Site-Directed Mutagenesis Reveals Critical Importance of the Catalytic Site in the Binding of α-Amylase by Wheat Proteinaceous Inhibitor

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ABSTRACT: A bacterial α-amylase from *Bacillus subtilis* was found to be strongly inhibited by wheat α-amylase inhibitors 0.53 and 0.19, which had previously been thought specific for animal α-amylase. Inhibition and gel filtration studies of site-directed mutants of *B. subtilis* α-amylase with the inhibitors indicated a direct correlation between the α-amylase activity and the inhibitory effect or inhibitor binding. A mutant enzyme His180 → Asn, which was 20 times less active in terms of k_{cat} than the wild type, was less sensitive to inhibition by similar degrees, while the specificity for 0.53 and 0.19 changed significantly as a result of the mutation. Catalytic-site mutants that were completely devoid of catalytic activity virtually lost the ability to bind inhibitors, even though they retained high affinities for substrates. The results show that the integrity of the catalytic site is crucial for inhibitor binding and, despite the previously observed tight binding, reveal a subtle nature of the interaction between α-amylase and the wheat inhibitor, which leads to a proposal of a two-step mechanism for the binding interaction.

To date a large number of proteinaceous inhibitors of α-amylase (EC 3.2.1.1) have been characterized with respect to their molecular properties and inhibitory effects, but the nature of their interaction with α-amylase remains unknown. For example, it is not yet clear whether inhibitor binds to the active site of α-amylase or it binds elsewhere and acts indirectly. To clarify this point, residues involved in the binding of the two proteins must be identified and the structure of the enzyme–inhibitor complex must be characterized. In the present study we tackled this problem by protein engineering.

In investigating the inhibitory effects for various combinations of inhibitors and α-amylases, we found that the wheat dimeric inhibitors designated 0.53 and 0.19 inhibited *Bacillus subtilis* N7 α-amylase quite effectively. These inhibitors belong to the so-called 24-kDa family, which is thought to act mainly on mammalian and insect α-amylases (Buonocore et al., 1977; Garcia-Olmedo et al., 1987). Their amino acid sequences differ at only seven positions out of 124 total amino acid residues, but they exhibit somewhat different specificities (Maeda et al., 1985). We studied the interactions of these wheat inhibitors with several site-directed mutants of *B. subtilis* α-amylase. These mutants have been previously characterized with respect to their kinetic and substrate-binding properties (Takase et al., 1992; Takase, 1992). Here we report that a single catalytic residue of α-amylase is essential for inhibitor binding and show that the inhibitor binding and specificity can be modified by mutation of a single residue in the active site. On the basis of these results, we propose a possible scheme for the α-amylase–inhibitor interaction, which could explain the different effects observed upon reversing the order of addition of inhibitor and substrate.

MATERIALS AND METHODS

Materials. Soluble starch (biochemical grade) was obtained from Wako Pure Chemicals. Starch solutions were prepared by boiling, filtered through 0.45-μm Millex-HA filters (Millipore), and used immediately. Human salivary α-amylase (Type IX-A) and *Bacillus licheniformis* α-amylase (Type XII-A) were purchased from Sigma, porcine pancreatic α-amylase and *Bacillus amyloliquefaciens* α-amylase were purchased from Boehringer Mannheim, and Taka-amylase A (*Aspergillus oryzae* α-amylase) was purchased from Sankyo Co. The enzyme preparations were used without further purification. *Bacillus stearothermophilus* A631 α-amylase was prepared and purified as described previously (Takase, 1993). Acarbose was a generous gift from Bayer. Molecular weight markers were obtained from Pharmacia and Sigma.

α-Amylase Inhibitors. Partially purified wheat α-amylase inhibitor was purchased from Oriental Yeast Co. and further purified on a column of Superdex 75 HR10/30 in 20 mM Tris-HCl (pH 7.5) using a FPLC system (Pharmacia). The pooled dilute solution was concentrated by Amicon (PM10 membrane). The purified inhibitor showed a single band in SDS-PAGE[1] (both 12.5% and 15% gels were used), from which the molecular weight was estimated to be about 16 kDa. It also migrated as a single band in native PAGE (7.5% gel) at a mobility of 0.44 relative to bromophenol blue. The elution profile from Superdex 75 HR10/30 in 20 mM Tris-HCl/0.1 M NaCl (pH 7.5) exhibited some trailing tendency, and the peak corresponded to a molecular weight of 23 kDa (Figure 2b). The molecular weight estimated in 6 M guanidine hydrochloride/50 mM Tris-HCl (pH 7.5) using a column of Superdex 200 HR10/30 was 12 kDa. Thus, this inhibitor is assumed to exist as a dimer under nondenaturing conditions. This preparation of the inhibitor was identified as the 0.53-inhibitor by coelectrophoresing in SDS-PAGE and native PAGE with standard samples of the 0.53- and 0.19-inhibitors provided by Nisshin Flour Milling Co. Our preparation and the standard 0.53-inhibitor had identical inhibitory effects on *B. subtilis* α-amylase. It was shown to strongly inhibit human salivary α-amylase while having little effect on porcine pancreatic α-amylase activity. The inhibition ratio for the two amylases was greater than 500–1000. The monomer molecular weight calculated from the amino acid sequence of the 0.53-inhibitor is 13 185 (Maeda et al., 1983a,b). Previ-

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[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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ously, the mobility in native PAGE was reported to be 0.53 (Maeda et al., 1982). The reason why our results of SDS-PAGE and native PAGE gave different molecular weight and mobility values is not clear. Treatment of the inhibitor with *N*-glycanase (from Genzyme) in the presence of SDS did not change the mobility in SDS-PAGE. The discrepancy of the mobility could be due to a difference in experimental conditions. In this study the dimer molecular weight of 26.4 kDa was used to calculate the molar quantities of the 0.53-inhibitor. Protein concentrations were determined by using $E^{1\%}_{1cm,280}$ = 12.4 (Maeda et al., 1982).

The wheat 0.19-inhibitor used was provided by Nisshin Flour Milling Co. Its mobility in native PAGE (7.5% gel) was 0.1, which disagrees with the value 0.19 reported previously (Silano et al., 1973). In SDS-PAGE (15% gel) it migrated slightly slower than the 0.53-inhibitor. The monomer molecular weight calculated from the amino acid sequence is 13 337 (Maeda et al., 1985); hence a value of 26.6 kDa was taken as the molecular weight of the dimer, the native form under nondenaturing conditions. Protein concentrations were determined by using $E^{1\%}_{1cm,280}$ = 12.6 (Buonocore et al., 1980). Purified common bean α-amylase inhibitor was supplied by Dr. M. Ishimoto of the National Agriculture Research Center, Japan.

B. subtilis α-Amylase (Wild-Type and Site-Directed Mutants). Wild-type and site-directed mutant enzymes of *B. subtilis* N7 α-amylase were prepared as described previously (Takase et al., 1988, 1992). The different N-terminal forms are distinguished by notations N34, N40, and N42 to indicate that the N-terminus is the 34th, 40th, and 42nd residue, respectively, from the initiator Met (Takase et al., 1988). The N42 form is the final mature enzyme, and the residue numbering for this form will be adopted also for N34 and N40 except for the extended sequences. There are no apparent differences in specific activity for the three forms (Takase et al., 1988). The wild-type and mutant enzymes with different N-termini were purified to homogeneity. The mutant enzymes DN176 [Asp176 → Asn], EQ208 [Glu208 → Gln], and DN269 [Asp269 → Asn] are catalytic-site mutants possessing no enzyme activity; HN180 [His180 → Asn] is a substrate-binding-site mutant that has a K_m 5-fold greater than that of wild type and a specific activity or k_{cat} (the catalytic rate constant) $^1/_{20}$ that of wild type; EQ203 [Glu203 → Gln] has a mutation at the site other than the active site and has the same specific activity as wild type (Takase et al., 1992). The protein concentration was determined by assuming $E^{1\%}_{1cm,280}$ = 20 (Takase et al., 1992). The molecular weights of the N40 and N42 forms (wild type) calculated from the amino acid sequence are 48 587 and 48 344, respectively, and are used to calculate molar quantities. When analyzed by gel filtration, *B. subtilis* α-amylase eluted at a position corresponding to a molecular weight of about 30 kDa under nondenaturing conditions on a column of Superdex 75 HR10/30 (Figure 2a) and of about 46 kDa in 6 M guanidine hydrochloride on Superdex 200 HR10/30. The apparent disagreement may be ascribed to a tendency of the enzyme to be adsorbed to gel filtration media, as observed for other amylases (Kauffman et al., 1970).

Assay of α-Amylase Inhibition. The inhibition of α-amylase activity by α-amylase inhibitor was assayed at 37 °C in 0.1 M sodium acetate/2 mM $CaCl_2$ (pH 6) as follows. α-Amylase (~0.3 units; 1 unit of enzyme hydrolyzes 0.1 mg of starch in 1 min) and inhibitor were preincubated at 37 °C in 67 μL of buffer containing 1 mg/mL bovine serum albumin (Sigma A7638) for 20 min; residual amylase activity was then measured by adding 133 μL of 0.5% soluble starch dissolved in the same buffer and allowing the reaction to proceed for an additional 5 min. Hydrolysis of starch was monitored by the iodine method (Fuwa, 1954). The percent inhibition was calculated from the difference between the amylase activities measured in the presence and absence of inhibitor.

The inhibition of both wild-type *B. subtilis* α-amylase and HN180 reached a plateau after 5–10-min preincubation with inhibitor; thus, the 20-min preincubation used in regular assays assured comparison after sufficient equilibrium.

For competition assays, an excessive amount of mutant enzyme was first mixed with the inhibitor and then wild-type enzyme was added for preincubation.

Gel Filtration. The FPLC system (Pharmacia) placed in a chromatochamber maintained at 4 °C was used for all the gel filtration experiments. Columns of Superdex 75 HR10/30 and Superdex 200 HR10/30 were used under nondenaturing conditions (20 mM Tris-HCl (pH 7.5) containing 0–1 M NaCl) and in 6 M guanidine hydrochloride buffered with 50 mM Tris-HCl (pH 7.5), respectively. The samples to be applied (in 100 μL) were filtered through Ultrafree C3-GV (0.22 μm) filters (Millipore).

The Superdex 75 column was run at a flow rate of 0.5 mL/min in most cases. The column was calibrated using the following molecular weight markers (molecular weight in parentheses): ribonuclease A (13.7 kDa); chymotrypsinogen A (25 kDa); carbonic anhydrase (29 kDa); ovalbumin (43 kDa); bovine serum albumin (67 kDa); aldolase (158 kDa). The values of K_{av} were calculated using the equation, K_{av} = (Ve − Vo)/(Vt − Vo), where Ve is the elution volume of the sample, Vo the void volume, and Vt the total bed volume. The Vo value was estimated by Blue Dextran 2000. Except for aldolase, a good straight line could be drawn for the plots of K_{av} vs log(molecular weight), which was used to estimate the molecular weights of native α-amylase, inhibitor, and their complex. In typical experiments of α-amylase–inhibitor complex formation, the two proteins were mixed and incubated at 25 °C for 20 min and then applied on the column and run at 4 °C.

The Superdex 200 column was run at 0.3 mL/min. Samples were pretreated in 6 M guanidine hydrochloride at 50 °C for 1 h before loading. The molecular weight markers used for estimating the monomer molecular weight of the 0.53-inhibitor containing four disulfide bonds were as follows (molecular weight; number of disulfide bond): ribonuclease A (13.7 kDa; 4); bovine α-lactalbumin (14.2 kDa; 4); soybean trypsin inhibitor (20.1 kDa; 2); chymotrypsinogen A (25 kDa; 5). For *B. subtilis* α-amylase, which contains no disulfide bonds, the following were used: cytochrome *c* (12.3 kDa; 0); myoglobin (17.8 kDa; 0); carbonic anhydrase (29 kDa; 0). Each calibration curve formed a straight line but differed from each other.

Gel Electrophoresis. Native PAGE was performed as described by Davis (1964). To observe complex formation of α-amylase and inhibitor, the two proteins were preincubated at 37 or 25 °C for 20 min in 20 mM Tris-HCl (pH 7.5) before being loaded onto the gel which was run at room temperature at 20 mA constant current. SDS-PAGE was performed according to the procedure of Laemmli (1970). The electrophoresis calibration kit of Pharmacia was used to estimate the molecular weights. The gels were stained with Coomassie Brilliant Blue. Since the inhibitors stained only faintly in native gel, it was also stained with silver when necessary.

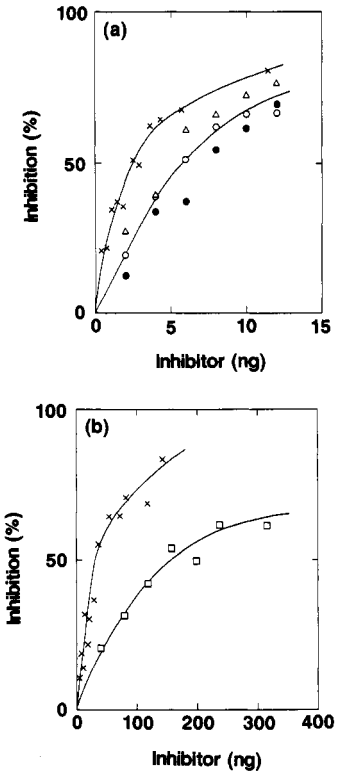

FIGURE 1: Inhibition of activity of *B. subtilis* and human salivary α-amylases by wheat inhibitors 0.53 and 0.19 at pH 6 and 37 °C. About 0.3 units of enzyme were used, which corresponded to 20 ng of *B. subtilis* α-amylase (200 ng in the case of HN180) and an estimated 10 ng of human salivary enzyme: (a) ○, *B. subtilis* wild type/N42 + 0.53; △, EQ203/N42 + 0.53; ●, human salivary α-amylase + 0.53; ×, *B. subtilis* wild type/N42 + 0.19. (b) □, HN180/N42 + 0.53; ×, HN180/N42 + 0.19. The curves were drawn arbitrarily along the points.

RESULTS

Inhibition of α-Amylase Activity by the 0.53-Inhibitor. Contrary to the known specificity of this class of wheat inhibitors, *B. subtilis* α-amylase was found to be inhibited by the 0.53-inhibitor as effectively as human salivary α-amylase (Figure 1a). On the other hand, the following α-amylases from bacterial or fungal origins were not inhibited appreciably: α-amylase from *B. stearothermophilus*, *B. amyloliquefaciens*, *B. licheniformis*, and *A. oryzae* (Taka-amylase A). Thus, the sensitivity of *B. subtilis* α-amylase to this inhibitor may place it in a special class among microbial α-amylases. However, it was not uniquely inhibited by common bean α-amylase inhibitor, which also had no significant inhibitory effects on the above-mentioned α-amylases of microbial origins.

There were essentially no differences in the inhibition pattern between the different N-terminal forms, N34, N40, and N42 (data not shown). EQ203 was also inhibited similarly (Figure 1a). All of these enzyme samples have almost identical specific activities of about 8000 units/A_{280} (Takase et al., 1992). On the other hand, the effect on HN180, which had a specific activity of 560 units/A_{280} (Takase et al., 1992), was greatly diminished, as 25 times more amounts of the 0.53-inhibitor were needed to achieve the same extent of inhibition as observed for the wild-type enzyme (Figure 1b). Because of the low specific activity of HN180, the result shown in Figure 1b was obtained using 0.2 μg of enzyme, which was 10 times more than the amount used for wild type. Therefore, possible effects of enzyme concentration on inhibition were investigated for both wild type and HN180. Varying the enzyme amounts from $^1/_4$ to 2.5 times the levels shown in Figure 1 only marginally affected inhibition, and the difference, as shown in Figure 1, between wild type and HN180 was confirmed by using the same amounts of the two enzymes: 0.05 and 0.1 μg of enzyme at low and high inhibitor concentrations, respectively. These results confirm that HN180 is less susceptible to the 0.53-inhibitor than the wild-type enzyme.

Table 1: Effect of Addition of Catalytic-Site Mutants on the Inhibition of Wild-Type α-Amylase Activity by Wheat α-Amylase Inhibitor 0.53[a]

wild-type enzyme (ng)	mutant enzyme [amount (ng)]	inhibitor (ng)	specific activity (units/A_{280})	inhibition (%)
20		0	7260	(0)
20		6	3710	49
20	EQ208 [12 100]	0	6730	7
20	EQ208 [12 100]	6	4120	43
20	DN269 [7820]	0	6990	4
20	DN269 [7820]	6	3530	51
20	DN176 [8440]	0	6970	4
20	DN176 [8440]	6	3360	54

[a] Assessed by first incubating the inhibitor with the mutant enzyme at 37 °C for 20 min and then adding wild-type enzyme and assaying the remaining α-amylase activity as described under Materials and Methods. The wild-type and mutant enzymes used were all N42 forms.

Application of the method of Bieth (1974) for analysis of mutual depletion systems to the data gave rough estimates of the (true) dissociation constant: 0.11 nM for *B. subtilis* wild-type α-amylase, 0.14 nM for EQ203, and 18 nM for HN180. The values (except for HN180) are comparable to those reported for other α-amylase–inhibitor complexes (Buonocore et al., 1980).

Comparison of the Inhibitory Effects between the 0.53- and 0.19-Inhibitors. The 0.19-inhibitor inhibited *B. subtilis* wild-type α-amylase more effectively than the 0.53-inhibitor (Figure 1a). The effect of the 0.19-inhibitor on HN180 was diminished (13-fold), but not as much (25-fold) as observed for the 0.53-inhibitor (Figure 1b). This resulted in an apparently larger difference in the inhibitory effects on HN180 between the two inhibitors than in those on the wild-type enzyme. Thus, a single point mutation in α-amylase has affected inhibitor specificity.

Effect of Catalytic-Site Mutants on Inhibition. Since the catalytic-site mutants EQ208, DN269, and DN176 are totally inactive, their possible interactions with inhibitor were investigated by competition assays. Table 1 summarizes the results of competition experiments in which the effect of addition of a large amount of mutant protein on the inhibition of wild-type enzyme by inhibitor (0.53) was examined. Six nanograms of inhibitor and 8–12 μg of each mutant protein were incubated together for 20 min followed by addition of 20 ng of wild-type enzyme. The preincubation was allowed to continue an additional 20 min, after which the α-amylase activity was assayed as described under Materials and Methods. As shown in Table 1, any of the catalytic-site mutants added in large excess (≥400-fold in weight) over the wild-type enzyme had negligible effects on the inhibition of wild-type activity by the inhibitor. The apparent inhibition of as much as 7% by EQ208 in the absence of inhibitor should be regarded as within the range of errors associated with the assay. Extending the incubation time of the initial mixture of inhibitor and mutant protein to 2 h did not reduce the extent of inhibition (data not shown). The same results were obtained using human salivary α-amylase as the active enzyme or when the 0.19-inhibitor was used (data not shown). The results show that these catalytic-site mutants do not compete

for the inhibitor with the wild-type enzyme, suggesting that they lost the ability to bind the inhibitor as a result of the mutations.

Gel Filtration Studies of the Complex Formation. The elution behaviors in gel filtration of *B. subtilis* α-amylase (the N40 form) and the 0.53-inhibitor were studied using a Superdex 75 HR10/30 column in 20 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl. The column was run at 4 °C at a flow rate of 0.5 mL/min unless otherwise stated. α–Amylase alone, either wild type or mutant, eluted at about 30 kDa (Figure 2a) (see Materials and Methods for their true molecular weights). The wheat inhibitor eluted as a 23-kDa dimer (Figure 2b).

(a) Wild-Type Enzyme. When the wild-type enzyme and inhibitor were premixed (preincubation at 25 °C for 20 min) at an approximate molar ratio of 1:1 (the dimeric form of the inhibitor is taken as one molecule), a bimodal pattern consisting of (99)-[2] and 57-kDa peaks appeared, while the peaks corresponding to each component disappeared (Figure 2c). Prolonging the preincubation at 25 °C to overnight (>16 h) did not affect the elution pattern. Reducing the flow rate to 0.05 mL/min resulted in merging of the two peaks at about 64 kDa (Figure 2c, broken line). When the wild-type enzyme and inhibitor were mixed at a 2:1 molar ratio, only one peak at (99) kDa was observed in addition to the 32-kDa α-amylase peak (Figure 2d). On the other hand, a 54-kDa peak with a little shoulder at (99) kDa and the 24-kDa inhibitor peak were observed using a 1:2 enzyme/inhibitor mixture (Figure 2e). These results suggest that the wild-type α-amylase and the wheat inhibitor form at least two different complexes, possibly differing in binding stoichiometry, which are in slow equilibrium with each other. Thus, the (99)-kDa form may be saturated with α-amylase, like a 2:1 α-amylase–inhibitor complex; the 54-kDa form may be saturated with inhibitor and exist as a 1:1 α-amylase–inhibitor complex (note that the apparent molecular weight, 54 kDa, is nearly equal to the sum of the values for each component).

(b) Mutant Enzymes That Are Active. The elution profile of EQ203 premixed with the inhibitor at a 1:1 molar ratio was nearly identical to that in the case of wild-type α-amylase (data not shown). Thus, EQ203, which is a fully active mutant, interacts with the wheat inhibitor similarly. On the other hand, HN180 gave different patterns. The elution profile of HN180 and inhibitor mixed at a 1:1 molar ratio (preincubation at 25 °C for 20 min) was rather broad, with a peak at 50 kDa (Figure 2h). Neither preincubation at 25 °C overnight nor running the column at a flow rate of 0.05 mL/min changed the pattern significantly, except that the peak was a bit sharper in the latter case (Figure 2h, broken line). Mixing HN180 and inhibitor at 2:1 gave a peak at 54 kDa in addition to the 32-kDa HN180 peak (Figure 2i). It should be noted that the 54-kDa peak position is the same as observed in the case of wild type–inhibitor (1:2) (Figure 2e). The elution profile of HN180–inhibitor mixed at 1:2 (1.5 μM HN180 and 3.8 μM inhibitor) was similar to that of the 1:1 mixture (data not shown). These results indicate that HN180 is greatly hindered in its ability to form the (99)-kDa complex but does form the 54-kDa complex, which dissociates in relatively rapid equilibrium. Thus, the data suggest an increased dissociation rate for HN180 as compared to the wild-type enzyme.

(c) Catalytic-Site Mutants. In contrast to the cases of the wild-type and above mutant enzymes which are catalytically

[2] The value 99 kDa is only a rough estimate because it is outside the range of accurate determinations.

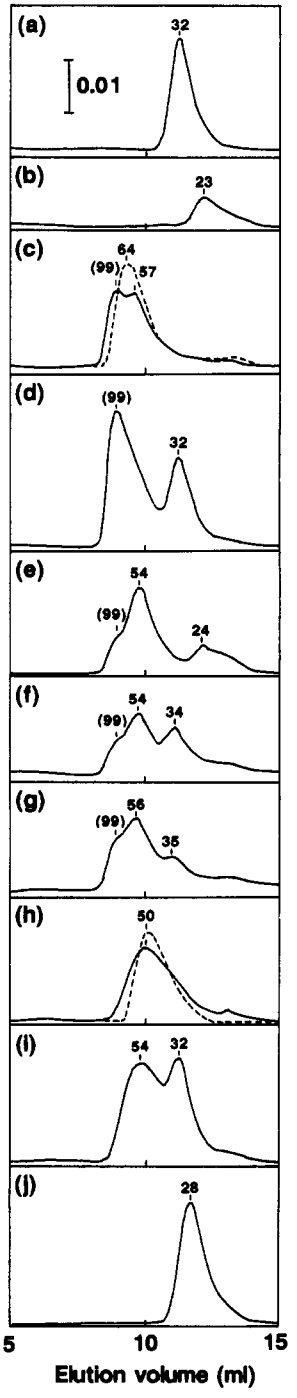

FIGURE 2: Gel filtration of *B. subtilis* α-amylase and wheat inhibitor 0.53 on a Superdex 75 HR10/30 column. The elution was monitored by absorbance at 280 nm (the same scale used for all profiles). The numbers shown above the peaks and shoulders indicate molecular weights in kDa (rough values in parentheses) estimated by calibration of the column with standard proteins of known molecular weights as described under Materials and Methods. The value of Vo was 8.00 mL. The samples were loaded in a volume of 100 μL containing each component at final concentrations as follows (WT, wild-type α-amylase/N40; HN, HN180/N40; EQ, EQ208/N40; I, wheat inhibitor; A, acarbose): (a) 3.1 μM WT; (b) 3.8 μM I; (c) 3.1 μM WT + 3.8 μM I; (d) 6.2 μM WT + 3.8 μM I; (e) 2.5 μM WT + 6.1 μM I; (f) 3.1 μM WT + 2 mM A + 3.8 μM I; (g) 3.1 μM WT + 3.8 μM I + 2 mM A; (h) 3.1 μM HN + 3.8 μM I; (i) 6.2 μM HN + 3.8 μM I; (j) 3.1 μM EQ + 3.8 μM I. The samples were mixed in 20 mM Tris-HCl/0.1 M NaCl (pH 7.5), incubated at 25 °C for 20 min, and then loaded on the column, which was run at 4 °C using the same buffer. In f and g the first two components were mixed and incubated first, followed by addition of the third component and incubation in the same way. The flow rate was 0.5 mL/min except that the broken curves in c and h were obtained at 0.05 mL/min.

active, we failed to observe any sign of complex formation for the catalytic-site mutants (EQ208, DN269, and DN176) under similar conditions. Thus, EQ208 premixed with the inhibitor at 1:1, 2:1, or 1:2 molar ratios and incubated at 25 °C for either 20 min or overnight showed only one apparent peak at about 28 kDa, which is close to that of EQ208 or inhibitor when applied separately (Figure 2j). The same results were obtained with DN269 or DN176 (data not shown). The results show that the ability to interact with the wheat inhibitor is substantially impaired in these mutants, which is the same conclusion obtained from the inhibition studies.

(d) Effect of NaCl. In addition to the standard buffer 20 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl, the effect of ionic strength on the complex formation was assessed using 20 mM Tris-HCl (pH 7.5) containing either 1 M NaCl or no NaCl. The elution profiles obtained in the presence of 1 M NaCl for the 1:1 mixtures of inhibitor and wild-type enzyme or HN180 were almost the same as those in 0.1 M NaCl (data not shown). Results in the absence of NaCl were similar to those obtained in 0.1 M NaCl with no indication of increased or decreased tendency of complex formation. In particular, EQ208 failed to form a complex with the inhibitor even in 20 mM Tris-HCl (pH 7.5). These results show that the interactions of α-amylase and the wheat inhibitor are not greatly influenced by ionic strength from 20 mM to 1 M.

(e) Effect of Acarbose. Acarbose is a pseudo-oligosaccharide competitive inhibitor of certain glycosidases (Truscheit et al., 1981). It is composed of three glucose units and a cyclitol ring and is thought to act as a transition-state analog. In our previous studies it was found to be a potent inhibitor of *B. subtilis* α-amylase (Takase et al., 1992). Therefore, the effect of addition of acarbose on the complex formation of the wild-type enzyme with the wheat protein inhibitor 0.53 was studied. Since the inhibitory effect of acarbose is time-dependent and depends on the order of addition with respect to substrate (Takase et al., 1992; Hanozet et al., 1981), the enzyme was either first preincubated with acarbose at 25 °C for 20 min and then with the wheat inhibitor for a further 20 min (Figure 2f) or first preincubated with the wheat inhibitor and then with acarbose (Figure 2g). The mixing molar ratio of enzyme and wheat inhibitor was approximately 1:1, and the final acarbose concentration was 2 mM, which is well above that required for 50% inhibition (Takase et al., 1992). Acarbose alone had no effect on the elution of α-amylase (data not shown). By comparing the results obtained in the absence of acarbose (Figure 2c–e), it is apparent that acarbose partially prevents the complex formation of amylase with the protein inhibitor. The effect of acarbose was more significant when the enzyme was preincubated with acarbose prior to the addition of the wheat inhibitor (Figure 2f) than when acarbose was added after preincubation of the enzyme with the wheat inhibitor (Figure 2g), as judged from the higher 34-kDa peak corresponding to free α-amylase and the lower (99)-kDa peak in the former case. It is noteworthy that the (99)-kDa complex is more vulnerable than the 54-kDa complex. Acarbose may deprive enzyme species capable of forming a complex with the wheat inhibitor, thus resulting in the same pattern as Figure 2e, excess wheat inhibitor over enzyme.

Gel Electrophoretic Studies. The interaction of *B. subtilis* α-amylase and the wheat inhibitor 0.53 was analyzed by 7.5% native PAGE at pH 8.3. Wild-type enzyme and EQ203 migrated as slower diffusing bands when each enzyme (1.4 μg) was premixed with inhibitor (0.7 μg), and a concomitant decrease in intensity of the inhibitor band was also observed (data not shown). HN180 exhibited such a tendency but to a much smaller degree. No effects of premixing with the inhibitor were seen for any of the three catalytic-site mutants EQ208, DN176, and DN269, as each mutant and inhibitor band migrated in the same way as when loaded separately. Preincubation of wild-type enzyme with 2 mM acarbose before addition of the wheat inhibitor resulted in two bands, one nearly corresponding to that of the amylase alone and the other to the retarding complex of the enzyme and wheat inhibitor. Addition of acarbose after preincubation of enzyme and wheat inhibitor had no apparent effect on the banding pattern. These results support the conclusions obtained from gel filtration studies.

DISCUSSION

The present unexpected finding that the wheat proteinaceous inhibitors 0.53 and 0.19 act on *B. subtilis* α-amylase with inhibition similar to that on human salivary α-amylase led to a study of the interactions between α-amylase and the inhibitors using site-directed mutagenesis, which focused on the binding site of the inhibitor on α-amylase. A single mutation that replaced the carboxyl group of Glu or Asp at the catalytic site of α-amylase with its amide form (EQ208, DN269, or DN176) resulted in a substantial loss (≫500-fold; Table 1 and Figure 2) of the ability to bind the inhibitor. This implies that any of the three residues Glu208, Asp269, and Asp176 is essential for inhibitor binding. A pair of these residues have been implicated by X-ray crystallographic studies (Matsuura et al., 1984) to be the catalytic residues that are directly involved in the catalytic reaction. Our previous site-directed mutagenesis studies (Takase et al., 1992) have demonstrated that these three residues are critically important in catalysis and thus confirmed that they constitute the catalytic site. These catalytic-site mutants are completely devoid of catalytic activity but retain high affinities for substrates (Takase, 1992). Thus, the present results reveal the critical importance of the catalytic site in inhibitor binding. Although this conclusion does not necessarily indicate that the catalytic site is the binding site of the inhibitor, more preference may be placed upon its rather direct involement, since a mutation of this kind is not likely to cause such a drastic structural change as to (almost) *completely abolish* the ability to bind a protein at the binding site remote from the mutation site.

HN180, which was about 20 times less active in terms of k_{cat} than the wild type as a result of mutation of His180 to Asn (Takase et al., 1992), was 25 and 13 times less sensitive than wild type to inhibition by inhibitors 0.53 and 0.19, respectively. HN180 has a K_m value 5-fold greater than that of wild type (Takase et al., 1992), which suggests that His180 takes part in substrate binding. Because the mutation also affected k_{cat}, it is likely to have disturbed the structure of the catalytic site and thus may have contributed to the decreased susceptibility of HN180 to the inhibitors. The altered specificity of HN180 to the 0.53- and 0.19-inhibitor is reminiscent of the relation between human salivary and pancreatic α-amylase (Maeda et al., 1985): the differential effects of the two inhibitors are much greater in the pancreatic enzyme than in the salivary enzyme, although the former enzyme is only poorly inhibited by both inhibitors. It should be noted that the change in inhibitor specificity was caused by mutation of a single amino acid residue in *B. subtilis* α-amylase, while human salivary and pancreatic α-amylases differ in their amino acid sequences by as much as 17%. Future structural and precise kinetic measurements of the α-amylase–inhibitor interactions will reveal the mechanism of recognition by the inhibitors.

The interfering effect of acarbose on the complex formation between α-amylase and the wheat inhibitor suggests that the two inhibitors, one a substrate analog and the other a protein, are mutually exclusive. Similar effects have been reported previously using maltose or starch (Buonocore et al., 1975; Petrucci et al., 1976). These observations are consistent with the results and conclusion obtained with mutant α-amylases.

The wheat inhibitors seem to recognize only the enzyme forms that are catalytically active, as a close correlation was observed between the catalytic activity (k_{cat}) of α-amylase and the inhibitory effect or the ability to bind the inhibitor. On the other hand, the fact that the inhibitor binding does not correlate with the substrate binding precludes a mechanism by which α-amylase binds the putative carbohydrate moiety of the inhibitor that has a structure analogous to that of the substrate. It is noteworthy that the proteinaceous inhibitor of α-amylase is apparently not a substrate analog and yet its interaction with α-amylase involves catalytic residues. Acarbose and protease inhibitors may also interact with the catalytic groups of the target enzymes but are substrate analogs. The overall nature of the α-amylase–proteinaceous inhibitor interaction must, therefore, be different from the latter.

Proteinaceous α-amylase inhibitors are known to bind α-amylase tightly. The data in Figure 1a are consistent with such tight binding. On the other hand, the present results show that the binding could be disabled by substitution of a single amino acid residue, which suggests a weak binding. This necessitates that we assume a two-step mechanism for the binding interaction: the first is a weak binding followed by a second tight binding. As noted also in this study, most proteinaceous α-amylase inhibitors including those from wheat are known to exhibit time-dependent manner of actions (Buonocore et al., 1980; Marshall & Lauda, 1975): the inhibitory effect increases with preincubation time prior to the addition of substrate; the inhibitory effect or binding is suppressed by preincubation of α-amylase with substrate (acarbose was used in this study), while the addition of substrate after preincubation of the enzyme and inhibitor has only a small effect. These facts suggest that a conformational change leading to a tight binding occurs upon complex formation of the two proteins. Thus, a picture of the α-amylase–inhibitor interaction may emerge as follows. The inhibitor first binds to α-amylase by weak interactions involving the catalytic site. This may be the step to recognize the catalytically active enzyme. Then, a time-dependent conformational change (in either or both enzyme and inhibitor) occurs, leading to a tight binding. This is to exert effective inhibition. The substrate and the inhibitor are mutually exclusive in binding to the enzyme. The initial interaction of enzyme and inhibitor may be so weak that it can hardly overcome substrate binding. The final complex should be so tight that it cannot be easily dissociated by substrate. Thus, the two-step model can explain the different effects of substrate added before and after inhibitor. This scheme may be rationalized by the nature's efficiency, as inhibitor would target only the *active* enzyme and still act effectively. It is assumed that the initial step is obligatory for inhibitory action to occur and may play a role in determining inhibitor specificity.

Aside from the identification of critical sites involved in the binding of α-amylase to its inhibitor, a number of interesting observations were made from gel filtration experiments. For example, it has become clear that there are at least two molecular forms of complex between wild-type α-amylase and inhibitor, possibly differing in binding stoichiometry. What are the structures of these complexes and how are they related to the mechanism? Is the complex formed by HN180 similar to one of those forms? These questions are beyond the scope of the present study and will be pursued in future studies.

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