Biochemistry, 32, 6259–6265 (1993)].

Human and porcine pancreatic α-amylases show maximal hydrolytic activity at pH 6.9 for high-molecular weight substrates such as starch and amylose.11 However, we have found that some low-molecular weight substrates such as p-nitrophenyl α-D-maltoside and maltopentaol exhibited the activity optima at pH 5.2,2,3 like microorganism α-amylases whose optimal pH values were 5–6 regardless of the substrate.4,5 Since the amino acid sequences relevant to the active site are highly conserved in both mammalian and microorganism α-amylases,5 the mechanism for the substrate-dependent pH activity specific to mammals is very interesting in terms of the biological evolution and protein engineering of α-amylases.

In the previous studies, it was revealed that the optimum pH values for the mammalian α-amylases were nearly neutral only when a substrate binding site remote from the catalytic center, subsite 5, received and recognized a component of the substrate, which must be a six-membered ring including a phenyl group.6 In this paper, we report the finding that another binding site located far from the catalytic center in the opposite direction to subsite 5 also plays an important role in controlling the pH activity of mammalian α-amylases, on the basis of the results of a study using porcine pancreatic α-amylase (PPA) and p-nitrophenyl maltoligosaccharides as the substrates.

PPA was purified from porcine pancreatin as isozyme I, as previously described.7,8 A TSK-G-2000 PW column (7.5φ × 600 mm) was from Toyo Soda Mfg. Co. Ltd. (Japan), and p-nitrophenyl α-D-maltotrioside (G₃Φ), p-nitrophenyl α-D-maltotetraoside (G₄Φ) and p-nitrophenyl α-D-maltopentaoside (G₅Φ) were from Calbiochem Corp. (U.S.A.). The other chemicals used were of reagent grade.

PPA-catalyzed reactions of the p-nitrophenyl maltoligosaccharides were carried out at 30°C, and the products were quantitatively analyzed by high-performance liquid chromatography (HPLC), using the TSK-G-2000 PW column (7.5φ × 600 mm). Sugar parameters were monitored by a differential refractometer, and p-nitrophenyl maltoligosaccharides were measured at 313 nm with a UV detector.9,10 The molarity of PPA was determined from the absorbance at 280 nm on the basis of a molecular weight of 55,000 and A₁% = 24.0 cm⁻¹.10 The reaction was initiated by mixing aliquots of the enzyme solution with the substrate (0.4–30.0 mM) in a 25 mM Tris-acetate buffer (pH 4.0–9.0) containing 30 mM NaCl and 0.1 mM CaCl₂ at 30°C, and terminated by adding the same volume of a 0.1 N acetic acid solution to the reaction mixture. The initial reaction rates and the hydrolytic patterns were determined from the products prior to 15% conversion. Michaelis parameters (kcat and Km) were determined by the non-linear

![Fig. 1. Cleavage Position of the p-Nitrophenyl Maltooligosaccharides (G₃Φ; (A), G₄Φ; (B), and G₅Φ; (C)), and the pH Profiles of Their Cleavage Activities.](image)

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least-squares method with the Taylor expansion.11) The substrate-binding pocket of PPA consists of five glucose-receptive subsites, and the catalytic residues are located between subsites 3 and 4.12) Using p-nitrophenyl-z-α-maltoligosaccharides (G₅-Φ) as the substrate of PPA, the hydrolytic bond-cleavage positions and pH profiles (plots of the activity vs. pH) for each cleavage reaction were determined with the results shown in Fig. 1. Under the conditions used, no transglycosylation products were observed, and the hydrolytic bond-cleavage patterns were independent of the substrate concentrations examined. G₅-Φ was cleaved at only one position, and the pH profile for the cleavage reaction showed maximum activity at pH 6.9, as illustrated in Fig. 1(A). G₅-Φ and G₄-Φ were cleaved at four and three positions, respectively (Fig. 1(B) and (C)). The $K_{m}$ values for the cleavage activity of these substrates were all independent of the pH value examined. The hydrolytic (productive) binding modes of G₅-Φ, G₄-Φ, and G₃-Φ to the binding pocket of PPA, and the optimum pH values obtained from the pH profiles for the corresponding cleavage activities are summarized in Fig. 2.

The optimum pH values for these substrates, except those of the following reactions, agree with expectations from the previously proposed mechanism.2,6) The acidic optima of the pH profiles at 5.3 for the hydrolytic activity, illustrated as ● in Fig. 1(B) and as ○ and △ in Fig. 1(C), cannot be explained by the proposed mechanism that the optimum pH for hydrolytic activity is controlled to be neutral when substrate 5 is occupied with a glucose residue (G) or p-nitrophenyl group (Φ). This fact suggests that the substrate binding to subsite 5 is not enough to cause optimal activity at neutral pH. Figure 2 and the previous studies2,3) indicate that the cleavage activity achieved at neutral optimum pH was observed when glucose residues occupy subsites 2 to 4 or 1 to 4, in addition to the occupancy of subsite 5 with G or Φ, respectively. This means that recognition of the substrate on both sides of the active center (subsites 1 and 2, and subsite 5) is important for controlling the optimum pH.

The $K_{m}$ values for neutral and acidic optimum pH were 1–2 mM and 3–10 mM, respectively. The affinity between the enzyme and the substrate showing optimum pH in the neutral region was a little higher than that showing optimum pH in the acidic region. Therefore, a small difference in the affinity contributed to the shift in optimum pH. This interaction between the bound substrates and the subsites is thought to induce a conformational change of the catalytic site to switch the catalytic mechanism that is effective at neutral pH values. The phenomenon of the optimum pH being controlled by the substrate has been observed in porcine pancreatic z-amylase,2,3) human pancreatic z-amylase,6) and human salivary z-amylase (unpublished data). For some of the z-amylases, the pH profile of the activity was independent of the substrate. Even for the quoted z-amylases, however, the similarity between the primary3) and tertiary structure13,14) among z-amylases suggests the possibility that the active center is influenced by the substrate binding sites without changing the optimum pH.

References