Structural and Enzymatic Analysis of Soybean β-Amylase Mutants with Increased pH Optimum*

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Comparison of the architecture around the active site of soybean β-amylase and Bacillus cereus β-amylase showed that the hydrogen bond networks (Glu380-Lys295-Met51 and Glu380-Asn340-Glu178) in soybean β-amylase around the base catalytic residue, Glu380, seem to contribute to the lower pH optimum of soybean β-amylase. To convert the pH optimum of soybean β-amylase (pH 5.4) to that of the bacterial type enzyme (pH 6.7), three mutants of soybean β-amylase, M51T, E178Y, and N340T, were constructed such that the hydrogen bond networks were removed by site-directed mutagenesis. The kinetic analysis showed that the pH optimum of all mutants shifted dramatically to a neutral pH (range, from 5.4 to 6.0–6.6). The $K_m$ values of the mutants were almost the same as that of soybean β-amylase except in the case of M51T, while the $V_{max}$ values of all mutants were low compared with that of soybean β-amylase. The crystal structure analysis of the wild type maltose and mutant-maltose complexes showed that the direct hydrogen bond between Glu380 and Asn340 was completely disrupted in the mutants M51T, E178Y, and N340T. In the case of M51T, the hydrogen bond between Glu380 and Lys295 was also disrupted. These results indicated that the reduced $pK_a$ value of Glu380 is stabilized by the hydrogen bond network and is responsible for the lower pH optimum of soybean β-amylase compared with that of the bacterial β-amylase.

β-Amylase (α-1,4-glucan maltohydrolase, EC 3.2.1.2) catalyzes the liberation of β-anomeric maltose from the non-reducing ends of starch and glycogen. β-Amylase has been classified into family 14 of 91 glycoside hydrolase families (last updated October 6, 2003) according to the method of Henrissat et al. (1, 2) and is distributed in higher plants and bacteria (3, 4). The cDNAs from higher plants, including soybean (5, 6), barley (7), rye (8), Arabidopsis thaliana (9), and sweet potato (10), and from bacteria, including Bacillus cereus (11), Bacillus polymyxa (12, 13), Clostridium thermosulfurogenes (14), and Bacillus megaterium DSM319 (15), have been cloned and sequenced. The three-dimensional structures of soybean (16, 17), barley (18), sweet potato (19), and B. cereus (20, 21) β-amylase have already been determined. All of the previously determined structures of β-amylase exhibit a well conserved (βα)8-barrel fold in the core domain and an active center in the cleft of the barrel. The structural architecture of the β-amylase (SBA)$^\dagger$-maltose complex indicated that Glu186 and Glu380 play important roles in the enzymatic reaction as a general acid and a base catalyst, respectively (16). This finding is supported by the results of site-directed mutagenesis (22) and affinity labeling (23). In the case of B. cereus β-amylase (BCB), Glu172 and Glu367 act as the general acid and base catalyst, respectively, corresponding to Glu186 and Glu380 in soybean β-amylase (20, 21). It has been reported that only bacterial β-amylase has the ability to digest raw starches (24–27), an activity that has been ascribed to its C-terminal raw starch-binding domain (11, 20, 28). Except for the raw starch digestive ability, properties of higher plant β-amylases differ from those of bacterial enzymes in terms of optimum pH, specific activity, isoelectric points, and the number of sulfhydryl/disulfide groups (29). The optimum pH of higher plant β-amylases is around 5.4, whereas that of bacterial enzymes is ~6.7. Fig. 1 shows the comparison of structures near Glu380 in both SBA and BCB. The hydrogen bond between the side chains of Glu380 and Lys396 (GLU and LYS in BCB), which is conserved in both enzymes, seems to be important for β-amylase catalysis. In SBA, the hydrogen bond networks (Glu380-Lys295-Met51 and Glu380-Asp176-Glu178-Asn340-Glu380) stabilize the negatively charged form of Glu380, therefore Glu380 in SBA may have a $pK_a$ lower than that of Glu380 in BCB as this type of stabilization was not found in BCB (20). The S of Met51 is located near N of Lys396 (3.5 Å) and Oe-2 of Glu380 (3.7 Å), possibly forming a hydrogen bond with N of Lys396. These hydrogen bond networks in SBA are not present in BCB except in the case of the isolated hydrogen bond between Tyr164 and Thr359. These five amino acid residues (Met51, Glu380, Asp176, Glu178, and Asn340 in SBA) that differ between SBA and BCB are conserved in each of the higher plant and bacterial β-amylases, respectively (16). This difference in architecture around the base catalytic site between SBA and BCB is thought to contribute to the difference in the pH optimum of the respective enzymes. To explore the role played by these amino acid residues, we focused on the Mut51, Glu178, and Asn340 of SBA. These three residues are located near Glu380 of the five residues and are considered as candidates for the control of the $pK_a$ of Glu380.

In this study, we characterized three SBA mutants, M51T, E178Y, and N340T, based on their enzymatic activity and x-ray diffraction coordinates and structure factors (code 1Q6C, 1Q6D, 1Q6E, 1Q6F, and 1Q6G) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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crystallography and examined the mechanism underlying their different pH optima.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Mutant SBA genes were constructed by a double PCR method. The PCR was performed in a reaction volume totaling 50 μl and containing 2.5 units of TAKARA EX Taq polymerase, 10× buffer, 200 μM dNTPs, two synthesized mutagenesis primers at 100 ng/ml, and 5 μl of pBRSA (pKK233-2 vector including the SBA gene), which had been constructed previously (6). The following primers, with the desired changes indicated in bold, were used in the mutagenesis procedures (F and R denote the upstream and the downstream primers, respectively): M51T-F, 5′-GTTGATGTGTGGTGGGGG-3′; M51T-R, 5′-GCCCTGCAGGAGAGC-3′; M9261T-F, 5′-GTTGGCCTCCAGGAGAGC-3′; E178Y-F, 5′-GGTTGGCCTCCAGGAGAGC-3′; E178Y-R, 5′-GTAAATGTCTATATAGTCCAGAT-3′; N340T-F, 5′-GCCATTCTTCATACCTGTCGC-3′; N340T-R, 5′-ATGATCTCCTACGCTGTGCA-3′. The mutagenesis primers were extended using a thermal cycler (model 2400, PerkinElmer) at 95 °C for 5 min, then annealing for 20 s at 55 °C, and elongation for 5 min at 72 °C for 18 cycles. The PCR products were separated by electrophoresis on a 1.2% (w/v) agarose gel, and the products were purified using glass powder (Bio-Rad). The resulting 1.5-kilobase pair fragments were blunted by a blunting kit, phosphorylated by T4 polynucleotide kinase at the 5′-segment, and self-ligated using a ligation kit, version 2 (Takara). The constructed mutant vectors were transformed into Escherichia coli strain JM105 using heat shock. The mutant DNA sequences were confirmed by a DNA sequencer.

Preparation of Mutant Proteins—Protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside to reach a final concentration of 1.0 mM in E. coli strain JM105. The purification and activity assay of the mutant SBA were performed by a method described previously (6).

Enzyme Kinetics and pH Dependence of β-Amylase Activity—The values of kcat and km for both SBA and the mutants were measured using potato amyllopectin as a substrate (30). Concentrations of the substrate were varied from 10 to 5 times the km value. Calculations of Vmax and km were performed using the Michaelis-Menten equation with KaleidaGraph Software (Synergy Software). The pH-dependent activities of SBA and the mutants were determined using potato amyllopectin at 37 °C in 0.05 M Britton-Robinson buffer at pH 3.0–9.0. The ionic strength of the buffer solution at each pH was adjusted to be 0.2 with NaCl. The apparent pK1 and pK2 values were calculated with the KaleidaGraph nonlinear curve-fitting program using Equation 1 (31),

\[
\nu = \frac{V}{1 + \frac{[H]}{K_{\text{H}} + K_{\text{L}}[H]}} \tag{1}
\]

where \(\nu\) and \(V\) are specific activity (units/mg) at each pH and pH-independent activity, respectively. \([H]\) is the concentration of hydrogen ions, and \(K_{\text{H}}\) and \(K_{\text{L}}\) are dissociation constants of catalytic groups of the enzyme.

Estimation of Dissociation Constants of the Mutant-Maltose Complexes—The dissociation constants \(K_{\text{d}}\) of SBA and the mutants respectively complexed with maltose were estimated by the titration of emission spectra based on tryptophan residues (excitation = 285 nm, emission = 330 nm) in 0.1 M acetate buffer at pH 5.4 and 25 °C; observations were made with a Hitachi F-3000 fluorescence spectrometer.

Crystallization and Data Collection—Based on the crystallized condition of the enzyme in a previous report (6), crystallization of wild-type and mutant SBA was performed at 4 °C by the hanging drop vapor diffusion method, i.e., mixing 5 μl of a 24 mg/ml protein solution in 0.1 M sodium acetate buffer (pH 5.4) with 5 μl of reservoir liquid of 48% ammonium sulfate containing 0.1 M sodium acetate buffer, 1 mM EDTA, and 18 mM 2-mercaptoethanol at pH 5.4. Crystals grew for 1 month. The wild-type and mutant crystals were soaked in the mother liquor in the presence of 200 mM maltose for 30 min at 20 °C, and then the samples were mounted in thin walled glass capillaries filled with the mother liquor and sealed with wax. We avoided crystal freezing to prevent unexpected changes, such as a shift in the pH, from occurring. X-ray data of the wild-type crystal was collected at up to 1.86-Å resolution at room temperature using CuKα radiation with a Rigaku RAXIS IIC detector coupled to a Rigaku rotating anode generator, and the data were processed with Rigaku software (Rigaku). X-ray data of the respective mutant SBA crystals were collected at up to 1.95–2.1-Å resolution at room temperature using CuKα radiation with a Bruker Hi-Star area detector coupled to a MAC Science M18XHF rotating anode generator, and the results were processed with the SADE and SAINT software packages (Bruker).

Phasing and Refinement—The initial model utilized the refined coordinates of SBA complexed with maltose (Protein Data Bank accession code 1BYC). The refinement program CNS (32) and the graphic program TURBO-FRODO (Architecture et Fonction des Macromolécules Biologiques-CNRS, Marseille, France) were used to refine and rebuild the SBA and the mutant models. The 2Fo − Fo and Fc − Fo maps were used to locate the correct models. Several rounds of minimization and B-factor refinement followed by manual model building were carried out to improve the model. The structures were refined against all reflections from 15.0 Å to the highest resolution available without any σ(F) cut-off (see Table I). The Rfree values of these mutants were calculated for randomly separated 10% data, respectively. The number of water molecules incorporated and the final refinement parameters are also indicated in Table I. The stereospecificity of the model was assessed using the program PROCHECK (33). The molecular models of SBA, the mutants, and BCB were superimposed using a fitting program implemented in TURBO-FRODO.

RESULTS AND DISCUSSION

Effect of pH on Mutant β-Amylase Activity—In the present study, the three mutants, M51T, E178Y, and N340T, were all shown to have an increased pH optimum as indicated in Fig. 2. The specific activity for the hydrolysis of potato amyllopectin at 37 °C in each optimum pH of the respective β-amylase mutants is given in Table II. M51T, E178Y, and N340T had decreased specific activities of 11, 43, and 32%, respectively, of the wild-type SBA. The relative pH activity profiles were bell-shaped except in the case of N340T (Fig. 2). Although we did not examine the pH dependence of the Vmax and km values separately, the relative pH activity profiles appeared to be mainly due to the Vmax value; notably the km value of the wild type was reported previously to be constant from pH 3.0 to 7.0 (34). Thus, the observed specific activities were fitted to Equation 1, assuming two pk values. Based on this curve-fitting, the op-
Data collection and refinement statistics of SBA mutants

| Mutant       | Wild type, pH 5.4 | M51T, pH 5.4 | E178Y, pH 5.4 | N340T, pH 5.4 |
|--------------|-------------------|--------------|---------------|---------------|
| Space group  | P3, 21            | P3, 21       | P3, 21        | P3, 21        |
| Maltose (mM) | 200               | 200          | 200           | 200           |
| a, b, c      | 86.2, 86.4        | 86.7         | 86.8          | 86.2          |
| Data collection |                  |              |               |               |
| Resolution range (Å) | 43.07–1.86       | 40.65–1.88   | 40.65–1.99    | 40.65–1.99    |
| No. of measured reflections | 122,982 (7,856) | 220,571 (7,247) | 247,346 (6,847) | 203,117 (4,842) |
| R-free (%)  | 17.8 (28.2)       | 17.7 (27.0)  | 17.2 (24.3)   | 17.4 (27.3)   |
| R-r.m.s.     | 1.89 (1.94)       | 1.99 (2.01)  | 1.94 (2.00)   | 1.86 (2.00)   |
| Refinement  |                  |              |               |               |
| Resolution range (Å) | 10–1.86 (1.93–1.86) | 15–2.0 (2.07–2.00) | 15–1.95 (2.02–1.95) | 15–2.0 (2.07–2.00) |
| No. of reflections | 39,261 (2,199)  | 39,375 (3,228) | 30,486 (2,290) | 36,693 (2,714) |
| R-free (%)  | 17.8 (28.2)       | 17.7 (27.0)  | 17.2 (24.3)   | 17.4 (27.3)   |
| R-r.m.s.     | 1.89 (1.94)       | 1.99 (2.01)  | 1.94 (2.00)   | 1.86 (2.00)   |
| Maltose-occupied sites | –2 to –1, +1 +2 | –2 to –1, +1 +2 | –2 to –1, +1 +2 | –2 to –1, +1 +2 |
| Alternate residues | 341–344, 379–381 | 341–344, 341–344 | 341–344, 341–344 | 341–344, 341–344 |
| Average B-factor (Å²) | 24.2           | 22.3         | 22.5          | 21.3          |
| r.m.s. deviation | 0.006          | 0.005        | 0.005         | 0.005         |
| Bond lengths (Å) | 1.29           | 1.62         | 1.26          | 1.25          |
| Bond angles (°) |               |              |               |               |
| R-free (%)  | 21.2 (40.0)       | 21.6 (31.7)  | 21.5 (29.0)   | 20.9 (25.5)   |
| R-factor (%) | 17.4 (37.4)       | 17.8 (28.2)  | 17.7 (27.0)   | 17.2 (24.3)   |

Specific activity and pKₐ values for the hydrolysis of potato amylopectin

| Specific activity | Wild type, pH 5.4 | M51T, pH 5.4 | E178Y, pH 5.4 | N340T, pH 5.4 |
|------------------|-------------------|--------------|---------------|---------------|
| Optimum pH       | 5.4               | 6.5          | 6.0           | 6.6           |
| units/mg         | 684 ± 0.18        | 75 ± 0.02    | 292 ± 0.28    | 222 ± 0.56    |
| Apparent pKₐ values |              |              |               |               |
| pK₁             | 3.6 ± 0.09        | 4.4 ± 0.08   | 4.4 ± 0.07    | 4.5 ± 0.14    |
| pK₂             | 8.1 ± 0.06        | 8.1 ± 0.07   | 7.9 ± 0.06    | 8.2 ± 0.13    |

Relative activity (%) vs. pH

![Relative activity (%) vs. pH](image)

β-Amylase Mutants with Increased pH Optimum

As shown in Table III, the apparent Kₐ values of E178Y and N340T were approximately the same as that of the wild type, whereas that of M51T was over 20 times larger than that of the wild-type enzyme. On the other hand, the V_max values of E178Y and N340T were approximately 2 and 3 times lower, respectively, than that of the wild-type enzyme. These results indicated that the mutations affected the environment of the base catalyst and that due to these mutations the changes in the base catalyst were more significant than those in the acid catalyst, resulting in the increased pKₐ characteristic of the mutant enzymes.

As shown in Table III, the apparent Kₐ values of E178Y and N340T were approximately the same as that of the wild type, whereas that of M51T was over 20 times larger than that of the wild-type enzyme. On the other hand, the V_max values of E178Y and N340T were approximately 2 and 3 times lower, respectively, than that of the wild-type enzyme. In contrast to the Kₐ values, the Kᵢ values of M51T for maltose was 10-fold lower than that of the wild type, whereas the Kᵢ values of the two mutants were both approximately equal to that of the wild-type enzyme. These results suggest that conformational changes occurred at the active site of M51T and consequently reduced...
the catalytic level and increased the unusual binding of substrates.

Quality of the Refined Models—To investigate the structural changes in the mutant SBA, we determined the crystal structures of the wild-type and mutant SBA complexed with maltose at 1.86–2.1-Å resolutions. The x-ray data collection and refinement statistics are summarized in Table I. From the Luzzati plots (38), the mean absolute positional errors were estimated to be 0.21–0.22 Å. Ramachandran plots of the main-chain conformation angles (39) showed that 88.8–88.9% of the residues lie within the core region, and 99.7–99.8% lie within the allowed region. With the exception of a small displacement of the main-chain region (residues 341–344) over the active site cleft, no significant changes occurred in the backbone structure of the mutants. The root mean square deviations between the wild-type and the mutant structures, calculated for all Ca
atoms, were less than 0.2 Å. The flexible loop (residues 96–103), which was reported to play an important role in the catalytic step (16, 17), was in the closed form (Table I). As shown in Fig. 3, the $2F_o - F_c$ maps of the active site and the bound maltose, except in the case of subsite +3, were sufficiently clear to provide a reliable interpretation of the structural changes in the mutants.

The Structure of Wild-type SBA-Maltose Complex—In a previous report concerned with the SBA-maltose complex (17), the electron density map of two tandem maltose molecules at subsites −2 to +2 was interpreted to indicate that one maltotetraose and two maltose molecules (β-anomer at subsites −2 to −1 and subsites +1 to +2, respectively) bind simultaneously. A better interpretation was considered to be that both $4^C_1$- and distorted $4^C_2$-anomeric maltoses can bind at subsite −2 to −1 without forming maltotetraose from the excess maltose. It is also assumed that due to steric hindrance, no glucose residue can bind at subsite +1 when an α-anomeric glucose residue binds at subsite −1. For the previous data, the ratio of the two anomers at subsite −1 was estimated to be 0.2 and 0.8 for α and β, respectively (40). However, the present data set of SBA-maltose complex could be refined by two maltoses at subsites −2 to −1 and +1 to +2, respectively, with a distorted sugar ring almost assuming the boat form with unknown anomer type at subsite −1 (Fig. 3A). It was suggested that the ratio of the distorted glucose and $4^C_1$ α-glucose residue at subsite −1 depended on the slight change in pH.

The Structure of M51T-Maltose Complex—Fig. 3B shows the $2F_o - F_c$ map and the residues around Glu380 of M51T with the bound maltose determined at pH 5.4. In the structure of M51T, the substitution of Thr51 created a space that had been occupied by $S^4$ and $C^6$ in the original Met51. Instead of being occupied by the side chain of Met51, this space was occupied by one water molecule, which was hydrogen-bonded to $Oe$-2 of Glu380 (2.8 Å) and $N_z$ of Lys295 (3.2 Å). The loss of the side chain of Met51 resulted in the disruption of the hydrogen bond between $N_z$ of Lys295 and $Oe$-2 of Glu380 (from 2.9 to 3.8 Å), which may have allowed for the splitting of the side chain of Glu380 into two alternate positions. Both of the positions were shifted by 0.6 and 2.1 Å from that of the Glu380 Oe-2 in the wild-type SBA. The disposition of Glu380 Oe-2 also disrupted the hydrogen bond between $Oe$-2 of Glu380 and $N_z$-2 of Asn340. The altered side-chain position of Glu380 was so close to O-1 of the distorted glucose at subsite −1 that it prevented the binding of $4^C_2$-anomeric glucose and the localization of a catalytic water molecule, which would be in accord with the low activity of M51T.

In contrast to the electron density map of the wild-type enzyme, that of maltose in M51T was interpreted to show a mixture of three maltose molecules bound at subsites −2 to −1.
(a distorted glucose and an α-1C1 glucose at subsite −1), at
subsites +1 to +2, and at subsites +2 to +3. It is assumed that
a maltose molecule can bind at subsites +1 to +2 only when a
distorted glucose residue binds at subsite −1. If an α-anomeric
residue binds at subsite −1, the second maltose will
bind at subsites +2 to +3 due to a collision between O-1 of the
residue at subsite −1 and O-4 of the glucose at subsite +1. The
occupancy refinement of the two alternate positions of maltose
coupled with the alternate position of the side chain of Glu380
showed that their respective occupancies were both about 0.5.

The Structure of E178Y-Maltose Complex—Fig. 3C shows the
$2F_o - F_c$ map and the residues around Glu380 of E178Y with
the bound maltose determined at pH 5.4. The substituted side
chain of Y178 created a novel hydrogen bond with the side
chain of Asn340 (O_{-1} Tyr_{275}-O_{6-1} AsnΔ340, 2.8 Å). The side chain
of Asn340 was found to flip toward O_{-1} of Tyr178 by rotating the
$\chi^2$ torsion angle about 53°, resulting in the disruption of the
hydrogen bond between Oε-2 of Glu380 and Oβ-1 of Asn340. Two
maltose molecules were found, one at subsite −2 to −1 and one
at subsite +2 to +3. The anomeric of the glucose residue at
subsite −1 was clearly identified as having the α-configuration,
suggesting that the collision of this O-1 with O-4 of the glucose
residue at subsite −1 prevented the second maltose from bind-
ing at subsite +1. Instead of a glucose residue at subsite +1, there
were four water molecules. One water molecule was
assigned as the catalytic water molecule, and it was found to be
in almost the same position as that of O-1 of the distorted
residue at subsite −1 in the wild-type SBA-maltose complex.
The alternative position of the α-anomeric glucose residue at
subsite −1 may be ascribed to the increased pK_{α}
value of Glu380, which was induced by the disruption of the
hydrogen bond with Asn340. It is thought that both distorted
maltose and normal maltose bind primarily at subsite −2 to
−1, depending on the protonated and deprotonated states of
Glu380.

To examine this hypothesis, the structure of the E178Y-
maltose complex was determined at pH 7.1 as shown in Fig. 3D.
The orientations of the side chains of Glu380, Asn340, and Tyr178
were the same as those found at pH 5.4. In contrast to the
results obtained at pH 5.4, both a distorted maltose and a
normal α-anomeric maltose bind at subsites −2 to −1, and the
second maltose binds to subsites +1 to +2 or subsites +2 to +3
as found in the case of M51T-maltose complex at pH 5.4 (Fig.
3B). It is suggested that only the deprotonated form of Glu380
can enable the binding of the distorted maltose to subsites −2
to −1.

The Structure of N340T-Maltose Complex—Fig. 3E shows the
$2F_o - F_c$ map and the residues around Glu380 of N340T
with the bound maltose determined at pH 5.4. Oγ-1 of the
substituted Thr340 was found to face the side chain of Glu178,creating a weak hydrogen bond with Oε-1 of Glu178 at a
distance of 3.4 Å and resulting in disruption of the original hydro-
gen bond between Oε-2 of Glu380 and Oβ-1 of Asn340. Two
separated maltose molecules were found, one at subsites −2 to
−1 (α-anomeric) and one at subsites +2 to +3 as was the case for
E178Y at pH 5.4. The distance between Oε-1 of Glu380 and
the catalytic water molecule was found to be 2.9 Å, which is slightly longer than that found in E178Y. The tem-
perature factor of this catalytic water molecule was also
slightly higher in N340T (35 Å^2) than in E178Y (31 Å^2). These parameters of the catalytic water molecule may account for the
decreased specific activity in these mutants and for the fraction
of bound distorted maltose at subsites −2 to −1 around their
respective pH optima.

Structural Changes Required for Shifts in pH Optimum—
Fig. 4 shows the superimposition of the residues around Glu380
in the mutants and the wild-type SBA onto those of BCB. In the
mutant M51T, the substituted side chain of Thr275 formed a
hydrogen bond with the side chain of Glu87 (Oγ-1 of Thr51-Oε-2
of Glu87, 2.8 Å) instead of the disrupted two interactions be-
tween S6 of Met51 and N_{ε} of Lys295 and N_{ε} of Lys295 and Oε-2 of
Glu380. The space was filled with a water molecule that formed a hydrogen bond with Oε-2 of Glu380 and N_{ε} of Lys295.
These changes led to the instability of the side chain of Glu380,
resulting in the two disordered alternate positions. The disrup-
tion of the hydrogen bond between Oε-2 of Glu380 and N_{ε}-2 of
Asn340 and the disordered side chain of Glu380 were in accord
with the increased pH optimum and the most decreased activity
of the mutant (Tables II and III). The situation in the case of
BCB was similar in that two water molecules occupied the
vacant space between the substituted Thr275 and the catalytic
base residue. But in the case of BCB, the other water molecule
was hydrogen-bonded to Oγ of Thr178 (3.2 Å), Oε-2 of Glu380 (3.2
Å), and N_{ε} of Lys287 (2.8 Å), indicating that these water-medi-
ated hydrogen bonds stabilized the position of Glu380. The
results for M51T suggest that the side chain of Met51 or the
water mediated-hydrogen bonds is important for fixing the
position of the base catalyst.

In contrast to M51T, E178Y and N340T have 42 and 32% of
the activity of the wild-type enzyme with increased pH opti-
umum, respectively. Their conformation changes are restricted
only around the mutated side chains, providing precise infor-
mation about the shift in optimum pH. In the case of E178Y,
despite the bulkiness of the side chain of Tyr178, the side chain
of Tyr178 was found to occupy almost the same orientation as
that of Glu178 in the wild-type SBA. Met51 also held the same

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**Fig. 4.** Stereodigrams of the residues around Glu380 in the mutants and the wild-type SBA superimposed onto those of BCB (yellow and black, SBA; cyan, BCB; green, M51T; blue, E178Y; and violet, N340T). The hydrogen bonds are indicated by broken lines. This figure was generated using Mol-
SCRIPT (54) and Raster3D (55).
position as it did in the wild-type SBA. However, the side chain of Asn340 was turned to face the side chain of Tyr178 by a 53° rotation of the \( \chi^2 \) torsion angle, thus forming a hydrogen bond (O\( \eta \) of Tyr178–O\( \epsilon \)-1 of Asn340, 2.8 Å), and this resulted in the disruption of the hydrogen bond between O\( \epsilon \)-2 of Glu380 and O\( \epsilon \)-1 of Asn340. In the case of N340T, the mutation did not affect the side-chain conformation of Met51 or Glu178. The mutated side chain of Thr340 created a hydrogen bond with the side chain of Glu178 as was the case with Tyr164 and Thr328 in BCB. As the interactions between N\( \epsilon \) of Lys295 and O\( \epsilon \)-2 of Glu380 and between S\( \delta \) of Met51 and N\( \epsilon \) of Lys295 were still maintained in E178Y and N340T, the disruption of the hydrogen bond between O\( \epsilon \)-2 of Glu380 and O\( \epsilon \)-1 of Asn340 was sufficient to increase the optimum pH from 5.4 to 6.0–6.6 (Table II).

In the structures of E178Y and N340T determined at pH 5.4, the binding mode of maltose was altered from subsites \( \epsilon \)-2 to \( \epsilon \)-1 and \( \epsilon \)-1 to \( \epsilon \)-2 (wild type) to \( \epsilon \)-2 to \( \epsilon \)-1 and +2 to +3, respectively, due to the predominant binding of an \( \alpha \)-anomeric glucose residue at subsite \( \epsilon \)-1. In every case in which a distorted glucose residue binds at subsite \( \epsilon \)-1, the sugar rings are distorted to form conformations that resemble boat or half-chair conformations. Alteration of the binding mode occurred due to changes in pH, as shown in Fig. 3D, in the case of E178Y, suggesting that the binding of a distorted glucose residue at subsite \( \epsilon \)-1 required the deprotonation of Glu380. The strong
nucleophile of the ionized side chain of Glu$_{380}$ may have enabled the distortion of the sugar ring at subsite −1. The position of O-1 in the distorted glucose residue at subsite −1 was very close to that of a catalytic water molecule found in the binding of α-glucose at the subsite, suggesting that the ionized side chain of Glu$_{380}$ can activate the catalytic water molecule to attack C-1 of the glucose residue, provided that a true substrate with an α-configuration binds at subsite −1. Thus, the catalytic activity is thought to be very sensitive to the nucleophilic nature of Glu$_{380}$ in terms of the distance between Oe-1 of the catalytic water molecule; this sensitivity may account for the decreased activity of E178Y and N340T relative to that of the wild-type enzyme.

Hydrogen Bond Networks Altering the pH OPTIMUM in Other Glycoside Hydrolases—Alteration of the pH optimum by changing the pK$_a$ of the acid/base catalyst in other members of the glycoside hydrolase family has been observed previously (41–43). In a study of Aspergillus awamori glucoamylase (GA) and Bacillus circulans xylanase (BCX), hydrogen bonds between the acid/base catalyst and residues at the active site were shown to play significant roles in regulation of the pH optimum (41, 43). To provide a general concept to describe the control of the pH optimum of enzymes, we compared the active site of SBA with those of GA and BCX. Fig. 5 shows the conformations of active site residues of SBA-maltose, GA-acarbose, and BCX-2FXb (2-deoxy-2-fluoro-xylobiose), which were arranged such that they had the same substrate orientation; in each case, the two catalytic residues were positioned above and below the substrate, respectively. Like SBA, GA (glycoside hydrolase family 15) is an inverting exoglycosidase (1, 2) that produces β-D-glucose by hydrolyzing α-1,4- and α-1,6-glucosidic linkage from the non-reducing ends of starch and related oligo- and polysaccharides (44, 45). Two carboxyl groups are known to be involved in the catalytic mechanism of GA in which Glu$_{179}$ and Glu$_{400}$ are the acid/base catalyst (A. awamori and Aspergillus niger numbering system) and correspond to Glu$_{186}$ and Glu$_{380}$ in SBA, respectively (46–49). Glu$_{400}$ in GA also forms hydrogen bond networks (Tyr$_{48}$–Glu$_{400}$–Ser$_{411}$ and Glu$_{400}$–Gln$_{401}$–Asn$_{415}$ including catalytic water) (Fig. 5B). Frandsen et al. (48) conducted a mutational analysis of Y48W, and suggested that Tyr$_{48}$ is functionally linked to Glu$_{400}$ and is important for maintaining the active site geometry and for the stabilization of an oxocarbonium ion intermediate. The k$_{cat}$ value of Y48W in GA was reduced 80–100-fold, while k$_{cat}$ was increased 2–3-fold. Y48W had unusually high activity at pH values below 4.0. In contrast, Fang et al. (41) reported that disruption of the hydrogen bond between Oγ of Ser$_{411}$ and Oe-1 of Glu$_{380}$ by site-directed mutagenesis of Ser$_{411}$ to either Ala (S411A) or Cys (S411C) reduced the catalytic efficiencies by only 46 and 26% that of the wild-type enzyme, respectively. Like SBA, GA and BCX share the same features responsible for controlling the pH optimum. It is concluded that hydrogen bonds, as well as networks between the acid/base catalyst and the residues near the active site, control the pH optimum despite the different reaction mechanisms used by inverting and retaining enzymes.

In this study, we demonstrated that the pH optimum of SBA mutants could be shifted toward the alkaline region by removing hydrogen bonds with the side chain of Glu$_{380}$, the catalytic base, possibly as a result of increasing its pK$_a$. The present study also suggested that the formation or deformation of hydrogen bonds between the catalytic residue and residues near the active site can control the pH optimum of enzymes; such changes in hydrogen bonding can lead to shifts in the pH optimum toward either the acidic or alkaline region.

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