Roles of the Aromatic Residues Conserved in the Active Center of *Saccharomycopsis* α-Amylase for Transglycosylation and Hydrolysis Activity

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Received July 6, 1993; Revised Manuscript Received October 12, 1993®

ABSTRACT: The molecular structure of *Saccharomycopsis fibuligera* α-amylase was predicted by a homology-based modeling technique, and the amino acid residues composing the active site were displayed with color codes according to their order of conservation. We noticed two highly conserved aromatic residues located in the active center, tyrosine 83 (Y83) and tryptophan 84 (W84), and examined their roles in catalytic activity by site-directed mutagenesis. The W, leucine (L), and asparagine (N) mutants at Y83 and the L mutant at W84 showed remarkable enhancement of transglycosylation activity and complementary decreases in native hydrolysis activity. The phenylalanine (F) mutant at Y83 and the F and Y mutants at W84 only decreased hydrolysis activity. Mechanistic and kinetic studies of these mutants using a reducing-end-blocked substrate and a hydrolysis-specific substrate revealed a probable transglycosylation mechanism and critical contributions of the 83rd and 84th aromatic residues to efficient hydrolysis. Given that aromatic residues stack against the faces of sugars, we assumed that Y83 and, presumably, W84 play roles in the binding of oligosaccharide substrates through the stacking interaction and in the indirect fixation of the catalytic water molecule through hydrogen bonding with the hydroxyl of the bound substrates. Mutations to nonaromatic residues could cause slight changes in the binding topology of substrates to favor transglycosylation over hydrolysis.

α-Amylase (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) catalyzes the hydrolysis of α-D-(1→4)glycosidic linkages of starch components (amylose and amylopectin), glycogen, and various oligosaccharides and is an important industrial enzyme. Many microorganisms can produce α-amylases, and several yeast species secrete them extracellularly. To improve this industrial enzyme, we conducted a protein engineering study using the yeast enzyme, *Saccharomycopsis fibuligera* α-amylase (Sfamy[1]), and have reported its enzymatic characteristics (Matsui et al., 1990), its subsite structure (Matsui et al., 1991a), and the alteration of its product specificity by site-directed mutation at the 210th lysine (Matsui et al., 1992a,b).

All α-amylases sequenced thus far possess six conserved regions, which constitute their active sites (Svensson, 1988). The 84th tryptophan residue (W84) of Sfamy is located in the first conserved region and is presumed to participate in substrate recognition. We examined the activities of mutants at W84 and found that one, W84L, in which W84 is replaced by a leucine (L) residue, greatly increased transglycosylation activity compared with the native enzyme (Matsui et al., 1991b). Since the 83rd tyrosine residue (Y83) adjacent to W84 was conserved in all α-amylases, we also targeted it. The present article reports the molecular modeling of Sfamy, including the identification of the amino acid residues evolutionally conserved in the active site and the presumed roles of Y83 and W84 in hydrolysis and transglycosylation.

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® Abstract published in *Advance ACS Abstracts,* December 15, 1993.

[1] Abbreviations: Sfamy, *Saccharomycopsis fibuligera* α-amylase; TAA, takaamylase A; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; G_2, maltose; G_3, maltotriose; G_4, maltotetrose; G_5, maltopentose; G_7, maltoheptose; $G_{\geq 10}$, maltooligosaccharides larger than maltodecose G_{10}; G_2-PNP, *p*-nitrophenyl α-D-maltoside; G_5-PNP, *p*-nitrophenyl α-D-maltopentoside; G_8-PNP, *n*-nitrophenyl α-D-maltooctoside.

MATERIALS AND METHODS

(*a*) *Energy Minimization of the Three-Dimensional Structure of Takaamylase A.* The three-dimensional (3D) structure of takaamylase A (TAA), a fungus α-amylase, was obtained from the Protein Data Bank (PDB) (Bernstein et al., 1977). After the positions of the hydrogen atoms and lone pairs of TAA were generated, energy minimization of the TAA molecule was carried out in an Amber united atom force field (Weiner et al., 1984), using the conjugated gradient method. The distance-dependent dielectric constant was $\epsilon = 4r$, and the nonbonded cutoff was 10 Å. A positional restraint energy of 1.0 kcal/mol/Å^2 was added for all atoms except hydrogen atoms and lone pairs. Calculations were made using a BIOCES (NEC Co., Ltd.) program system, an EWS workstation 4800/20 (NEC), and a PS 340 (Evans & Sutherland) graphics terminal. The BIOCES system has been used in many protein modeling studies (Miyata et al., 1992; Kajihara et al., 1993).

(*b*) *Construction of the Molecular Model of Sfamy Involving Partial Structures Searched by Computer.* The 3D model of Sfamy was constructed with the understanding that the tertiary structure is generally more conservative than the primary structure. Since the sequence of the target protein had considerable homology with that of the reference protein of known structure, its 3D structure was predicted theoretically by the following procedure.

First, an alignment of the target and reference protein sequences was formed, using hydrophobic core scores (Kanao-

0006-2960/94/0433-451$04.50/0

ka et al., 1989) to improve the accuracy of the alignment. Scoring restrains amino acid insertions/deletions in the sequence of the hydrophobic core region of the target protein. A SCROIL program (Umezawa & Umeyama, 1988) was used to search for the hydrophobic core region of the reference protein and to calculate the hydrophobic core scores.

Second, the initial coordinates for the main-chain atoms of the target protein were determined. For the main-chain regions without insertions/deletions, we used the coordinates of the corresponding atoms of the reference protein for those of the target protein. For the regions containing insertions/deletions, partial structures possessing equal residue numbers and similar sequence features, including the bond orientations of both end residues, were collected from the data base (PDB). The most preferable structures were selected from the collected partial structures by superimposed comparison with the corresponding structures of the reference protein using a graphic display. The whole main-chain structure of the target protein was constructed by substituting the selected partial structures for the corresponding parts of the reference protein.

Third, the atomic coordinates of the side chains were obtained. Those for the conserved regions were identical with those for the corresponding regions of the reference protein, whereas the atomic coordinates for the regions where amino acid replacements or insertions/deletions occur were generated using the standard torsion angles of amino acids, which were determined by X-ray crystallography. The steric hindrance of the side chains was then removed with a random search composed of 200 steps.

(*c*) *Energy Minimization of the Constructed 3D Structure of Sfamy*. The calculation for the energy minimization of the constructed Sfamy molecular structure was made using the same method as that for TAA.

(*d*) *Evolutionally Invariant Amino Acids in the Active Site of Sfamy*. On the basis of the alignment of the primary sequences of 10 different α-amylases derived from microorganisms, animals, and one plant (Holm et al., 1990), eight sequence regions constituting the active sites of these enzymes were specified The corresponding eight sequences of Sfamy range from the following positions: 16–41, 61–98, 114–128, 151–177, 201–219, 226–252, 289–302, and 338–350. The conservation of the amino acid residues forming these regions was scored and displayed with seven colors, varying from warm to cold to white, on the Sfamy 3D structure. The amino acid residues are distinguished by their order of conservation with the colors red, orange, yellow, green, blue, cyan, and white (Figure 3). Red represents the most conserved and invariant residue whereas white represents the least conserved residues, for variations of more than seven kinds of amino acids or with insertion/deletion at the position. Other amino acid residues are indicated by the color white.

(*e*) *Construction of the Mutated Gene and Preparation of the Native and Mutant Sfamy Enzymes*. An *Eco*RI–*Pst*I DNA fragment (2.5 kb) containing an Sfamy gene was isolated from the plasmid pSfα1 (Yamashita et al., 1985; Itoh et al., 1987) and subcloned to the M13 phage vector. Replacement of Y83 with phenylalanine (F), tryptophan (W), leucine (L), asparagine (N), and arginine (R) was carried out using synthetic oligonucleotides, as previously reported (Kunkel, 1985). The native, Y83F, Y83W, Y83L, Y83N, and Y83R Sfamy genes were inserted into the multicloning site of the vector YEp351 (Hill et al., 1986), and the formed plasmids were used to transform *Saccharomyces cerevisiae* KK4 cells (α, ura3, his1/3, trp1, leu2, gal80), as previously reported (Hinnen et al., 1978). The W84F and W84Y Sfamy genes were prepared and treated in a similar manner.

The transformant cells were cultured in YPD medium (1% yeast extract, 2% bactopeptone, and 2% glucose), at 30 °C for 6 days. The native and mutant enzymes were purified from the culture supernatants by ion-exchange chromatography on DE-52 cellulose (Whatman) (a linear gradient elution with 0–1.0 M sodium chloride solution), hydrophobic chromatography on butyl-Toyopearl 650S (a linear gradient elution with 40–0% saturated ammonium sulfate solution), and gel filtration on Superose 12, as described previously (Matsui et al., 1992a).

The purified native and mutant enzymes were electrophoresed in duplicate on a sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE), as previously reported (Laemmli, 1970). One gel was stained by Coomassie Brilliant Blue (Bio-Rad) and another was used for Western immunoblotting. Western blotting was performed with a blotting detection kit (Amersham). The blotted membrane was blocked by treatment with 10 mL of TBS-T buffer (20 mM Tris, 137 mM NaCl, and 0.1% Tween 20, pH 7.5) containing 0.5 g of dried milk at room temperature for 1 h. The blocked membrane was incubated in 10 mL of TBS buffer (20 mM Tris/137 mM NaCl, pH 7.5) containing 50 μg of mouse anti-Sfamy antibody at room temperature for 1 h. The membrane was then washed with TBS-T buffer and incubated in 10 mL of TBS buffer containing 20 μg of a biotinylated anti-mouse antibody and a streptavidin–alkaline phosphatase conjugate at room temperature for 30 min. The membrane was rewashed with TBS-T buffer and then incubated at room temperature for 15–30 min in 10 mL of 100 mM diethanolamine buffer containing a dye, nitro blue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate, a substrate for the alkaline phosphatase. The colored membrane was washed with water and dried on filter paper.

(*f*) *Analysis of the Products from Maltoheptose (G_7) and p-Nitrophenyl α-D-Maltopentoside (G_5-PNP)*. The activitis of native, Y83F, Y83W, Y83L, Y83N, W84F, and W84Y enzymes for the maltooligosaccharide substrate (G_7) were examined by analyzing their reaction products using paper chromatography. The enzyme reaction was carried out in a solution (0.4 mL) containing the substrate and the enzyme in 50 mM acetate buffer (pH 5.5) at 30 °C. The concentrations of the native, Y83F, Y83W, Y83L, Y83N, W84F, and W84Y enzymes were 4×10^{-8}, 2×10^{-7}, 1×10^{-7}, 8×10^{-7}, 8×10^{-7}, 1×10^{-7}, and 1×10^{-7} M, respectively, and that of the G_7 substrate was 1.6×10^{-3} M. The native and Y83W enzyme reactions were also performed in solutions with a 10-fold higher concentration of G_7. Aliquots (0.1 mL) of the reaction mixtures were taken out at appropriate time intervals, and the reactions were stopped with 30 μL of glacial acetic acid. The mixtures were boiled for 5 min and concentrated. The residues were dissolved in 5 μL of water, and 2.5 μL of each solution was spotted on 3MM paper (Whatman). The spotted papers were developed three times by an ascending method with a solvent system using ethyl acetate:methanol:water = 37:40:23 (v/v) at 55 °C. The developed papers were treated with glucoamylase (Seikagaku Kogyo) according to the method of Kainuma and French (1969) and colorized with $AgNO_3$ (Trevelyan et al., 1950). The relative amounts (%) of G_7 and the products were determined from the densities of the colored spots on the paper chromatogram, as measured by a chromatoscanner (type CS-930, Shimadzu).

The activities of native and mutant enzymes for a maltooligosaccharide derivative, *p*-nitrophenyl α-D-maltopentoside

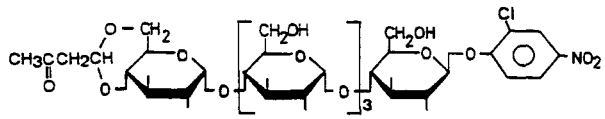

FIGURE 1: Structure of 3-oxobutylidene β-2-chloro-4-nitrophenyl maltopentoside (3KB-G5-CNP).

(G_5-PNP), were examined by analyzing their reaction products with HPLC. The enzyme reaction was carried out in a solution (40 μL) containing the enzyme and G_5-PNP in 50 mM acetate buffer (pH 5.25) at 37 °C. The substrate concentration was 7×10^{-3} M, and the concentrations of the native, Y83F, Y83W, Y83L, and Y83N enzymes were 1.4×10^{-7}, 2.7×10^{-7}, 2.7×10^{-7}, 2.7×10^{-6}, and 2.7×10^{-6} M, respectively. Aliquots (12 μL) of the reaction mixtures were taken out at appropriate time intervals, and their reactions were stopped by adding 6 μL of glacial acetic acid. Reactants and products were analyzed on a high-performance liquid chromatograph (LC-6A, Shimadzu) equipped with a TSK-GEL oligo-PW column (Toso, 7.8 × 300 mm) by elution with distilled water at a flow rate of 1 mL/min (Ishikawa & Hirata, 1989). Sugars and *p*-nitrophenyl compounds were monitored with a differential refractometer and an ultraviolet detector at 313 nm, respectively. The standard solution containing 1% of G_1, G_2, G_3, G_5, G_2-PNP, G_3-PNP, G_4-PNP, and G_5-PNP in 50 mM acetate buffer (pH 5.25) was used to identify and calibrate the reaction products separated with the column.

(*g*) *Measurement of the Kinetic Parameters for the Hydrolysis of 3-Oxobutylidene β-2-Chloro-4-Nitrophenyl Maltopentoside (3KB-G5-CNP).* The substance 3KB-G_5-CNP cannot be a substrate for a transglycosylation reaction since both ends of the oligosaccharide are blocked by modifiers, as illustrated in Figure 1. The hydrolysis of 3KB-G_5-CNP was carried out at pH 7.0 and 37 °C, and the products were colorized with an assay kit (Diacolor, Ono Pharmaceutical Co.) (Makise, 1984) and quantified by the absorbance at 400 nm using a spectrometer (UV-260, Shimadzu). The initial velocities (v) were obtained directly from the initial slopes of the time course plots of the reaction. The k_{cat} and K_m values for the substrate were calculated from the plots of v against the substrate concentrations using the Michaelis–Menten equation and the least-squares method (Sakada & Hiromi, 1976).

RESULTS

(*a*) *Energy Minimization of the Three-Dimensional Structure of Takaamylase A (TAA) and Molecular Modeling of Sfamy.* The energy minimization calculation of the X-ray structure of TAA was carried out using an Amber united atom force field for a refined structure of the preference protein. The calculation was continued until the average gradient reached 0.6 kcal/mol/Å, indicating the refined structure.

Figure 2 shows the target Sfamy and reference TAA sequences, their hydrophobic core regions, and the insertion/deletion places in the Sfamy sequence based on the TAA sequence. Five deletion places were identified in the Sfamy sequence at positions 401–403, 417, 448–449, 467–468, and 477–479, and only one insertion place was observed at the sixth position. Eight cysteine residues relevant to the four cystine bridges were all completely conserved at positions 31, 39, 151, 165, 241, 384, 440, and 475 and contributed to the hydrophobic core formation. The whole main-chain structure of Sfamy was constructed by replacing the insertion/deletion regions of TAA with adequate partial structures searched by a computer from PDB. The five partial structures of TAA

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     Part        1
(   1- 50) ....+....*....+....*....+....*....+....*....+....*....+....*
   T.P   : ETNADKWRSQSIYQIVTDRFARTDGDTSASCNTEDRLYCGGSFQGIIKKL
           : :: ::::::::  ::::::::: : : ::: :  :::: :::: ::
   R.P   : ATPAD-WRSQSIYFLLTDRFARTDGSTTATCNTADQKYCGGTWQGIIDKL
   H-CORE        +    ++++++ +          +       +  +  ++  +

     Part
(  51- 100) ....+....*....+....*....+....*....+....*....+....*....+....*
   T.P   : DYIKDMGFTAIWISPVVENIPDNTAYGYAYHGYWMKNIYKINENFGTADD
           :::  :::::::: ::    :  ::: :: :::   ::  ::: :::::
   R.P   : DYIQGMGFTAIWITPVTAQLPQDCAYGDAYTGYWQTDIYSLNENYGTADD
   H-CORE  ++  + + +++++ +   ++  +         +   ++ +   +  +

     Part
( 101- 150) ....+....*....+....*....+....*....+....*....+....*....+....*
   T.P   : LKSLAQELHDRDMLLMVDIVTNHYGSDGSGDSIDYSEYTPFNDQKYFHNY
           :: :   :: : : :::: : :: : :: : : :::   ::  : :::
   R.P   : LKALSSALHERGMYLMVDVVANHMGYDGAGSSVDYSVFKPFSSQDYFHPF
   H-CORE  + +   +    +++++ + +  + +      + +  + ++    ++  +

     Part
( 151- 200) ....+....*....+....*....+....*....+....*....+....*....+....*
   T.P   : CLISNYDDQAQVQSCWEGDSSVALPDLRTEDSDVASVFNSWVKDFVGNYS
           : : :: :: ::  :: ::  : :::: :   :      ::   : :::
   R.P   : CFIQNYEDQTQVEDCWLGDNTVSLPDLDTTKDVVKNEWYDWVGSLVSNYS
   H-CORE  +++        +  +++    + ++ +      +   +  ++  ++  +

     Part
( 201- 250) ....+....*....+....*....+....*....+....*....+....*....+....*
   T.P   : IDGLRIDSAKHVDQGFFPDFVSASGVYSVGEVFQGDPAYTCPYQNYIPGV
           :::::::  :::   : :   : :::  :::  ::::::::::::   ::
   R.P   : IDGLRIDTVKHVQKDFWPGYNKAAGVYCIGEVLDGDPAYTCPYQNVMDGV
   H-CORE  +  + + ++  +   +++ +   + ++++  ++       +    ++  +

     Part
( 251- 300) ....+....*....+....*....+....*....+....*....+....*....+....*
   T.P   : SNYPLYYPTTRFFKTTDSSSSELTQMISSVASSCSDPTLLTNFVENHDNE
           ::: :::    :: :  :   :  ::  : : : : :::  :::::::
   R.P   : LNYPIYYPLLNAFKSTSGSMDDLYNMINTVKSDCPDSTLLGTFVENHDNP
   H-CORE  + ++++  ++ ++      +  +  ++  +   +   + + +++

     Part
( 301- 350) ....+....*....+....*....+....*....+....*....+....*....+....*
   T.P   : RFASMTSDQSLISNAIAFVLLGDGIPVIYYGQEQGLSGKSDTNNREALWL
           :::: : :  :  :  ::  : :: : :: ::::   :  :  :::: ::
   R.P   : RFASYTNDIALAKNVAAFIILNDGLPIIYAGQEQHYAGGNDPANREATWL
   H-CORE   +  ++  + ++  +  ++++   ++++++     +          ++++

     Part
( 351- 400) ....+....*....+....*....+....*....+....*....+....*....+....*
   T.P   : SGYNKESDYYKLIAKANAARNAAVYQDSSYATSQLSVIFSNDHVIATKRG
           :::   :  ::::: ::: :: :  :   :     :          :
   R.P   : SGYPTDSELYKLIASANAIRNYAISKDTGFVTYKNPYIKDDTTIAMRKGT
   H-CORE    +     ++ ++     +  + +   + ++     ++     +++

     Part    2              3                              4
( 401- 450) ....+....*....+....*....+....*....+....*....+....*....+....*
   T.P   : ---SVVSVFNNLGSSG-SSDVTISNTGYSSGEDLVEVLTCSTVSGSS--D
                :   : : :: :     :   :  :  : ::  : ::   :
   R.P   : DGSQIVTILSNKGASGDSYTLSLSGASYTAGQQLTEVIGCTTVTVGSDGN
   H-CORE      +++++    +    + + +  + +     +  ++ +  + +

     Part                  5        6
( 451- 479) ....+....*....+....*....+....*....+....*....+....*....+....*
   T.P   : LQVSIQGGQPQIFVPA--KYASDICS---
             :   :: :   :      : :::
   R.P   : VPVPMAGGLPRVLYPTEKLAGSKICSDSS
   H-CORE  + + +    + +++    +    ++
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FIGURE 2: Sequence alignment of the target (Sfamy) and reference (TAA) proteins, taking into account the hydrophobic core regions. T.P., R.P., and H-core designate the target and reference proteins and the hydrophobic core, respectively. The amino acid residues composing the hydrophobic core of TAA are indicated by a + at the H-core line. Amino acids identical between Sfamy and TAA are marked by a colon. Gaps are denoted by –.

corresponding to the partial sequences of positions 1–9, 396–407, 412–422, 444–452, and 463–473, which are indicated as

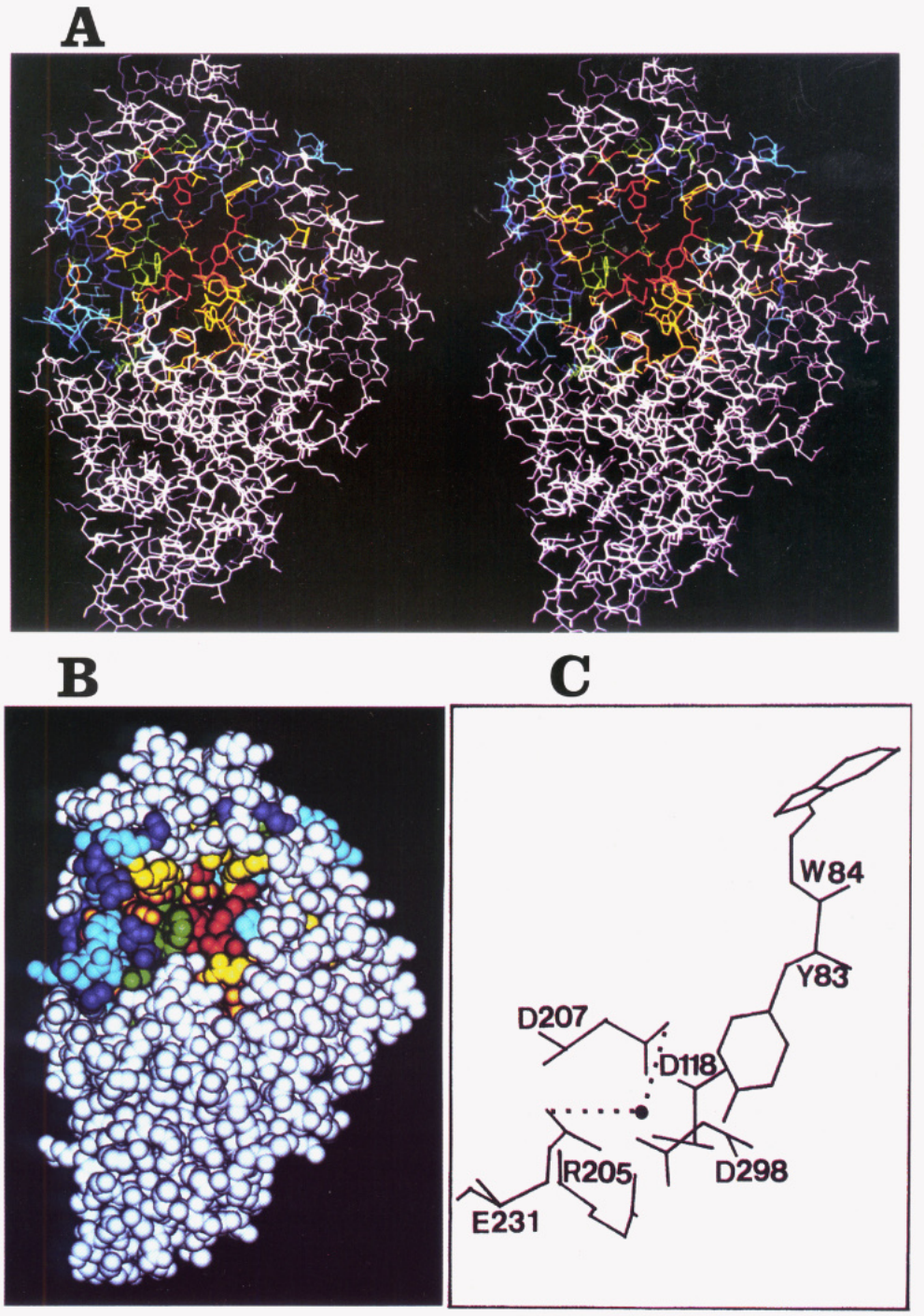

FIGURE 3: Molecular model of Sfamy, where the active amino acid residues conserved in 10 different α-amylases are displayed with distinctive colors. (A) Stereoview of the predicted three-dimensional (3D) structure of Sfamy, shown as a wire model. (B) The same 3D model of Sfamy shown as a space-filling model. (C) Detailed view of the six red residues (Y83, D118, R205, D207, E231, and D298), which are the most conserved and localized in the center of the active site, and W84, the yellow residue next to Y83. A black point and dotted lines denote the position of a water molecule trapped between the two possible catalytic residues, D207 and E231, and the hydrogen bonding between the water molecule and the residues, respectively, which was estimated from the steric structure of the catalytic center of cyclomaltodextrin glucanotransferase (CGTase), an α-amylase family enzyme (Klein et al., 1992).

parts 1–5 in Figure 2, were replaced by those derived from the following PDB file proteins, respectively: 151–159 of 1CTS, 422–430 of 1F19, 647–656 of 4RHV, 388–394 of 2TAA, and 207–217 of 3PGK. All side chains were generated on the constructed main-chain structure of Sfamy, followed by a random search to remove steric hindrance among the side chains. The constructed structure was then optimized by an energy minimization procedure. The minimized molecular energy of the Sfamy structure finally obtained was −2233.9 kcal/mol, indicating no steric hindrance. Figure 3 shows the predicted structure of Sfamy.

The Sfamy molecule (Figure 3) is composed of main (M) and carboxy-terminal (C) domains. The M and C domains correspond to the sequence regions of positions 1–383 and 384–479 in Figure 2 and are folded into an $(\alpha/\beta)_8$ barrel structure and an eight-stranded antiparallel β-sandwich structure, respectively. Since the M domain alignments of Sfamy and TAA are identical, except for one amino acid insertion at the sixth position of Sfamy, their main-chain structures are well conserved to less than 1 Å of the relative atomic positions. The C domains showed only 28% homology between the Sfamy and TAA sequences, whereas the M

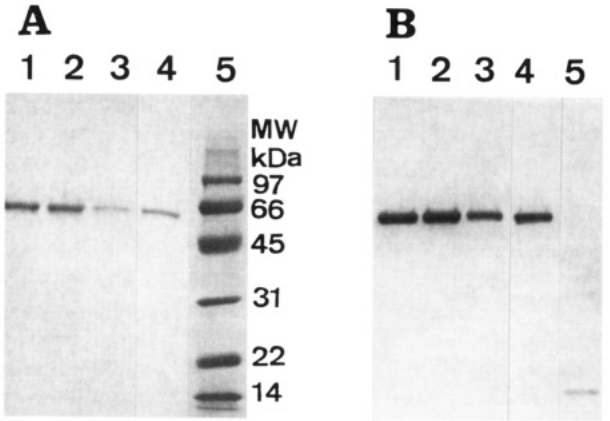

FIGURE 4: SDS–PAGE and Western analysis of the native, Y83W, Y83L, and Y83N Sfamy enzymes. The enzyme solutions (5 μL) mixed with 5 μL of SDS buffer solution were boiled for 5 min. The sample solutions were mixed with 4 μL of a marker dye solution and charged to duplicated slab gels. After electrophoresis, one gel was stained with Coomassie Brilliant Blue R-250 and another gel was used for Western immunoblotting analysis. (A) SDS–PAGE of the purified enzymes: lanes 1–4 are the Y83N, Y83L, Y83W, and native enzymes, respectively, and lane 5 is molecular weight markers. (B) Western immunoblotting patterns: lanes 1, 2, 3, 4, and 5 correspond to those in A.

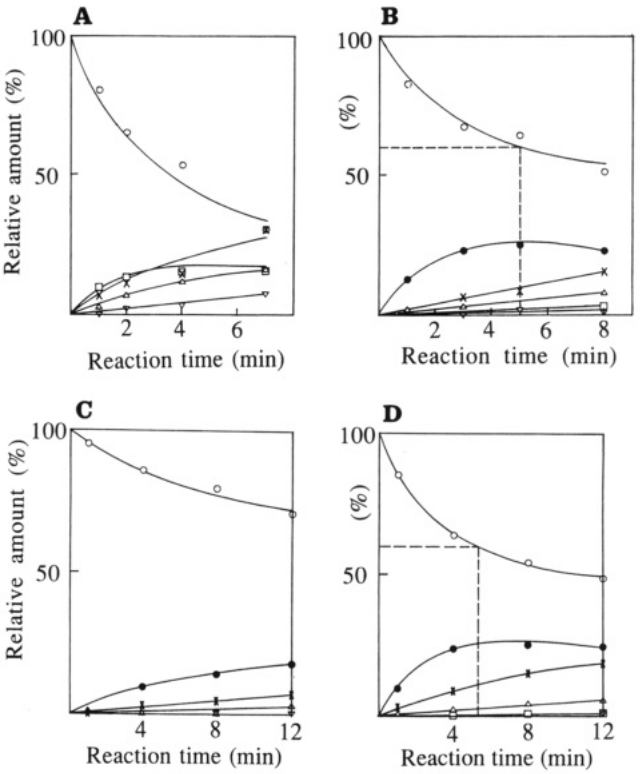

FIGURE 5: Time course plots of the native and mutant enzyme reactions for the substrate G_7. The initial concentration of the substrate G_7 was 1.6×10^{-3} M. The other reaction conditions are described in the text. A–D show the time course plots of the native, Y83W, Y83L, and Y83N enzyme reactions, respectively: ●, $G_{\geq 10}$; ○, G_7; □, G_5; △, G_4; ×, G_3; ▽, G_2. The dashed lines represent the reaction times at the 40% digestion point of starting G_7.

domains showed 58% homology. However, two cysteine residues forming a disulfide bond were conserved between Sfamy and TAA at the same positions in their C domains. Although the Sfamy C domain had four deletions at turn regions that join eight antiparallel β-sheets and one deletion at the C-terminal α-helix, the lengths and relative positions of the β-sheets, which determine the whole structure of the C domain, were well conserved between the two enzymes in spite of low sequence homology.

(*b*) *Evolutionally Invariant Amino Acids in the Active Site of Sfamy*. The active site amino acids of Sfamy are displayed in Figure 3A with warm to cold colors in the order of conservation in the 10 different α-amylases examined. Nine red residues, tyrosine 83 (Y83), glycine 96 (G96), aspartate 118 (D118), histidine 123 (H123), D176, arginine 205 (R205), D207, glutamate 231 (E231), and D298, were completely conserved in all of the α-amylases. Among them, Y83, D118, and R205 were located in the vicinity of the supposed catalytic residues, D207, E231, and D298, which correspond to D206, E230, and D297 of TAA, respectively (Matsuura et al., 1991). The Y83 residue was especially close to these proposed catalytic residues. A space-filling model illustration (Figure 3B) indicates that the above six red residues are located in the center of the active site. Highly and moderately conserved residues, colored with orange and yellow, respectively, were observed around the red active center. The steric structure of the active center composed of these red invariant amino acids was presumed to be essential for the hydrolysis function of α-amylases.

(*c*) *Production and Purification of the Native and Mutant Sfamy Enzymes*. The mutant enzymes at the Y83 and W84 positions were secreted into the medium and were purified completely, as was the native enzyme. Figure 4A,B shows the SDS–PAGE and the Western blotting results of the native and Y83 mutant enzymes, respectively, indicating they were purified into a single band on the SDS gel and were immunologically indistinguishable. Similarly, the purity and antigenicity of the W84 mutant enzymes were also confirmed.

(*d*) *Analysis of the Transglycosylation Products in the Native and Mutant Enzyme Reactions for the Substrate G_7 and G_5-PNP*. Reactivity features of the mutant enzymes were examined on the substrate G_7 by paper chromatographic analysis of the products. The enzyme reactions were carried out in solutions containing 1.6×10^{-3} M G_7. As shown in Figure 5, the time course plots of the Y83W, Y83L, and Y83N enzyme reactions indicated that the major products were maltooligosaccharides larger than maltodecose (G_{10}), due to transglycosylation, and that the hydrolysis products smaller than maltopentose (G_5), G_3, G_4, and G_5, which were major products in the native enzyme reaction, were minor components in these mutant enzyme reactions. The Y83W and Y83N enzyme reactions gave the transglycosylation products $G_{\geq 10}$ which amounted to 63% and 64% of the total product, respectively, at the 40% digestion point of G_7, whereas the native enzyme produced almost no $G_{\geq 10}$. Similar results were obtained when the substrate G_7 concentration was increased to 1.6×10^{-2} M; the Y83W enzyme produced $G_{\geq 10}$ which amounted to 59% of the total product at the 40% digestion point of G_7, but the native enzyme produced trace amounts of $G_{\geq 10}$. The transglycosylation products from these mutant enzyme reactions were analyzed by mass spectrometry and identified to be G_{10}, G_{11}, and G_{12} (I. Matsui, unpublished data). The reactivity features of the other mutant enzymes, Y83F, W84F, and W84Y, were similar to the reactivity feature of the native enzyme, and hydrolysis predominated over transglycosylation.

The products from *p*-nitrophenyl α-D-maltopentoside (G_5-PNP) in the native and mutant enzyme reactions were analyzed by HPLC to examine the effect of blockage in the reducing-end reactivity of the substrate. Figure 6 shows the elution profiles of the native and Y83W enzyme reaction mixtures at the 48% and 44% digestion points of the substrate, respectively. In the Y83W enzyme reaction, *p*-nitrophenyl α-D-maltooc-

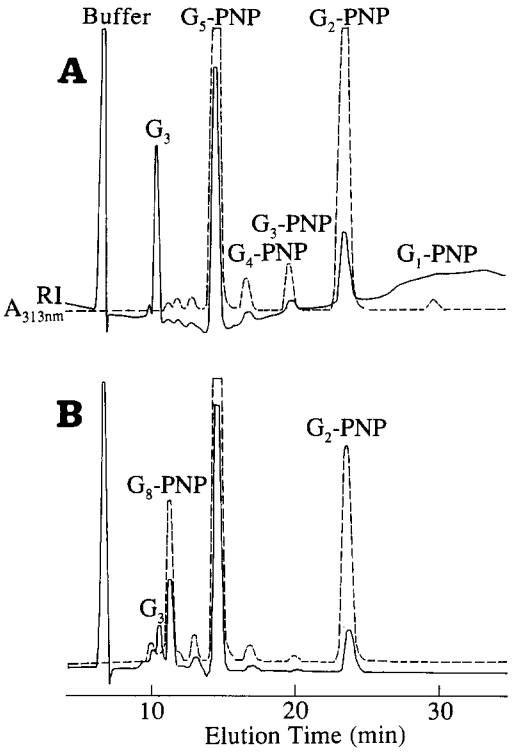

FIGURE 6: HPLC analysis of the products from G_5-PNP in the native and Y83W enzyme reactions. (A) Product distribution at the 48% digestion point of G_5-PNP in the native enzyme reaction. (B) Product distribution at the 44% digestion point of G_5-PNP in the Y83W enzyme reaction. RI (—) and A_{313nm} (- - -) denote refractive index and ultraviolet spectrometries, respectively.

Table 1: Comparison of the Transglycosylation Activity and the Kinetic Parameters in the Native and Mutant Enzyme Reactions

	K_m[a] ($\times 10^{-3}$ M)	k_{cat}[a] ($\times 10^3$ min^{-1})	k_{cat}/K_m[a] ($\times 10^7$ min^{-1} M^{-1})	transglycosylation activity[b]
native	0.087	1.59 (100%)	1.83 (100%)	–
Y83F	0.123	0.44 (28)	0.36 (20)	–
Y83W	0.150	0.22 (14)	0.15 (8)	+
Y83L	0.120	0.02 (1)	0.02 (1)	+
Y83N	0.190	0.03 (2)	0.02 (1)	+
W84F	0.035	0.24 (15)	0.69 (38)	–
W84Y	0.120	0.41 (26)	0.34 (19)	–
W84L	0.072	0.43 (27)	0.61 (34)	+

[a] These kinetic parameters were determined for the substrate 3KB-G_5-CNP. [b] The transglycosylation activity was evaluated by the amount of $G_{\geq 10}$ produced from G_7.

toside (G_8-PNP) was one of the major products and amounted to 40% of the total product, while the hydrolysis products, G_3 and G_2-PNP, were reduced to 26% and 77%, respectively, of those in the native enzyme reaction where the hydrolysis products were major and the transglycosylation products were very minor. The Y83L and Y83N enzyme reactions also afforded G_8-PNP as one of the major products. On the other hand, Y83F, W84F, and W84Y showed the same reaction profiles as the native.

(*e*) *Kinetic Parameters for the Hydrolysis Activities of the Native and Mutant Enzymes and Evaluation of the Transglycosylation Activities.* Table 1 summarizes the k_{cat} and K_m values for the hydrolysis activities of the native and mutant enzymes determined using a hydrolysis-specific substrate, 3KB-G_5-CNP. Also shown are the transglycosylation activities of the mutants evaluated by comparison with the activity of the native enzyme.

The k_{cat} values of the mutants at Y83 were much more dependent on the substituents than were the K_m values. Replacement of Y83 by nonaromatic residues caused a more drastic decrease in the k_{cat}/K_m value than replacement by aromatic residues; the k_{cat}/K_m values of Y83L and Y83N were 1% of that of the native enzyme, whereas those of the Y83F and Y83W enzymes were 20% and 8%, respectively. All of these mutant enzymes, except Y83F, showed enhancement in transglycosylation activity.

By contrast, the k_{cat}/K_m values of the mutants at W84 were almost independent of the substituents, although the values were relatively smaller than the value for the native enzyme. The hydrolysis activities of W84F, W84Y, and W84L were 38%, 19%, and 34% of the activity of the native enzyme, respectively, and only the W84L enzyme showed increased transglycosylation activity, as reported previously (Matsui et al., 1991b).

DISCUSSION

While the 3D structures of several α-amylases have been elucidated by X-ray crystallography, i.e., TAA (Matsuura et al., 1984), porcine pancreatic α-amylase (PAA) (Buisson et al., 1987), and an acid α-amylase from *Aspergillus niger* (Boel et al., 1990), the structure of Sfamy has not been known. We predicted the 3D structure of the Sfamy molecule using a biochemical expert system, BIOCES [E], with TAA as the reference protein. On the basis of the best matched alignment, and given the hydrophobic core scores for the Sfamy and TAA sequences shown in Figure 2, we developed the wire and space-filling models shown in Figure 3. The structures of both the highly homologous M domain and the less homologous C domain resemble those of TAA, although the C domain of Sfamy has a more compact structure due to sequential deletions. This suggests that the tertiary structures of proteins are more highly conserved than the primary structures. The functional importance of the C domain is uncertain, although the characteristics of some α-amylase mutants lacking C domains have been reported (Holm et al., 1990; Imanaka, 1992). Our earlier attempts to produce the C domain deletion mutant of Sfamy were unsuccessful, since the protein was poorly secreted from the transformant cells (I. Matsui, unpublished data). The location at the C-terminal end and the unique shape comprising only β-sheets and turns suggest that the C domain is essential for stable folding of α-amylase.

α-Amylases are widely distributed enzymes found in plants, animals, and microorganisms. Holm et al. (1990) reported a sequence alignment of 10 distantly related α-amylases derived from different sources. Eight sequence regions composing the active sites of these 10 enzymes were identified on the basis of the predicted structure of Sfamy. The amino acid residues forming these regions of Sfamy were scored according to their level of conservation and were indicated in the Sfamy 3D structure by distinctive colors (Figure 3). Six completely conserved residues (red) are located in the center of the active site. Three red residues, Y83, D118, and R205, are located in close proximity to the other three red residues, D207, E231, and D298, which are presumed to be catalytic residues. The Y83 residue is especially close to these catalytic residues.

All α-amylases sequenced so far possess six conserved regions, which probably constitute the active sites of the enzymes (Svensson, 1988). The 84th residue (W84) of Sfamy is located in the first conserved region. Replacement of the residue with a leucine (L) residue greatly increased the transglycosylation activity of Sfamy (Matsui et al., 1991b).

On the other hand, the 83rd residue (Y83) adjacent to W84 was thought to play an important role in hydrolysis because of its strong conservation in all α-amylases examined. Thus, the roles of Y83 and W84 were deduced from the activities of the site-directed mutants of Sfamy at the residues.

The replacement of Y83 with W, L, and N enhanced the transglycosylation activity for substrate G_7 more markedly than did the substitution of W84 with L. The W84F and W84Y enzymes did not show such increases in transglycosylation activity. The results of the Y83W, Y83L, and Y83N enzyme reactions for a reducing-end-blocked substrate, G_5-PNP, the drastic decrease in one of the hydrolysis products, G_3, and the complementary increase in the transglycosylation product, G_8-PNP, strongly suggest that transglycosylation results from the linking of the nonreducing end of the starting substrate (G_5-PNP) and the reducing end of the hydrolysis fragment (G_3). The transglycosylation mechanism that a hydrolysis fragment in the non-reducing-end side and the starting substrate combine, respectively, at their reducing and nonreducing ends is reasonable and it must be applicable to ordinary substrates, including maltooligosaccharides.

The kinetic parameters of the native and mutant enzymes for the hydrolysis-specific substrate, 3KB-G_5-CNP, listed in Table 1 show that the substituent dependence of the K_m value of the mutants at Y83 is not as great as that of the k_{cat} value. This suggests that replacement of the Y83 residue does not cause drastic changes in the binding manner of the substrate. Table 1 also shows that the k_{cat}/K_m values of the Y83 mutants for the nonaromatic substituents are much smaller than those for the aromatic substituents. It is presumed that the aromatic side chain of the 83rd residue is critical for achieving high hydrolytic efficiency. Conversely, the nonaromatic substituent residues, such as L and N, cause an increase in transglycosylation activity. The decreases in the k_{cat} values and the increases in the K_m values for W84 were not as marked, since they are independent of whether the substituents are aromatic or aliphatic. Further, only the W84L enzyme enhanced transglycosylation activity (Matsui et al., 1991b). These results suggest that the Y83 residue contributes more directly to the hydrolytic activity of Sfamy than does the W84 residue.

Mutagenic studies on α-amylases from different origins have shown that three carboxylic acids are critical for catalysis (Holm et al., 1990; Takase et al., 1992; Nagashima et al., 1992). In a proposed hydrolysis mechanism for TAA, the D206, E230, and D297 residues are possible catalytic residues (Matsuura et al., 1991), which correspond to the D207, E231, and D298 residues in the Sfamy molecule. α-Amylase is known to catalyze not only hydrolysis but also transglycosylation and condensation reactions. These α-amylase activities can be understood comprehensively in terms of the reaction mechanism involving a carbonium ion intermediate. This intermediate is formed from the reaction of a substrate fragment in the non-reducing-end side with a catalytic residue (probably E231 in Sfamy) and is stabilized by another catalytic residue, D298. Hydrolysis occurs when the carbonium ion intermediate is nucleophilically attacked by a water molecule close to the catalytic residues, and transglycosylation occurs when it is attacked by an intact substrate molecule. The enhanced transglycosylation activity of the mutant enzymes of Sfamy could occur through such a mechanism. As suggested by the well-ordered water molecule in a cyclomaltodextrin glucanotransferase (CGTase) molecule (Klein et al., 1992) or a cereal lectin–sugar complex (Wright, 1990; Sharon, 1993), the catalytic water molecule in the Sfamy–substrate complex may be trapped between the two possible catalytic residues, D207 and E231 (Figure 3C), and is stabilized by hydrogen bonding with the nucleophilic hydroxyl of the oligosaccharide substrate. Y83 and, presumably, W84 could stabilize the binding of the substrate through nonpolar stacking interactions between their aromatic side chains and the faces of substrate sugars, which were noted as a feature of protein–carbohydrate interactions (Vyas, 1991). The replacement of these residues, especially with nonaromatic residues, might slightly change the geometry of binding of the oligosaccharide substrates to affect the position and stability of the water molecule in the active center, thereby favoring transglycosylation over hydrolysis.

Our search for Y residues in other α-amylase-like enzymes, which correspond to the Y83 in Sfamy, has revealed that they are completely conserved at the same positions in many α-amylase family enzymes (Jespersen et al., 1991). These enzymes include maltotetrahydrolase (Zhou et al., 1989), CGTase (Takano et al., 1986), maltase (Hong & Marmur, 1986), branching enzyme (Baecker et al., 1986), pullulanase (Katsuragi et al., 1987) neopullulanase (Kuriki & Imanaka, 1989), oligo-1,6-glucosidase (Watanabe et al., 1990), isoamylase (Amemura et al., 1988), and β-amylase (Uozumi et al., 1989). Like the Y83 in Sfamy, the Y residues located at the active centers of these enzymes must help to hold sugar substrates and, indirectly, the catalytic water molecules in place in the active centers through stacking interactions. The present results will help to elucidate the mechanism of hydrolysis of many α-amylase family enzymes and may improve these important industrial enzymes.

ACKNOWLEDGMENT

The authors thank Dr. K. Takase of the National Institute of Agrobiological Resources for his helpful advice and Dr. M. Yamaoka of our institute and Dr. K. Ishikawa of the National Institute of Materials and Chemical Research for valuable collaboration on these experiments.

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