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Carbohydrate Research 257 (1994) 155–161

CARBOHYDRATE RESEARCH

Note

Effect of modifying histidine residues on the action of *Bacillus amyloliquefaciens* and barley-malt α-amylases

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(Received October 5th, 1992; accepted in revised form September 14th, 1993)

Modification of porcine pancreatic α-amylase (PPA) and Taka-amylase A(TAA) with diethyl pyrocarbonate (DEP) causes activation of the release of *p*-nitrophenol from *p*-nitrophenyl α-maltoside (G2PNP), and a decrease in amylase activity (hydrolysis of α-1,4 glucosidic bonds in starch) [1–4]. Among the possible sites of modification, attention focuses on three histidine residues present around the active site of α-amylases of many different origins [5–8]. In PPA these are His 101, His 201, and His 299, with His 101 and His 299 being very close to the site of catalysis and thus perhaps directly or indirectly involved in the catalysis. On the other hand, His 201 is located on the aglycon side of the catalytic site, and we have suggested that it is involved in the increase of PNP release after chemical modification [1–3]. Investigations of site-directed mutagenesis of the histidine residues of human pancreatic α-amylase support this identification [9].

Although the degree of sequence similarity among α-amylases of different origins is low, there are several conserved short regions [8,10]. Most belong to the structural components of the active site in PPA and TAA. Furthermore, there is a close similarity in the three-dimensional structures of PPA and TAA [5–7]. The conserved residues around the active site in all α-amylases suggest some universal structural similarities in these active sites. Therefore, we examined the effects of the chemical modification of histidine residues in *Bacillus amyloliquefaciens* α-amylase(BLA) with DEP and made a comparison with modification of barley α-amylase isozyme II(BAII), using identical substrate systems. These two α-

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SSDI 0008-6215(93)E0396-I

amylases have more substrate binding subsites than PPA and TAA, and have similar action patterns with malto-oligosaccharides [11,12].

1. Experimental

Bacillus amyloliquefaciens α-amylase(BLA) was from Seikagaku Kogyo Co. α-Amylase from barley malt, type VIII-A was purchased from SIGMA CHEMICAL Co., and isozyme-II (BAII) was purified from the commercial product by chromatographic procedures [13] and corn starch absorption methods [14]. Purified BAII showed a single PAGE band with a migration corresponding to that reported by Weselake et al. [13]. The concentration of enzyme was determined spectrophotometrically at 280 nm, assuming $E^{1\%,1\ cm} = 25.6$ and a molecular weight of 58 000 for BLA [15,16], and $E^{1\%,1\ cm} = 25$ and a molecular weight of 45 005 for BAII [17], respectively.

p-Nitrophenyl derivatives of α-malto-oligosaccharides, namely *p*-nitrophenyl α-maltoside (G2PNP), *p*-nitrophenyl α-maltotrioside (G3PNP), *p*-nitrophenyl α-maltotetraoside (G4PNP), *p*-nitrophenyl α-maltopentaoside (G5PNP), *p*-nitrophenyl α-maltohexaoside (G6PNP), and *p*-nitrophenyl α-maltoheptaoside (G7PNP), were purchased from CALBIOCHEM Co. Diethyl pyrocarbonate (DEP) and other chemicals were purchased from Nacalai Tesque.

Amylase activity, as international units (μmol α-1,4 glucosidic bonds hydrolyzed per min), was measured at 30°C by the Somogyi–Nelson method [18], using soluble starch (0.27%) in 20 mM acetate buffer with 1 mM $CaCl_2$, at pH 6.0 for BLA and pH 5.5 for BAII.

PNP-release was measured at 30°C and pH 6.9 (20 mM phosphate buffer containing 1 mM $CaCl_2$), using various *p*-nitrophenyl α-malto-oligosaccharides as substrates. Release of *p*-nitrophenol was measured by the increase of absorbance at 400 nm (molar absorbance of 9470 $M^{-1}\ cm^{-1}$ at pH 6.9) in a Shimadzu UV2200 spectrophotometer. Kinetic parameters for native and modified BLA were determined using G6PNP as a substrate under the above conditions. The concentration of the substrate was varied between 1.01 and 6.05 mM (5 points), and the enzyme concentration was 1.0 μM. The experimental data were analyzed by Sakoda's method [19].

Modifications of the enzymes with DEP were carried out essentially as described previously [1–3]. The reaction was started at 20°C and pH 6.0 (20 mM phosphate buffer), by adding DEP (20% solution in ethanol) from a microsyringe to 3 mL of the enzyme solution in a quartz cuvette, to give a final DEP concentration of 4.5 mM. The reaction was monitored by measuring the increase of the absorbance at 240 nm due to formation of N^{im}-ethoxycarbonylhistidine [20]. Excess histidine (12 mg/mL) was added to remove unreacted DEP, and the mixture was then dialyzed repeatedly at 4°C against 20 mM phosphate buffer (pH 6.9) containing 1 mM $CaCl_2$.

The number of modified histidine residues per enzyme molecule was determined by the change of absorbance at 240 nm (3200 $M^{-1}\ cm^{-1}$) [20]. Modifica-

tions were carried out in the presence of 15 mM maltotetraose in the manner just described.

2. Results and discussion

PNP-release from α-PNP-maltodextrins is described in the following reaction scheme,

$$\overset{K_P}{\leftrightarrows} E(GnPNP)_P \overset{k_P}{\rightarrow} E + PNP + Gn$$

$$E + GnPNP \overset{K_A}{\leftrightarrows} E(GnPNP)_A \overset{k_A}{\rightarrow} E + GiPNP + Gn - i$$

$$\overset{K_N}{\leftrightarrows} E(GnPNP)_N$$

$$(n = 2 \sim 7, i < n)$$

where E, $E(GnPNP)_P$, $E(GnPNP)_A$, and $E(GnPNP)_N$ are free enzyme, the productive complex for PNP-release, productive complexes to produce oligosaccharides smaller than G*n*, and nonproductive complexes, respectively; G*n*PNP is a *p*-nitrophenyl derivative of G*n*; K_P, K_A, K_N are the respective formation constants. Intrinsic catalytic rate constants from $E(GnPNP)_P$ and $E(GnPNP)_A$ are shown as k_P and k_A, respectively. Michaelis parameters (k_{cat} and K_m) for PNP-release activity are formulated as follows,

$$k_{cat} = \frac{K_p k_p}{K_p + K_A + K_N} \quad (1)$$

$$K_m = \frac{1}{K_p + K_A + K_N} \quad (2)$$

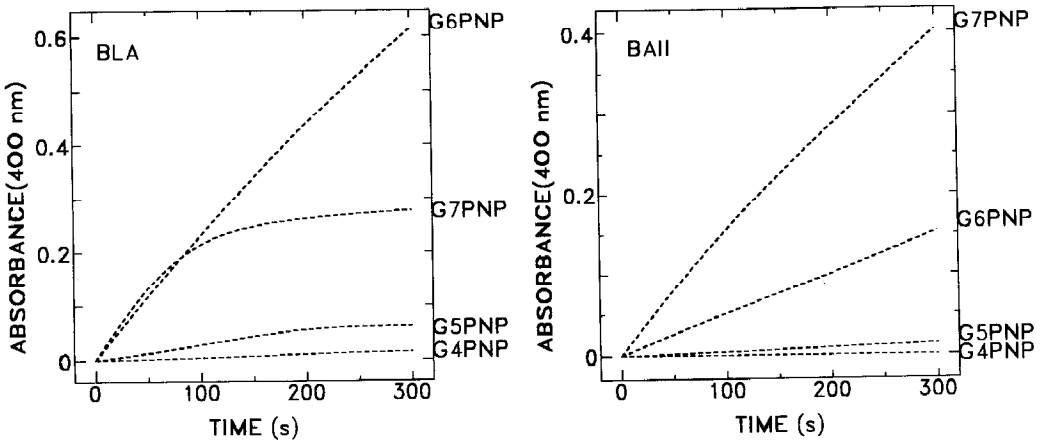

Fig. 1. Relative activities for the release of PNP from *p*-nitrophenyl α-malto-oligosaccharides. Substrate concentrations were 0.225% (thus, G4PNP 2.86 mM, G5PNP 2.37 mM, G6PNP 2.02 mM, and G7PNP 1.77 mM). Enzyme concentrations were: BLA, 1.0 μM; BAII, 0.18 μM.

Table 1
Relative activities of native and modified BLA and BAII

Sample [a]		Amylase activity, U/mg (%)	PNP-release activity, U/mg (%)
Native	BLA	1670 (100)	0.24 (100)
Modified	BLA	1370 (81)	1.38 (575)
Native	BAII	362 (100)	0.40 (100)
Modified	BAII	311 (86)	0.23 (58)

[a] Conditions are described in the Experimental section.

The PNP-release activities of BLA and BAII are shown in Fig. 1. The relative activities of BLA for G6PNP and G5PNP were ca. 1:0.1. There was only a slight activity on G4PNP (less than 2% of G6PNP), and no discernible activity on G3PNP and G2PNP by 1.0 μM BLA. The relative activities of BAII for G6PNP and G5PNP were also ca. 1:0.1. Discernible activity was not observed with G4PNP, G3PNP, and G2PNP in 0.18 μM BAII. The release of *p*-nitrophenol from G6PNP or G5PNP was linear in the initial stage of hydrolysis by BLA or BAII, but that from G7PNP deviated from linearity very early, particularly in the case of BLA (Fig. 1). Therefore, comparisons of PNP-release activities from native and modified BLA or BAII were carried out with G6PNP as a substrate.

DEP modification of BLA was completed within 50 min, with 3.9 histidine residues derivatized per enzyme molecule. In the case of BAII, DEP modification was completed within 100 min, with derivatization of 2.4 histidine residues per molecule. Amylase and PNP-release activities (substrate G6PNP) of both modified enzymes were compared with the activities of the native proteins. The results are shown in Table 1. The PNP-release activity of BLA was increased (575% vs. native BLA) by the modification of the histidine residues, but the amylase activity was slightly decreased (81% vs. native BLA). Thus, the behavior of BLA upon modification of its histidine residues is qualitatively the same as published for PPA and TAA (substrate G2PNP) [1–4]. By treatment of the modified BLA with 0.5 M HCl–hydroxylamine [2] the properties of native enzyme were recovered, as a result of the removal of the ethoxycarbonyl substituents. Both the amylase and the PNP-release activities of BAII were only slightly decreased by modification of the histidine residues (86 and 58%, respectively, of those of the native BAII). Consequently no discernible effect was observed on treatment of modified BAII with HCl–hydroxylamine.

Michaelis parameters for the PNP-release activities of native and modified BLA were determined and are given in Table 2. The results of the protection of the active site by 15 mM maltotetraose are also shown in Table 2. Only 1.7 histidine residues per enzyme molecule were modified in the presence of 15 mM maltotetraose. The PNP-release and amylase activities of the maltotetraose-protected enzyme were almost the same as those of the unprotected enzyme. The k_{cat} was increased ca. ten-fold and the K_m was increased ca. two-fold compared to native

Table 2
Kinetic parameters for PNP-release activity of native and modified BLA

Sample [a]	k_{cat} (s^{-1})	K_m (mM)
Native	0.57 ± 0.04	2.3 ± 0.3
Modified [b]	6.8 ± 0.1	4.8 ± 1.3
Modified/G4[b,c]	5.9 ± 0.9	3.6 ± 1.1

[a] Conditions and analytical procedures are described in the Experimental section.
[b] Average numbers of derivatized histidine residues were 3.9 (Modified) and 1.7 (Modified/G4).
[c] Modified/G4 means derivatization in the presence of 15 mM maltotetraose.

BLA. In the case of BAII, 1.3 histidine residues per enzyme molecule were modified in the presence of 15 mM maltotetraose but there were no discernible changes in either amylase or PNP-release activities. Overall, DEP modification of histidine residues in BAII is without significant effect.

The subsite patterns of BLA [11] and BAII [12], determined from catalytic activities and product analyses, are shown in Fig. 2. Subsites are numbered from the catalytic site (indicated by a thick upward arrow) toward the nonreducing or reducing end as N*i* or R*i* ($i = 1, 2, 3, \ldots$ etc.), respectively. Subsites toward the nonreducing end are extended to N6 for both BLA and BAII. Therefore, the productive binding mode for the formation of *p*-nitrophenol from G6PNP is from N6 to R1 as shown in Fig. 2. Robyt [21] suggested that the nonreducing ends of small substrates tend to occupy the terminal N6 site of BLA, sometimes giving productive and sometimes nonproductive complexes.

There is a remarkable difference between BLA and BAII in the effects of histidine modification on their PNP-release activities. The difference must reflect differences in the location of histidine residues at the active sites of the two enzymes. There are three histidine residues around N1, R1, and R2 (His 101, His 201, and His 299) in PPA and TAA [5,6], and probably most other mammalian and microbial α-amylases. His 201 is located near R1 and R2 of PPA and TAA [5,6]. In the productive binding mode for PNP-release the *p*-nitrophenyl group occupies the R1 site, and therefore, His 201 may engage in noncovalent interactions with the p-nitrophenyl group. In our previous studies on PPA, we suggested that modification of His 201 results in steric and partial hydrophobic effects around R1,

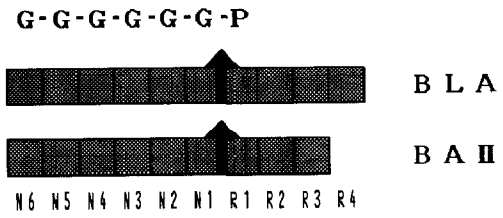

Fig. 2. The subsite patterns of BLA and BAII and the productive binding mode for PNP-release from G6PNP. Subsites are numbered as described in the text. G = glucose residue and P = *p*-nitrophenylate.

and these effects may be advantageous for PNP-release activity on G2PNP. Increase of PNP-release activity is due to an increase of K_P or decrease of both K_A and K_N; and/or increase of k_P in Eq. 1. These effects may correspond to stabilization of the productive binding mode or destabilization of other binding modes; and/or stabilization of the transition state during catalysis.

Although TAA and PPA are produced by widely different organism, the three-dimensional structures of the catalytic sites of the two enzymes resemble each other [5,6]. It is assumed that, in general, parts of the active site composed of conserved homologous regions have similar structures in α-amylases from different origins. The most highly conserved homologous regions are around N1, R1, and R2. Therefore, it is reasonable to consider that His 201 is located near R1 and R2 in the majority of α-amylases. However, His 201 is replaced by Gly in barley α-amylases [8,22]. This replacement may be regarded as a point mutation. The slight decrease of the activity for PNP-release resulting from histidine modification in BAII is interpreted as an indirect effect due to the derivatization of histidine residues located at positions other than N1, R1, and R2. In our previous studies on mutants of human pancreatic α-amylase, the substitution of aspacagine for His 101 and His 299 caused decrease of both amylase (substrate maltohexaitol) and PNP-release (substrate G2PNP) activities, and His 201 → Asn caused increase of PNP-release activity [9]. Therefore, His 101 and His 299 in most α-amylasaes are assumed to be inactive toward DEP under our conditions.

Since derivatization in the presence of 15 mM maltotetraose reduced the number of modified histidine residues in both BAII and BLA, histidines other than the three conserved residues are near the maltotetraose binding site. Because the main binding site of maltotetraose is subsites N2–N5 of the active site [12], histidine residues around R1 and R2 may not be effectively protected by maltotetraose. However, the modification of histidine residues other than His 201 does not result in the activation of PNP-release catalysis. His 201 is replaced by different residues in other plant α-amylases (e.g., from rice and mung bean) [23,24], from which it is expected that in general the PNP-release activities of microbial and animal α-amylases would be increased by histidine modification with DEP, whereas those of plant α-amylases, lacking His 201, would not be.

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