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Comparison of the Wild-Type α-Amylase and Its Variant Enzymes in *Bacillus amyloliquefaciens* in Activity and Thermal Stability, and Insights into Engineering the Thermal Stability of *Bacillus* α-Amylase

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The starch hydrolysis activity and thermal stability of *Bacillus amyloliquefaciens* α-amylase (wild-type enzyme or WT) and its variant enzymes, designated as M77, M111, and 21B, were compared. All have an optimal pH at around 6, as well as almost the same reaction rates and K_m and k_{cat} values. The optimal temperature in the absence of Ca^{2+} ions is 60°C for WT and M77 and 40°C for M111 and 21B. Those of M111 and 21B rose to 50–60°C upon the addition of 5 mM $CaCl_2$, while those of WT and M77 did not change. The dissociation constants K_d for Ca^{2+} to WT and M77 are much lower than those of M111 and 21B. Asp233 in WT is replaced by Asn in M111 and 21B, while it is retained in M77, suggesting that Asp233 is involved in the thermal stability of the enzyme through Ca^{2+} ion binding. These findings provide insight into engineering the thermal stability of *B. amyloliquefaciens* α-amylase, which would be useful for its applications in the baking industry and in glucose manufacturing.

Key words: α-amylase, *Bacillus amyloliquefaciens*, calcium ion, starch hydrolysis, thermal stability.

Abbreviation: BAA, *Bacillus amyloliquefaciens* α-amylase.

α-Amylase ([EC 3.2.1.1] 1,4-α-D-glucan glucanohydrolase, endoamylase) is an amylolytic enzyme that hydrolyzes starch, glycogen, and related polysaccharides by cleaving internal α-1,4-glycosidic linkages to form dextrin and oligosaccharides of relatively low molecular weight (*1*). α-Amylases are produced by various sources including plants, mammals, and microorganisms. In particular, α-amylases from *Bacillus* species, such as *licheniformis*, *amyloliquefaciens*, and *stearothermophillus* have been studied widely, and are known to have common features.

Bacillus α-amylases contain 3 or 4 calcium ions and 1 sodium ion, and a calcium-sodium-calcium metal triad bridge between domains A and B (*2*, *3*). This metal triad has been considered to be important for maintaining the protein structure in the correct conformation and for resisting thermal inactivation of enzyme (*4–6*). The active site of α-amylase is located in a cleft at the interface between domains A and B. Three amino acid residues (Asp262, Glu292, and Asp359) are known to be essential for the catalytic activity of *Bacillus amyloliquefaciens* α-amylase (hereinafter designated BAA). Asp262 is the catalytic nucleophile, while Glu292 is the catalytic hydrogen donor. A third residue (Asp359) is believed to assist in the catalysis by hydrogen bonding to the substrate and by increasing the pK_a value of Glu292 (*5*).

Amylases have a large number of commercial applications, for example, starch hydrolysis in the starch liquefaction process that converts starch into fructose and glucose syrups. Amylases are also used as a substitute for the expensive malt in the brewing industry, to improve flour in the baking industry, to produce modified starch in the paper industry, and to remove stains that contain starch in the detergent industry. Each of these processes takes place under physical and chemical conditions that are quite diverse (*7–9*). α-Amylases isolated from bacterial, cereal, and fungal sources have been used as anti-salting agents in bread-making, but their effectiveness for this purpose varies depending on their enzymatic properties. Thermostable and intermediately-thermostable bacterial α-amylases show different activities in their rates of bread firming and the extent of starch retrogradation in bread and starch gels (*10*). These differences in firming rate are believed to be related to differences in the thermal stabilities of the α-amylases used (*11*). *Aspergillus oryzae* α-amylase is widely used in baking processes, because it is completely inactivated at 75°C within 10 min. On the other hand, *Bacillus* α-amylases are more thermostable and require higher temperatures and longer times for inactivation than those needed for the *Aspergillus* enzyme. The content and characteristics of the dextrin in the crumb affect the texture of the bread. α-Amylase activity remaining during the baking process results in a sticky crumb, and thus *Bacillus* α-amylases are not suitable for baking because of their high thermal stabilities. *Aspergillus* α-amylase acts primarily on damaged starch in which the starch granules are ruptured, but the *Bacillus* enzyme acts favorably on non-damaged starch. This means that *Bacillus* α-amylase may be more effective in baking

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processes if *Bacillus* α-amylase variants could be generated that possess lowered thermal stabilities but with enough starch-hydrolysis activity retaining. From this point of view, efforts have been made to examine factors controlling the thermal stability of α-amylase and to generate variant enzymes with lowered thermal stability by means of various mutational trials. Recently, three variants (designated 21B, M77, and M111) of *Bacillus amyloliquefaciens* α-amylase (BAA) were developed [S. Tamagawa, M. Yoshida, and M. Minoda (1999) Application of new amylase containing novel gene for breadmaking. Int. Patent Cl[7], C12N15/09]. The variants are not transgenic but self-cloning enzymes with various degrees of thermal stability. Although their enzyme chemical characterization remains to be undertaken, their effects on the rheological properties of wheat dough and bread have been examined (*12*). In the present paper, we describe comparative studies on the starch hydrolysis activity and thermal stability of the wild-type BAA (hereinafter designated WT) and its variant enzymes. Particularly, we discuss the remarkable effect of calcium ions on their thermal stabilities.

EXPERIMENTAL PROCEDURES

Materials—Soluble starch as substrate and maltose as the standard for the activity assay were purchased from Nacalai Tesque (Kyoto, Japan). Neocuproine-HCl (2,9-dimethyl-1,10-phenanthroline, Lot 21K1148) as coloring reagent B in the neocuproine method was obtained from Sigma(St. Louis, MO). Coloring reagent A (0.38 M Na_2CO_3, 1.8 mM $CuSo_4$, and 0.2 M glycine) in the neocuproine method was purchased from Nacalai Tesque. All other chemicals were of reagent grade and were purchased from Nacalai Tesque.

Preparation of Enzymes—The wild-type BAA (WT) and its variants (21B, M77, and M111) were obtained from Daiwa Kasei (Osaka, Japan); the preparations of the variants have been described previously (*12*). WT was supplied by the manufacturer in the powder form (commercial name: Kleistase M1) adsorbed onto starch used as an excipient. The powder (40 mg) was dissolved in 20 ml of 20 mM Tris-HCl buffer, pH 9.0 (buffer A). The dissolved BAA solution was centrifuged at 10,000 × *g* for 15 min at 4°C, and the recovered supernatant was applied to a TSKgel QAE-Toyopearl 550C column [9.0 mm (inner diameter) × 5.0 cm] (Tosoh, Tokyo) equilibrated with buffer A. After washing the column with buffer A to eliminate the starch, BAA was eluted from the column by 20 mM Tris-HCl buffer, pH 9.0, containing 0.5 M NaCl (buffer B). The recovered BAA fractions were dialyzed thoroughly against 20 mM sodium phosphate buffer, pH 7.0, (buffer C) at 4°C. The BAA variants (21B, M77, and M111) were purified in a similar way. Variant enzymes in buffer A (2 mg/ml, 20 ml) were centrifuged at 10,000 × *g* for 15 min at 4°C. The supernatant was dialyzed against buffer C at 4°C, concentrated with Centriprep-YM 30 (Millipore, Bedford, MA, USA), and applied to gel-filtration HPLC on a TSKgel $G2000SW_{XL}$ [7.8 mm (inner diameter) × 30 cm] column (Tosoh) equilibrated with buffer C. The protein concentration during purification was evaluated by the Lowry method or by absorbance at 280 nm using bovine serum albumin (BSA) as the standard (*13*). α-Amylases were purified homogeneously on SDS-PAGE (*14*). They were identified using their molar absorption coefficient (ϵ_{280}) of 1.30×10^5 (M^{-1} cm^{-1}) at 280 nm, which was estimated from the numbers of tyrosyl and tryptophyl residues (29 and 19 residues, respectively, per mole) contained in the enzymes (see Fig. 1; *15*, *16*). All spectrophotometric measurements were done with a Shimadzu UV-240 spectrophotometer (Kyoto). The molecular mass of 46.14 kDa was used for WT, 21B and M77, and 46.17 kDa was used for M111 based on the amino acid compositions (Fig.1, see "RESULTS AND DISCUSSION").

DNA Sequencing and Analysis—DNA sequence analysis was performed by the dideoxy chain termination method (*17*) with the AutoRead sequencing kit (Pharmacia-LKB Biotechnology, Uppsala, Sweden). After sub-cloning fragments of appropriate sizes in M13mp18,19 or pUC18,19, both strands were sequenced with fluorescent dye-linked universal primer or, in some cases, with the aid of internally priming oligonucleotides using an automated laser fluorescent DNA sequencer (A.L.F. Sequencer; Pharmacia-LKB Biotechnology). Insert-containing plasmid vectors used for double stranded-sequencing were purified on a CsCl/etidium bromide gradient.

Activity Assay—α-Amylase activity was evaluated by the rate of increase in the reducing ends produced by the hydrolysis of soluble starch by the enzyme. The reducing end was measured by the neocuproine method and calibrated with maltose as the standard (*18*, *19*). The reaction was initiated by the addition of enzyme solution (10–20 μl) into 5 ml of substrate solution that had been incubated at 30°C. The assays under the standard conditions in 20 mM sodium malate buffer, pH 6.0, (standard buffer) at 30°C were done without the addition of $CaCl_2$.

Temperature-Dependence of the Activity and Irreversible Thermal Inactivation of α-Amylases—Temperature-dependence of α-amylase activity was examined over a temperature range of 30–90°C in the standard buffer. Ten microliters of enzyme solution (500 nM) was added to 5 ml of the pre-heated substrate solution, and the activity was measured by the neocuproine method. The effect of Ca^{2+} ion on the enzyme activity was examined in the presence of 5 mM $CaCl_2$. The thermal inactivation of α-amylase was examined over a temperature range of 30–90°C in the standard buffer, and the effect of Ca^{2+} ion on the thermal inactivation was observed in three experimental systems (systems 1–3) in the absence and presence of 5 mM $CaCl_2$. In system 1, the thermal treatment of the enzyme and the assay were done without $CaCl_2$; in system 2, the thermal treatment of the enzyme was done in the absence of $CaCl_2$ and the assay was done with substrate solution containing 5 mM $CaCl_2$; and in system 3, the thermal treatment of enzyme and the assay were done in the presence of 5 mM $CaCl_2$. One hundred microliters of enzyme solution (2 μM) was added to 1.9 ml of pre-heated standard buffer at each temperature, and 100 μl of the solution was withdrawn at incubation times of 5, 10, 20, and 30 min. The solution was placed on ice for 5 min, and the residual starch hydrolysis activity was determined by measuring the reaction product in the standard buffer at 30°C for 3 min. T_{50} is defined as the temperature at which 50% of the enzyme activity is lost following heat-treatment for 30 min. The apparent first-order rate constant (k) of thermal inactivation was determined from the plot of the logarithm of the residual α-amylase activity against the time of heat-treatment. The activation energy (E_a) for

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   1 ATG ATT CAA AAA CGA AAG CGG ACA GTT TCG TTC AGA CTT GTG CTT ATG TGC ACG CTG TTA  60
   1  M   I   Q   K   R   K   R   T   V   S   F   R   L   V   L   M   C   T   L   L  20

  61 TTT GTC AGT TTG CCG ATT ACA AAA ACA TCA GCC GTA AAT GGC ACG CTG ATG CAG TAT TTT 120
  21  F   V   S   L   P   I   T   K   T   S   A   V   N   G   T   L   M   Q   Y   F  40

 121 GAA TGG TAT ACG CCG AAC GAC GGC CAG CAT TGG AAA CGA TTG CAG AAT GAT GCG GAA CAT 180
  41  E   W   Y   T   P   N   D   G   Q   H   W   K   R   L   Q   N   D   A   E   H  60

 181 TTA TCG GAT ATC GGA ATC ACT GCC GTC TGG ATT CCT CCC GCA TAC AAA GGA TTG AGC CAA 240
  61  L   S   D   I   G   I   T   A   V   W   I   P   P   A   Y   K   G   L   S   Q  80

 241 TCC GAT AAC GGA TAC GGA CCT TAT GAT TTG TAT GAT TTA GGA GAA TTC CAG CAA AAA GGG 300
  81  S   D   N   G   Y   G   P   Y   D   L   Y   D   L   G   E   F   Q   Q   K   G 100

 301 ACG GTC AGA ACG AAA TAC GGC ACA AAA TCA GAG CTT CAA GAT GCG ATC GGC TCA CTG CAT 360
 101  T   V   R   T   K   Y   G   T   K   S   E   L   Q   D   A   I   G   S   L   H 120

 361 TCC CGG AAC GTC CAA GTA TAC GGA GAT GTG GTT TTG AAT CAT AAG GCT GGT GCT GAT GCA 420
 121  S   R   N   V   Q   V   Y   G   D   V   V   L   N   H   K   A   G   A   D   A 140

 421 ACA GAA GAT GTA ACT GCC GTC GAA GTC AAT CCG GCC AAT AGA AAT CAG GAA ACT TCG GAG 480
 141  T   E   D   V   T   A   V   E   V   N   P   A   N   R   N   Q   E   T   S   E 160

 481 GAA TAT CAA ATC AAA GCG TGG ACG GAT TTT CGT TTT CCG GGC CGT GGA AAC ACG TAC AGT 540
 161  E   Y   Q   I   K   A   W   T   D   F   R   F   P   G   R   G   N   T   Y   S 180

 541 GAT TTT AAA TGG CAT TGG TAT CAT TTC GAC GGA GCG GAC TGG GAT GAA TCC CGG AAG ATC 600
 181  D   F   K   W   H   W   Y   H   F   D   G   A   D   W   D   E   S   R   K   I 200

 601 AGC CGC ATC TTT AAG TTT CGT GGG GAA GGA AAA GCG TGG GAT TGG GAA GTA TCA AGT GAA 660
 201  S   R   I   F   K   F   R   G   E   G   K   A   W   D   W   E   V   S   S   E 220

 661 AAC GGC AAC TAT GAC TAT TTA ATG TAT GCT GAT GTT GAC TAC GAC CAC CCT GAT GTA GTG 720
 221  N   G   N   Y   D   Y   L   M   Y   A   D   V   D   Y   D   H   P   D   V   V 240

 721 GCA GAG ACA AAA AAA TGG GGT ATC TGG TAT GCG AAT GAA CTG TCA TTA GAC GGC TTC CGT 780
 241  A   E   T   K   K   W   G   I   W   Y   A   N   E   L   S   L   D   G   F   R 260

 781 AAT GAT GCC GCC AAA CAT ATT AAA TTT TCA TTT CTG CGT GAT TGG GTT CAG GCG GTC AGA 840
 261  I   D   A   A   K   H   I   K   F   S   F   L   R   D   W   V   Q   A   V   R 280

 841 CAG GCG ACG GGA AAA GAA ATG TTT ACG GTT GCG GAG TAT TGG CAG AAT AAT GCC GGG AAA 900
 281  Q   A   T   G   K   E   M   F   T   V   A   E   Y   W   Q   N   N   A   G   K 300

 901 CTG GAA AAC TAC TTG AAT AAA ACA AGC TTT AAT CAA TCC GTG TTT GAT GTT CCG CTT CAT 960
 301  L   E   N   Y   L   N   K   T   S   F   N   Q   S   V   F   D   V   P   L   H 320

 961 TTC AAT TTA CAG GCG GCT TCC TCA CAA GGA GGC GGA TAT GAT ATG AGG CGT TTG CTG GAC 1020
 321  F   N   L   Q   A   A   S   S   Q   G   G   G   Y   D   M   R   R   L   L   D 340

1021 GGT ACC GTT GTG TCC AGG CAT CCG GAA AAG GCG GTT ACA TTT GTT GAA AAT CAT GAC ACA 1080
 341  G   T   V   V   S   R   H   P   E   K   A   V   T   F   V   E   N   H   D   T 360

1081 CAG CCG GGA CAG TCA TTG GAA TCG ACA GTC CAA ACT TGG TTT AAA CCG CTT GCA TAC GCC 1140
 361  Q   P   G   Q   S   L   E   S   T   V   Q   T   W   F   K   P   L   A   Y   A 380

1141 TTT ATT TTG ACA AGA GAA TCC GGT TAT CCT CAG GTG TTC TAT GGG GAT ATG TAC GGG ACA 1200
 381  F   I   L   T   R   E   S   G   Y   P   Q   V   F   Y   G   D   M   Y   G   T 400

1201 AAA GGG ACA TCG CCA AAG GAA ATT CCC TCA CTG AAA GAT AAT ATA GAG CCG ATT TTA AAA 1260
 401  K   G   T   S   P   K   E   I   P   S   L   K   D   N   I   E   P   I   L   K 420

1261 GCG CGT AAG GAG TAC GCA TAC GGG CCC CAG CAC GAT TAT ATT GAC CAC CCG GAT GTG ATC 1320
 421  A   R   K   E   Y   A   Y   G   P   Q   H   D   Y   I   D   H   P   D   V   I 440

1321 GGA TGG ACG AGG GAA GGT GAC AGC TCC GCC GCC AAA TCA GGT TTG GCC GCT TTA ATC ACG 1380
 441  G   W   T   R   E   G   D   S   S   A   A   K   S   G   L   A   A   L   I   T 460

1381 GAC GGA CCC GGC GGA TCA AAG CGG ATG TAT GCC GGC CTG AAA AAT GCC GGC GAG ACA TGG 1440
 461  D   G   P   G   G   S   K   R   M   Y   A   G   L   K   N   A   G   E   T   W 480

1441 TAT GAC ATA ACG GGC AAC CGT TCA GAT ACT GTA AAA ATC GGA TCT GAC GGC TGG GGA GAG 1500
 481  Y   D   I   T   G   N   R   S   D   T   V   K   I   G   S   D   G   W   G   E 500

1501 TTT CAT GTA AAC GAT GGG TCC GTC TCC ATT TAT GTT CAG AAA TAA 1545
 501  F   H   V   N   D   G   S   V   S   I   Y   V   Q   K   *  514
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Fig. 1. **The nucleotide sequence of the wild-type α-amylase of *Bacillus amyloliquefacience* and the amino acid sequence deduced from the nucleotide sequence, and a comparison of the sequences of the variant enzymes (M77, M111, and 21B).** The upper line with the numbering of 1–1545 is for the nucleotide sequence of the wild-type enzyme (WT), and the lower line with the numbering of 1–514 is for its amino acid sequence. 89C in the nucleotide sequence of WT is changed to T, and 583G to A in M77; 575C to T and 697G to A in M111; and 697G to A in 21B. Accordingly, Ser30 in the amino acid sequence of WT is converted to Leu, and Asp195 to Asn in M77; Ala192 to Val, and Asp233 to Asn in M111; and Asp233 to Asn in 21B. The amino acid sequence 1–31 is the signal sequence.

the thermal inactivation of the wild-type BAA and variant enzymes was determined from the Arrhenius equation (Eq. 1).

$$\ln k = A - E_a/RT \quad (1)$$

The Gibbs energy change of activation $\Delta G^{\ddagger}$, the enthalpy change of activation $\Delta H^{\ddagger}$, and the entropy of activation $\Delta S^{\ddagger}$ were determined according to the Eyring equations (Eq. 2) (*17*, *20*):

$$\Delta G^{\ddagger} = -RT[\ln(hk/k_{\mathrm{B}}T)] \quad (2)$$

$$\ln(hk/k_{\mathrm{B}}T) = (\Delta H^{\ddagger}/\mathrm{RT}) + (\Delta S^{\ddagger}/R) \quad (3)$$

where k_{B}, h, and R are the Boltzmann, Plank, and gas constants, respectively. T is the temperature in Kelvin.

Effect of pH on α-Amylase Activity—The pH dependence of α-amylase activity for WT and its variant enzymes was examined at enzyme and soluble-starch concentrations of 1.0 nM and 3.0 mg/ml respectively and at 30°C in the following buffers: 20 mM glycine-HCl buffer (pH 3.0); 20 mM sodium acetate buffer (pH 4.0 and 5.0); 20 mM sodium phosphate buffer (pH 6.0, 7.0, and 8.0); and 20 mM Tris-HCl buffer (pH 9.0).

Determination of Ca^{2+}-Binding Parameters—One hundred microliters of enzyme solution (2 μM) was added to 1.9 ml of standard buffer (pH 6.0) including 2–200 μM $CaCl_2$ that had been incubated at 50°C. Aliquots of the heated solution were withdrawn at 2, 4, 6, 8, and 10 min, and added to 100 μl of the same buffer cooled on ice. The apparent first-order rate constant (k_{app}) for thermal inactivation was estimated from semi-logarithmic plots of the residual activity against the heat-incubation time. The thermal inactivation in the presence of Ca^{2+} was analyzed by assuming that the inactivation may follow the following mechanism (Scheme 1) according to Tomazic and Klibanov (*21*);

$$\mathrm{U} \xleftarrow{k_{\mathrm{den}}'} \mathrm{N} \overset{K_{\mathrm{d}}}{\rightleftharpoons} \mathrm{N}^* \xrightarrow{k_{\mathrm{den}}} \mathrm{U}^* \qquad \textbf{Scheme 1}$$

where N and U show the native and inactivated enzyme forms containing Ca^{2+} ions, respectively; N* and U* is are the native and inactivated enzyme forms not containing Ca^{2+} ions, respectively. The rate constants for conversions from N to U and N* to U* are given by k_{den}' and k_{den}, respectively. The dissociation constant of Ca^{2+}-binding to N is K_{d} (Eq. 4):

$$K_{\mathrm{d}} = [\mathrm{N}^*][\mathrm{Ca}^{2+}]/[\mathrm{N}] \quad (4)$$

From this equation, it is obvious that the conversion from N to U has no effect on the dissociation constant K_{d}. After the first-order rate constants were estimated, the dissociation constants (K_{d}) for Ca^{2+}-binding to the enzyme and rate constant (k_{den}) for the thermal denaturation were calculated from Eq. 5:

$$1/k_{\mathrm{app}} = (1/k_{\mathrm{den}}) + [\mathrm{Ca}^{2+}]/(K_{\mathrm{d}}k_{\mathrm{den}}) \quad (5)$$

RESULTS

Nucleotide and Amino Acid Sequences of the Wild-Type BAA (WT) and Its Variant Enzymes (M77, M111, and 21B)—The nucleotide sequences of WT and its variant enzymes and their deduced amino acid sequences are shown in Fig. 1. The total nucleotide sequences for all enzymes comprised 1545 bases, and 89C and 583G of WT were replaced with T and A, respectively, in M77; 575C and 697G were replaced with T and A, respectively, in M111; and 697G was replaced with A in 21B. The numbering of the amino acid residues is applied to the pro-sequence of 514 residues, which contains the signal sequence at positions 1–31. Accordingly, the mature enzyme comprises the sequence between positions 32 and 514. The amino acid substitutions in the variants are as follows: for M77, Ser30 of WT is replaced with Leu, and Asp195 with Asn; for M111, Ala192 is replaced with Val, and Asp233 with Asn; and for 21B, Asp233 is replaced with Asn. It is interesting to note that the substitution of Asn for Asp is observed in all variants, and the replacement is at Asp233 in M111 and 21B. This suggests that a negative charge carried on the aspartate is eliminated in the variants as compared with WT. The replacement of Ala192 with Val in M111 suggests that the hydrophobic interaction at the region around position 192 is increased in M111 in comparison with 21B. The replacement of Ser30 with Leu in M77 occurs in the signal sequence, and thus this substitution should have no effect on the enzyme characteristics. Therefore, M77 and 21B are single-mutation variants, and M111 is a double-mutation variant.

Comparison of the Kinetic Parameters of Starch Hydrolysis of WT and Its Variant Enzymes in the Absence and Presence of $CaCl_2$—The starch-hydrolyzing activity of α-amylases was examined in 20 mM sodium malate buffer at pH 6.0 (standard buffer) at 30°C. The Michaelis constant K_{m} and molecular activity k_{cat} values for WT, M77, M111, and 21B were as follows: for WT, 0.34 ± 0.04 mg/ml and 586 ± 0.04 16 s^{-1}; for M77, 0.24 ± 0.04 0.03 mg/ml and 503 ± 14 s^{-1}; for M111, 0.80 ± 0.11 mg/ml and 656 ± 30 s^{-1}; and for 21B, 0.63 ± 0.06 mg/ml and 722 ± 18 s^{-1}. The kinetic parameters determined under the same conditions in the presence of 5 mM $CaCl_2$ were substantially the same as those in the absence of $CaCl_2$. Seemingly Ca^{2+} ions have no effect on the structure and function of BAA at pH 6.0 and 30°C. The respective k_{cat} values were 61.5 ± 1.7% for WT, 63.0 ± 1.8% for M77, 74.5 ± 1.9% for M111, and 81.0 ± 3.7% for 21B, so that the respective maximum k_{cat} values given at 60°C for WT and M77 and at 40°C for M111 and 21B were set to 100% of the relative-activity scale (see Fig. 2).

Temperature Dependence of α-Amylase Activity—The temperature-dependence of the starch-hydrolyzing activity of α-amylases was examined over the temperature range of 30–90°C. The relative activity of each enzyme in the absence and presence of calcium ions is shown in Fig. 2. In the absence of $CaCl_2$, the optimal temperatures (T_{opt}) were estimated to be around 60°C for WT and M77 and 40°C for M111 and 21B (Fig. 2A). The temperature ($T_{\mathrm{opt/2}}$) at which 50% of the maximal activity observed at the optimal temperature is observed was introduced as a parameter indicating stability at higher temperatures than T_{opt}. The $T_{\mathrm{opt/2}}$ values were estimated to be 71–73°C for WT and M77, and 55°C for M111 and 21B. The four enzymes examined were grouped into two groups with respect to the thermal stability, and WT and M77 are

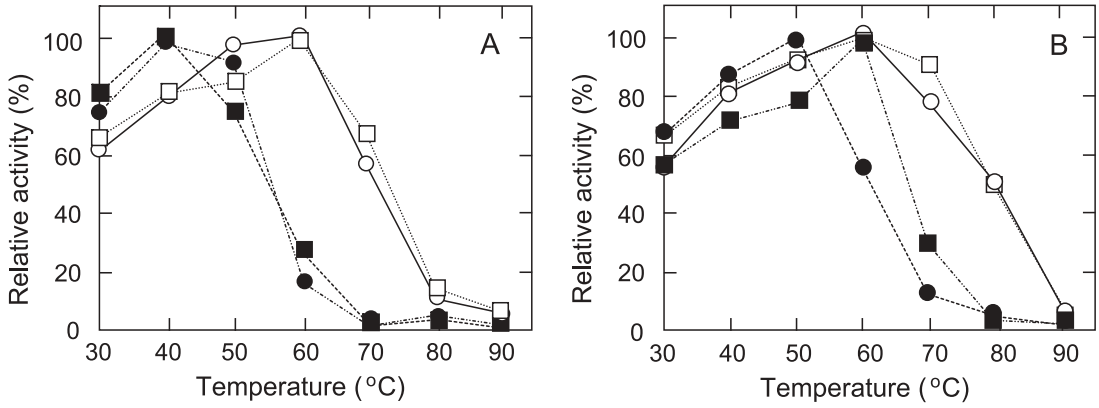

Fig. 2. **Effect of temperature on the activity of the wild-type α-amylase and its variant enzymes.** The activity was assayed in the standard buffer at pH 6.0 in the absence (A) or presence (B) of 5 mM $CaCl_2$. WT, open circles; M77, open squares; M111, solid squares; and 21B, solid circles. The assay conditions are described in "EXPERIMENTAL PROCEDURES," and the reaction rates for the respective enzymes are shown in the text.

evidently more stable than M111 and 21B. In the presence of 5 mM $CaCl_2$, the optimal temperatures (T_{opt}) of WT and M77 were estimated to be 60°C and those of M111 and 21B were 60°C and 50°C, respectively (Fig. 2B). The $T_{opt/2}$ values were 79°C for WT and M77, 62°C for 21B, and 67°C for M111. It was shown that WT and M77 are more stable than M111 and 21B even in the presence of 5 mM $CaCl_2$. The optimal temperatures of WT and M77 did not change much following the addition of $CaCl_2$, while those of M111 and 21B were significantly increased. The effect of temperature on the enzyme activity can be visualized more clearly by comparing $T_{opt/2}$ values than T_{opt} values. The $T_{opt/2}$ values for WT and M77 were increased by 6–8°C by the addition of 5 mM $CaCl_2$, and those for 21B and M111 increased by 7°C and 12°C, respectively. The thermal stability of all enzymes as evaluated by $T_{opt/2}$ increased to some extent by the addition of 5 mM $CaCl_2$, and the stabilities of WT and M77 were significantly higher than those of 21B and M111 in the absence and presence of 5 mM $CaCl_2$. It should be noted that the stability of M111 in the presence of 5 mM $CaCl_2$ is intermediate between WT (M77) and 21B. The activities of M111 and 21B at 40°C and of WT and M77 at 60°C in the absence of $CaCl_2$ were 610 ± 60, 760 ± 40, 810 ± 40, and 900 ± 45 nM s^{-1}, respectively. Those of 21B at 50°C and of M111, WT, and M77 at 60°C in the presence of 5 mM $CaCl_2$ were 770 ± 40, 560 ± 60, 1,120 ± 60, and 950 ± 50 nM s^{-1}, respectively. These values were designated as 100% for determining relative activities.

Irreversible Thermal Inactivation of α-Amylases— Irreversible thermal inactivation of the starch-hydrolyzing activity of α-amylase was observed over the temperature range of 30–90°C. The T_{50} values were calculated from the residual activities obtained after 30 min of heat treatment (Table 1). When the BAA enzymes were heat-treated in the absence of Ca^{2+} ions, the T_{50} values for all enzymes were in the range of 38–45°C, and the values were not much affected by the presence of 5 mM $CaCl_2$ in the assay system. The value for WT is apparently slightly higher than those for the variants. The T_{50} values observed when the enzymes were heat-treated in the presence of 5 mM $CaCl_2$ were considerably higher than those in the absence of $CaCl_2$, and the values for WT, M77, M111, and 21B increased by 30–31, 22–23, 14–20, and 11–16°C, respectively, in the presence of 5 mM $CaCl_2$. The addition of $CaCl_2$ is most effective for increasing the thermal stability of WT, and less so for the variants.

Table 1. **Thermal stability of the wild-type BAA and its variant enzymes.**

Enzyme	T_{50} (°C)		
	System 1 $CaCl_2$ (–)	System 2 $CaCl_2$ (–)	System 3 $CaCl_2$ (+)
WT	44 ± 4	45 ± 4	75 ± 8
M77	39 ± 4	40 ± 4	62 ± 6
M111	39 ± 4	45 ± 4	59 ± 6
21B	38 ± 4	43 ± 4	54 ± 5

T_{50} is the temperature at which 50% of the enzyme activity is lost when the enzyme is incubated for 30 min in the standard buffer (pH 6.0) in the absence or presence of 5 mM $CaCl_2$. The enzyme activity was measured by starch hydrolysis in the standard buffer at 30°C. $CaCl_2$ (–) and $CaCl_2$ (+): the absence and presence of 5 mM $CaCl_2$ respectively, when the enzyme was heat treated. $CaCl_2$ was not present in the assay conditions in system 1; and present in systems 2 and 3.

Thermodynamic activation parameters for the thermal inactivation of BAA enzymes were calculated from the thermal inactivation curves. Apparent first-order rate constants (k_{app}) for the thermal inactivation of each enzyme were determined in the presence and absence of Ca^{2+} ion. Time-dependent progress curves for the thermal inactivation of WT at pH 6.0, Arrhenius plots and Eyring plots of the apparent first-order rate constant of inactivation are shown in Fig. 3. The activation energy (E_a) and thermodynamic parameters ($\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$) of thermal inactivation of the BAA enzymes were calculated from the slope of Arrhenius plots and Eyring plots, respectively (Table 2).

It was shown that the thermal stability of the enzymes increases remarkably by the addition of 5 mM $CaCl_2$. The positive values of $\Delta H^{\ddagger}$ suggest that the activation process is endothermic. To determine the Ca^{2+}-binding parameters of α-amylase, irreversible thermal inactivation of α-amylase was examined in the presence of various Ca^{2+} concentrations at 50°C and at pH 6.0. Progress curves of the thermal inactivation of WT at a given temperature are shown in Fig. 4A. Semi-logarithmic plots of the enzyme activity

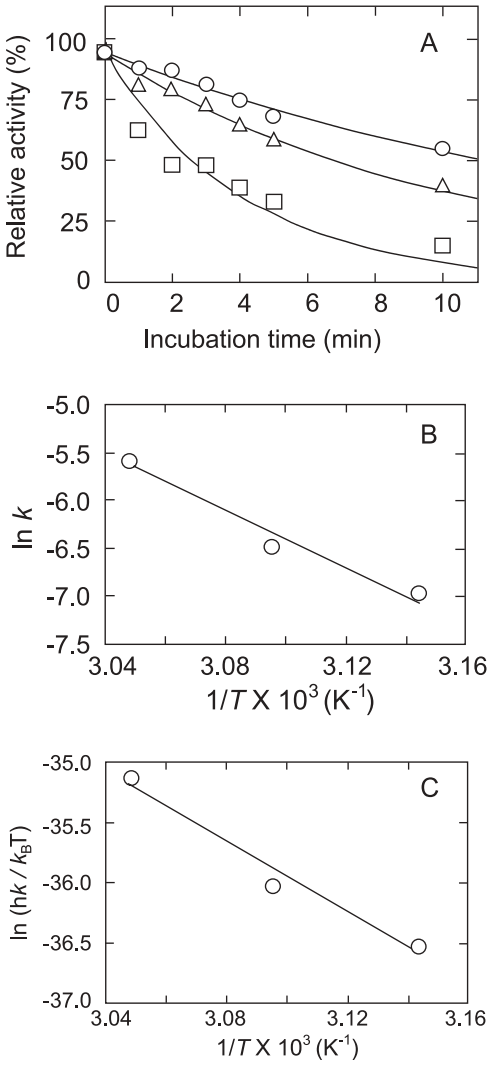

Fig. 3. **Thermal inactivation of the wild-type BAA (WT).** Panel A: Progress curves of the thermal inactivation of WT. WT was incubated at 45°C (circles), 50°C (triangles), or 55°C (squares) at pH 6.0 for the times indicated. The apparent first-order rate constants (k) for the thermal inactivation at 45, 50, and 55°C were determined to be 0.9×10^{-3}, 1.5×10^{-3}, and 3.8×10^{-3} s^{-1}, respectively, by semi-logarithmic plot of the residual activity against the time of heat-treatment. The solid lines are the theoretical curves drawn using the respective k values. Panel B: Arrhenius plots of k. The activation energy E_a of the thermal inactivation was determined to be 124 ± 22 kJ mol^{-1}. Panel C: Eyring plots of k. The enthalpy and entropy changes of activation of the thermal inactivation were determined to be 121 ± 22 kJ mol^{-1} and 76.3 ± 67.5 J mol^{-1} K^{-1}, respectively.

against time are shown in Fig. 4B. From the slope of the plots, a first-order rate constant (k_{app}) of thermal inactivation was calculated according to Eq. 5. Plots of k_{app}^{-1} against calcium concentration are shown in Fig. 4C. The plots show a liner relationship, suggesting that the model proposed in Scheme 1 is suitable for interpreting the inactivation; the dissociation constants (K_d) for Ca^{2+} binding and rate constants (k_{den}) for thermal inactivation were determined (Table 3). The k_{den} and K_d values for WT and M77 are almost identical, whereas they are significantly higher for M111, and much more higher for 21B. The k_{den} value is positively correlated with the K_d value. This indicates that the equilibrium between the native state (N) and the Ca^{2+}-depleted state (N*) shifts more to the N* side as the rate constants of thermal inactivation, k_{den}, increase. With respect to the k_{den} and K_d values, the four enzymes could be grouped into two groups as well as shown by thermal stability (Fig. 2), but it is interesting to note that M111 shows an intermediate behavior between WT (M77) and 21B. This corresponds well to the behavior of M111 in the thermal stability in the presence of 5 mM $CaCl_2$ (Fig. 2B).

Effect of pH on α-Amylase Activity of the Wild-Type BAA (WT) and Variant Enzymes—The reaction rates (v) for the respective α-amylases showed bell-shaped pH-dependent profiles, with optimal pH around pH 5.8–6.0 for all enzymes (Fig. 5). The reaction rate (v_{opt}) at the optimal pH and the acidic and alkaline pK_a values (pK_{a1} and pK_{a2}), which correspond to the pH values giving v of 0.5 v_{opt}, were estimated from the profiles (Table 4). A slight variation in v_{opt} was observed, with the values in the order: WT > 21B > M77 > M111. The v_{opt} value of the double-mutation variant M111 is 85% of the v_{opt} for WT. The proton dissociation constant (pK_{a1}) on the acidic side changed drastically in comparison with that (pK_{a2}) on the alkaline side; namely, pK_{a1} was observed for WT and M77 at around 3.6, whereas it was increased 0.6 pH units for M111 and 21B. On the other hand, the change in pK_{a2} is much smaller, in the range of 7.9–8.1. These findings suggest that the replacement of Asp195 with Asn hardly affects α-amylase activity, while the replacement of Asp233 with Asn significantly suppresses the ionization of the catalytic residue with pK_{a1}. Under the reaction conditions [1.0 nM enzyme and 3.0 mg/ml substrate soluble-starch] used, the reaction rate (v) is considered to be approximately the maximal reaction rate (V_{max}) attained at the substrate concentration much higher than the Michaelis constant K_m. Accordingly, the pH-dependence of the reaction rate (v) could be analyzed by an active-site model in the enzyme bound to substrate, which has two catalytically-functional

Table 2. **Thermodynamic parameters for thermal inactivation of the wild-type BAA (WT) and its variant enzymes (M77, M111, and 21B) at 50°C and pH 6.0.**

Enzyme	E_a (kJ mol^{-1})		$\Delta G^{\ddagger}$ (kJ mol^{-1})		$\Delta H^{\ddagger}$ (kJ mol^{-1})		$T\Delta S^{\ddagger}$ (kJ mol^{-1})	
	Ca (–)	Ca (+)	Ca (–)	Ca (+)	Ca (–)	Ca (+)	Ca (–)	Ca (+)
WT	124 ± 22	249 ± 27	96 ± 0	115 ± 2	121 ± 22	246 ± 27	25 ± 22	131 ± 24
M77	181 ± 5	203 ± 22	94 ± 0	104 ± 1	178 ± 5	200 ± 22	84 ± 5	97 ± 21
M111	141 ± 21	157 ± 9	94 ± 0	103 ± 0	139 ± 22	154 ± 9	42 ± 20	51 ± 9
21B	127 ± 4	146 ± 5	91 ± 3	98 ± 0	124 ± 5	143 ± 5	34 ± 2	45 ± 5

The enzyme activity was measured by starch hydrolysis in the standard buffer at 30°C. Ca (–) and Ca (+): the absence and presence of 5 mM $CaCl_2$ respectively, when the enzyme was heat treated.

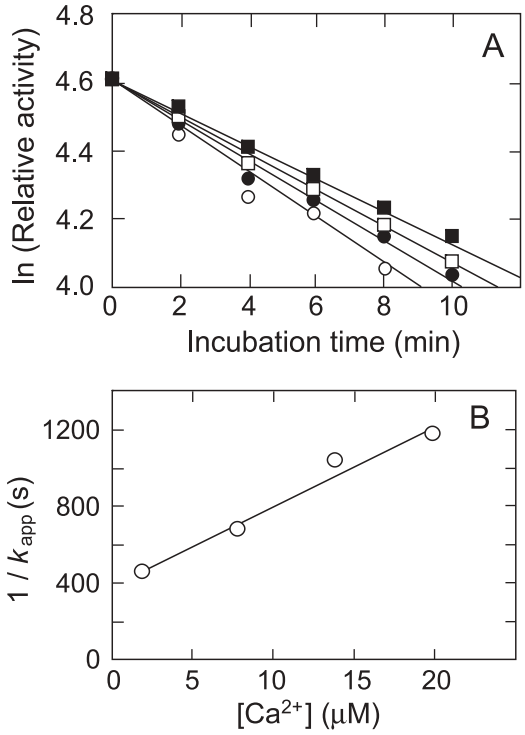

Fig. 4. **Effect of $CaCl_2$ on the thermal inactivation of the wild-type BAA (WT).** Panel A: Semi-logarithmic plots of the progress curves of the thermal inactivation of BAA. BAA was incubated at 50°C at pH 6.0 for the times indicated. The $CaCl_2$ concentrations were 2 μM (open circles), 8 μM (solid circles), 14 μM (open squares), and 20 μM (solid squares). The apparent first-order rate constants (k_{app}) for the thermal inactivation in the presence of $CaCl_2$ at 2, 8, 14, and 20 μM were determined to be 2.3×10^{-3}, 1.5×10^{-3}, 1.0×10^{-3}, and 0.9×10^{-3} s^{-1}, respectively. Panel B: Plot of k_{app}^{-1} *versus* $CaCl_2$ concentration. The dissociation constant (K_d) for calcium ion from BAA and the rate constant for thermal inactivation (k_{den}) were determined to be 9.2 ± 1.3 μM and 0.026 ± 0.009 s^{-1}, respectively.

Table 3. **Comparison of the rate constants (k_{den}) for the thermal inactivation of the wild-type BAA (WT) and its variant enzymes (M77, M111, and 21B) and dissociation constants (K_d) of Ca^{2+} ions with the BAA enzymes at pH 6.0.**

Enzyme	k_{den} (s^{-1})	K_d (μM)
WT	0.026 ± 0.009	9.2 ± 1.3
M77	0.027 ± 0.002	7.4 ± 0.3
M111	0.052 ± 0.003	31 ± 3
21B	0.270 ± 0.008	57 ± 12

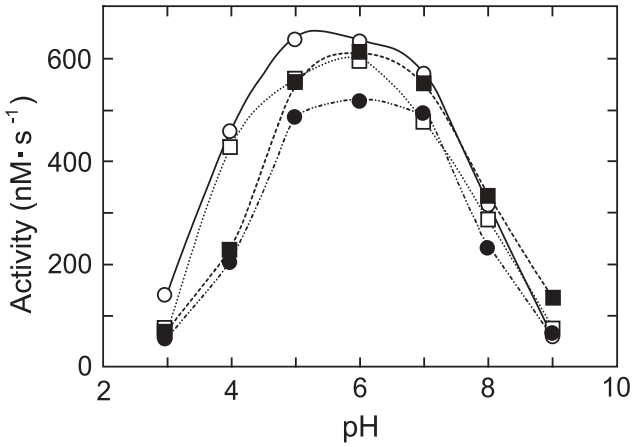

Fig. 5. **pH-dependence of α-amylase activity of wild-type BAA (WT) and its variant enzymes (M77, M111, and 21B).** WT, open circles; M77, open squares; M111, solid squares; and 21B, solid circles. The reaction conditions are described in "experimental procedures." The theoretical lines were drawn by postulating a reaction mechanism in which the enzyme activity is controlled by two functional ionizing groups (*22*).

ionizing groups with proton dissociation constants K_{es1} and K_{es2} (*17*, *22*). pK_{a1} and pK_{a2} are considered to be pK_{es1} and pK_{es2}, respectively, and v_{opt} can be regarded as an intrinsic V_{max} [$(V_{max})_o$], which is the maximum reaction rate independent of pH.

DISCUSSION

Role of Asp233 in the Thermal Stability of BAA—The optimal temperature (40°C) for the activity of M111 and 21B was significantly lower than that (60°C) for WT and M77 in the absence of calcium ions (Fig. 2). That of M111 and 21B was increased to 50–60°C by the addition of 5 mM $CaCl_2$, while that of WT and M77 was not much affected. The starch-hydrolysis activity of M111 and 21B decreased following incubation at 40°C in the absence of $CaCl_2$, although the decrease was prevented in the presence of 5 mM $CaCl_2$. Asp at position 233 in WT and M77 is replaced by Asn in M111 and 21B. Therefore, the substitution from Asp233 to Asn decreases the thermal stability of BAA enzymes, but the decreased stability of the substituted BAA containing Asn at this position (Asn233-BAA) is recovered to the level of BAA containing Asp at position 233 (Asp233-BAA) in the presence of 5 mM $CaCl_2$. The thermal stability of BAA should be maintained in part by Asp233, and the instability derived by the substitution is cancelled by the addition of $CaCl_2$. It is noted that the increase in thermal stability in the presence of 5 mM $CaCl_2$ is observed only with Asn233-BAA, and the stability of Asp233-BAA is not enhanced further.

Effect of Ca^{2+} Ions on the Thermal Stability of BAA—The 3D-structure of BAA is not yet available, and thus the states of the Asp233 and Ca^{2+}-binding sites in BAA are not identified. However, the structure of a chimeric protein constructed from genes encoding BAA and *Bacillus licheniformis* α-amylase (BLA) provides information about Asp233 and Ca^{2+}-binding sites in BAA (*2*). From the sequence alignment of BAA and BLA, it is considered that the 3D-structure and Ca^{2+}-binding sites of BAA might closely resemble those of BLA (*3*). Asp233 in BLA is located in the C-terminal region of the β-strand in domain B, and one Ca^{2+} ion is identified in the neighborhood of Asp 233. This Ca^{2+} ion consists of the Ca^{2+}-Na^{+}-Ca^{2+} metal triad, and lies at the junction of domains A and B. This cluster is known to stabilize the protein structure (*6*). From the studies on the thermal stability of BLA and its mutant, it is suggested that mutations that affect the triadic metal-binding site either directly or indirectly are usually highly detrimental to the thermal stability (*6*). In contrast, most of them do not interfere with α-amylase activity at moderate temperature (*6*). Because the amino acid sequence of BAA (Fig. 1) is similar to that of the chimeric α-amylase and

Table 4. **Effect of pH on the α-amylase activity of the wild-type BAA (WT) and its variant enzymes (M77, M111, and 21B).**

Enzyme	WT	M77	M111	21B
v_{opt} (nM s^{-1})	647 ± 8	582 ± 25	549 ± 16	617 ± 30
pK_{a1}	3.6 ± 0.0	3.6 ± 0.1	4.2 ± 0.1	4.2 ± 0.1
pK_{a2}	8.0 ± 0.0	8.0 ± 0.1	7.9 ± 0.1	8.1 ± 0.1

The soluble-starch hydrolysis activity was measured at an enzyme concentration of 1.0 nM and substrate concentration of 3.0 mg/ml at pH 6.0, 30°C. v_{opt}: the reaction rate at the optimum pH.

BLA, the 3D-structure of BAA is also considered to be similar to that of the chimeric one and BLA (*3*). The state of Asp233 in BAA might be similar to that of BLA, and thus it must be interacting with a Ca^{2+} ion tightly bound to the protein. This interaction may give the thermal stability corresponding to a T_{opt} of 60°C to BAA even without Ca^{2+} ions added to the reaction medium. The decreased thermal stability of Asn233-BAA (M111 and 21B) can be explained by the lack of an interaction between Asp233 and a Ca^{2+} ion. From these lines of evidence, we propose a scheme illustrating the interaction of Asp233 with a Ca^{2+} ion bound at the triadic metal-binding site (Fig. 6).

The interaction between Asp233 and a Ca^{2+} ion in the metal triad can be formed in Asp233-BAA, but not in Asn233-BAA. However, this interaction seems not to be so strong, because the K_d values of Asp233-BAA and Asn233-BAA are 7–9 μM and 30–60 μM, respectively (Table 3). From these K_d values, no Ca^{2+} ion is thought to be bound at the triadic metal-binding site without addition of $CaCl_2$. This is in good agreement with the data showing that when BAA enzymes were heat-treated in the absence of $CaCl_2$, they had almost the same T_{50} values (Table 1). However, the optimal temperature (T_{opt}) for Asp233-BAA is considerably higher than that for Asn233-BAA (Fig. 2). This suggests that the carboxyl group of Asp233 might contribute to the stabilization of the enzyme, probably through an electrostatic interaction with a positive charge on the enzyme surface. Because of this possible interaction, the thermal stability of Asp233-BAA as evaluated by T_{opt} and $T_{opt/2}$ is higher than that of Asn233-BAA even in the presence of 5 mM $CaCl_2$ (Fig. 2). On the other hand, when the enzymes were heat-treated in the presence of 5 mM $CaCl_2$, all of them were stabilized remarkably, and the degree of stability enhancement [(ΔT_{50} = (T_{50} at 5 mM $CaCl_2$) – (T_{50} at 0 M $CaCl_2$)] seems to be larger for Asp233-BAA than Asn233-BAA (Table 1). However, it should be noted that the enhancement and T_{50} of M77, an Asp233-BAA, are much lower than those of WT, another Asp233-BAA, and rather closer to the values of Asn233-BAAs. This suggests that there might be factors that control the thermal stability of BAA other than the interaction of Asp233 with the Ca^{2+} ion in the metal triad. It should be remembered that there is a correlation between the stability-enhancement (ΔT_{50}) by 5 mM $CaCl_2$ and affinity (K_d^{-1}) of Ca^{2+} for the enzyme (Table 3). The metal triad has been considered to play an important role in maintaining the protein structure and in resistance to thermal inactivation (*4–6*). However, it is suggested in the present study that the role of the metal triad is not crucial but only limiting.

Comparison of Thermodynamic Parameters for Thermal Inactivation—The $\Delta G^{\ddagger}$ values for the respective cases are

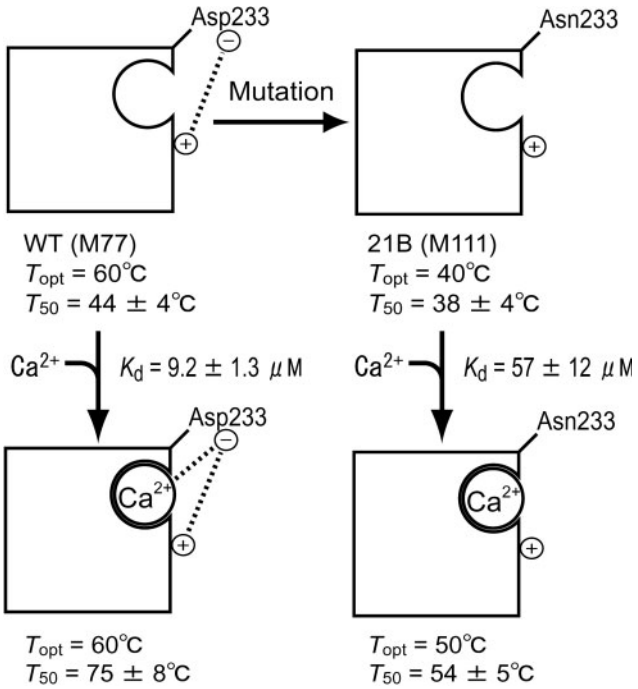

Fig. 6. **Illustration of the relationship between the interaction of Asp233 with the Ca^{2+} ion of the metal triad and the stability of BAA enzymes.**

lower than the $\Delta H^{\ddagger}$ values (Table 2). This is due to the positive entropic contribution during the inactivation process, suggesting an increase in local disorder in the transition state as compared with the ground state (*21*, *23*, *24*). The $\Delta G^{\ddagger}$ value in the presence of 5 mM $CaCl_2$ is higher than in its absence (Table 2), indicating that the thermal inactivation is suppressed by the addition of Ca^{2+} ions. The increase in $\Delta G^{\ddagger}$ is largely due to the increase in $\Delta H^{\ddagger}$, which might be due to the disruption of hydrogen bonds formed in the folded protein molecules so as to reach the transition state for unfolding. The $\Delta H^{\ddagger}$ value increases with increasing [Ca^{2+}] in the reaction medium. The increase in $\Delta H^{\ddagger}$ with [Ca^{2+}] suggests that Ca^{2+} ions enhance and stabilize the hydrogen-bonding network formed in the ground state (*23*). However, the increase in $\Delta H^{\ddagger}$ is compensated for totally by the increase in $\Delta S^{\ddagger}$. Accordingly, $\Delta G^{\ddagger}$ is not changed by the addition of Ca^{2+} ions, and thus the thermal stability of Asn233-BAA is not significantly increased by Ca^{2+} ions.

The linear relationship shown in Fig. 4B indicates that the binding of Ca^{2+} ion(s) to the protein enhances the stabilization of the protein. The K_d values of WT and M77 (both are Asp233-BAA) do not differ, whereas those of M111 and 21B (Asn233-BAA) are larger than those of Asp233-BAA. Hence, Asp233 seems to be effective in Ca^{2+}-binding. The k_{den} value correlates positively with the K_d value, indicating that the shift in equilibrium between the native (N) and calcium-depleted (N*) states to the N* side is accompanied by an increase in the rate constants (k_{den}) of thermal inactivation. This suggests that the Ca^{2+}-binding site may be located close to position 233, or that there might be an allosteric change between position 233 and the site so that the affinity of Ca^{2+} for the site increases more in Asp233-BAA than in Asn233-BAA.

In the present paper, we have examined the effects of $CaCl_2$ on the activity and stability of BAA by assuming that the effect are derived from Ca^{2+} ions and not Cl^- ions (Fig. 4), although we have no information on the effect of Cl^- on BAA. It is well known that Cl^- ions greatly activate the activities of human and porcine α-amylases (*20*, *25*, *26*). This suggests that that possibility that Cl^- ions may play unexpected roles in the structure and function of BAA can not be excluded. We also assumed that the Ca^{2+}-binding sites should be conserved in the variant BAA enzymes as well as WT, and tightly-bound Ca^{2+} ions such as those in the metal triad, should remain in the protein molecule even under conditions without $CaCl_2$ in the reaction mixture. Recently, a new type of *Bacillus* α-amylase, a calcium-free α-amylase, was reported, and sodium ions, instead of calcium ions, are used to retain the structure and function of this enzyme (*27*). This report suggests that the interaction modes of BAA enzymes with ions are various and diverse. An investigation into this point is currently under way. The present study provides insights into engineering the thermal stability of *Bacillus* α-amylase and strategies to generate *Bacillus* α-amylase with lower thermal stability, useful in the baking industry. Most recently, we found a variant BLA enzyme with improved stability and activity as compared with the parent BLA enzyme, and shows high activity even at pH 4.5 (*28*). This BLA variant has proved to be useful for glucose production from starch with a combination of β-amylases. These BLA and BAA variants not only provide a suitable tool for investigating the structure-function relationship of *Bacillus* α-amylase, but also useful for industrial applications.

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REFERENCES

1. Kuriki, T. and Imanaka, T. (1999) The concept of the α-amylase family: Structural similarity and common catalytic mechanism. *J. Biosci. Bioeng.* **87**, 557–565
2. Brozozowski, A.M., Lawson, D.M., Turkenburg, J.P., Bisgaard-Frantzen, H., Svendsen, A., Borchert, T.V., Dauter, Z., Wilson, K.S., and Davies, G.J. (2000) Structural analysis of a chimeric bacterial α-amylase. High-resolution analysis of native and ligand complexes. *Biochemistry* **39**, 9099–9107
3. Machius, M., Wiegand, G., and Huber, R. (1995) Crystal structure of calcium-depleted *Bacillus licheniformis* α-amylase at 2.2 Å Resolution. *J. Mol. Biol.* **246**, 545–559
4. Khajeh, K., Ranjbar, B., Naderi-Manesh, H., Habibi, A.E., and Nemat-Gorgani, M. (2001) Chemical modification of bacterial α-amylases: changes in tertiary structures and the effect of additional calcium. *Biochim. Biophys. Acta* **1548**, 229–237
5. Nielsen, J.E. and Borchert, T.V. (2000) Protein engineerng of bacterial α-amylases. *Biochim. Biophys. Acta* **1543**, 253–274
6. Declerck, N., Machius, M., Wiegand, G., Huber, R., and Gaillardin, C. (2000) Probing structural determinants specifying high thermostability in *Bacillus licheniformis* α-amylase. *J. Mol. Biol.* **301**, 1041–1057
7. Richardson, T.H., Xuqiu Tan, Frey, G., Callen, W., Campbell, M., Lam, D., Macomber, J., Short, J.M., Robertson, D.E., and Miller, C. (2002) A Novel, high Performance enzyme for starch liquefaction. *J. Biol. Chem.* **277**, 26501–26507
8. Crabb, W.D. and Mitchinson, C. (1997) Enzymes involved in the processing of starch to sugars. *Trends. Biotechnol.* **15**, 349–352
9. Vielle, C. and Zeikus, G. (1996) Thermozyme: identifying molecular determinants of protein structural and functional stability. *Trends. Biotechnol.* **14**, 183–190
10. Palacios, H.E., Schwarz, P.B., and D'Appolonia, B.L. (2004) Effect of α-Amylases from different sources on retrogradation and recrystallization of concentrated wheat starch gels: Relationship to bread salting. *J. Agric. Food Chem.* **52**, 5978–5986
11. Palacios, H.E., Schwarz, P.B., and D'Appolonia, B.L. (2004) Effect of α-amylases from different sources on the firming of concentrated wheat starch gels: Relationship to bread staling. *J. Agric. Food Chem.* **52**, 5987–5994
12. Maeda, T., Hashimoto, T., Minoda, M., Tamagawa, S., and Morita, N. (2003) Effects of mutant thermostable α-amylases on rheological properties of wheat dough and bread. *Cereal Chem.* **80**, 722–727
13. Lowry, o. H., Rosebrough N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol.Chem.* **193**, 265–275
14. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
15. Goodwin, T.W. and Morton, R.A. (1946) The spectrophotometoric determination of tyrosine and tryptophan in proteins. *Biochem. J.* **40**, 628–632
16. Pace, C.N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.* **4**, 2411–2423
17. Segel, I.H. (1975) *Enzyme Kinetics, Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, pp. 926–942, John Wiley and Sons, New York
18. Dygert, S., Li, L.H., Florida, D., and Thomas, J.A. (1965) Determination of reducing sugar with improved precision. *Anal. Biochem.* **13**, 367–374
19. Brown, M.E. and Boston M.S. (1961) Ultra-micro sugar determinations using 2,9-dimethyl-1,10-phenanthroline hydrochloride (neocuproine). *Diabetes* **10**, 60–63
20. Oneda, H., Lee, S., and Inouye, K. (2004) Inhibitory effect of 0.19 α-amylase inhibitor from wheat kernel on the activity of porcine pancreas α-amylase and its thermal stability. *J. Biochem.* **135**, 421–427
21. Tomazic, S.J. and Klibanov, A.M. (1988) Why is one *Bacillus* α-amylase more resistant against irreversible thermoinactivation than another? *J. Biol. Chem.* **263**, 3092–3096
22. Muta, Y., Oneda, H., and Inouye, K. (2005) Anomalous pH-dependence of the activity of human matrilysin (matrix metalloproteinase-7) as revealed by nitration and amination of its tyrosine residues. *Biochem. J.* **386**, 263–270
23. Chen, X. and Matthews, R. (1994) Thermodynamic properties of the transition state for the rate-limiting step in the folding of the subunit of tryptophan synthase. *Biochemistry* **33**, 6356–6362
24. Stein, R.A. and Staros, J.V. (1996) Thermal inactivation of the protein tyrosine kinase of the epidermal growth factor recepter. *Biochemistry* **35**, 2878–2884
25. Levitzky, A. and Steer, M. (1972) The allosteric activation of mammalian α-amylase by chloride. *Eur. J. Biochem.* **41**, 171–180.
26. Yamashita, H., Nakatani, H., and Tonomura, B. (1991) Substrate-selective activation of histidine-modified porcine pancreatic α-amylase by chloride ion. *J. Biochem.* **110**, 605–607
27. Nonaka, T., Fujihashi, M., Kita, A., Hagihara, H., Ozaki, K., Ito, S., and Miki, K. (2003) Crystal structure of calcium-free α-amylase from *Bacillus* sp. strain KSM-K38 (AmyK38) and its sodium ion binding sites. *J. Biol. Chem.* **278**, 24818–24824
28. Lee, S., Oneda, H., Minoda, M., Tanaka, A., and Inouye, K. (2006) Comparison of starch hydrolysis activity and thermal stability of two *Bacillus licheniformis* α-amylases and insights into engineering α-amylase variants active under acidic conditions. *J. Biochem.* **139**, 997–1005