Alteration of Bond-Cleavage Pattern in the Hydrolysis Catalyzed by *Saccharomycopsis* α-Amylase Altered by Site-Directed Mutagenesis

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Received December 17, 1991; Revised Manuscript Received March 20, 1992

ABSTRACT: The 210th lysine (K) residue in the *Saccharomycopsis* α-amylase (Sfamy) molecule was replaced by arginine (R) and asparagine (N) residues by site-directed mutagenesis. The influences of the replacements on the bond-cleavage pattern for several substrates were analyzed. Both mutant enzymes, K210R and K210N, cleave mainly the first glycosidic bond from the reducing end of maltotetraose (G_4), while the native enzyme hydrolyzes mainly the second bond from the reducing end. We changed successfully the major cleavage point in the hydrolysis reaction of G_4. The 8th subsite affinities of the K210R and K210N enzymes are calculated to be +2.52 and −0.01 kcal/mol, respectively, whereas that of the native enzyme is +3.32 kcal/mol as reported in the previous paper. These affinity values suggest that the K210 residue composes the 8th subsite, one of major subsites, and that a positively charged amino residue is necessary for the 8th subsite affinity. The K210N enzyme is found to be less active for short substrates like maltotetraose (G_4) than for long substrates like amylose A ($\sim G_{18}$). The reduced catalytic activity specifically for the short substrates is also attributable to the remarkable decrease in the affinity of the 8th subsite.

Polysaccharases including endoglucanase, glucoamylase, and cellobiohydrolase are widely used in various industries such as strach processing, brewing, paper manufacture, and pharmacy. Improvement in the stability, the substrate and product specificities, and the productivity of those enzymes is very important and effective not only in those industries but in many other fields (Yoshizumi & Ashikari, 1989). From the standpoint of the improvement of these industrial enzymes, we undertook the protein engineering study on *Saccharomycopsis fibuligera* α-amylase (Sfamy)[1] and reported its enzymatic characteristics and molecular structure (Matsui et al., 1990), its subsite structure in which the 5th and 8th subsites are major (Matsui et al., 1991a), and the activity of a site-directed mutant at the 84th tryptophan (Matsui et al., 1991b). In this paper, we have reported the preparation of site-directed mutants of Sfamy at the 210th lysine residue (K210), one of the assumed components of the 8th subsite, and the comparison of their hydrolytic activities for maltooligosaccharides with those of the native enzyme. We found that the mutation alters the major cleavage point of the substrates.

Materials and Methods

Construction of the Mutated Gene and Transformation of Saccharomyces cerevisiae. An *Eco*RI–*Pst*I DNA fragment (2.5 kb) containing Sfamy gene was isolated from the plasmid pSfα 1 (Yamashita et al., 1985; Itoh et al., 1987) and subcloned to the M13 phage vector. Replacement of K210 with arginine (R) and asparagine (N) was carried out using oligonucleotides synthesized by the reported procedure (Kunkel, 1985). The native, K210R, and K210N Sfamy genes were inserted into the multicloning site of the vector YEp351 (Hill et al., 1986), and the formed plasmids were designated as pSA5N, pSA5K210R, and pSA5K210N, respectively. *S. cerevisiae* strain KK4 cells (α, ura3, his1/3, trp1, leu2, gal80) were transformed with the plasmids as reported (Hinnen et al., 1978).

Production and Purification of the Native and Mutant Sfamy's. The transformant cells were cultured in the YPD medium (1% yeast extract, 2% bacto-peptone, and 2% glucose) at 30 °C for 6 days. After filtration of the culture medium, 25 g of DE52-cellulose (Whatman) was added to 1 L of the filtrate, and the mixture was stirred for 1.5 h at 4 °C. The adsorbent was collected by decantation, washed 3 times with 1 L of 50 mM acetate buffer (pH 5.2) containing 2.5 mM $CaCl_2$, poured into a column (i.d. 20 × 100 mm), and then eluted with a sodium chloride linear gradient (0–1.0 M) in the same buffer. Amylase activity of the eluates was assayed for soluble starch by the I_2/KI method (Bhella & Altosaar, 1985). The active fractions were collected and 90% saturated with ammonium sulfate. The precipitate was collected by centrifugation and dissolved in 1 mL of the same buffer. After dialysis against the same buffer, the solution was put on a Superose 12 column (i.d. 1.0 × 100 cm, Pharmacia LKB) and eluted with the same buffer. The active fractions (20 mL) were collected and 90% saturated with ammonium sulfate, and the precipitate was dissolved in 1 mL of the same buffer. Then the solution was 40% saturated with ammonium sulfate, charged to a butyl-Toyopearl 650S column (Toso, i.d. 3 × 20 cm), and eluted with an ammonium sulfate linear gradient (40–0% saturation) in the same buffer. The active fractions were collected, dialyzed against the same buffer, and concentrated to 1 mL by ultrafiltration with Centricon 30 (Amicon). The concentrated solution was stored at 4 °C. The purified native and mutant enzymes were electrophoresed in duplicate on a sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) according to the reported method (Laemmli, 1970). Then one gel was stained by a protein-specific dye, Coomassie Brilliant Blue (Bio-Rad), and another gel was used

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[1] Abbreviations: Sfamy, *Saccharomycopsis fibuligera* α-amylase; G_5-PNP, *p*-nitrophenyl α-D-maltopentaoside; 3KB-G_5-CNP, 3-ketobutylic chloronitrophenyl maltopentaose; G_4*, reducing end-labeled maltotetraose.

0006-2960/92/0431-5232$03.00/0

for Western immunoblotting. Western blotting was performed as described in the literature (Burnette, 1981). The blotted membrane was blocked by treating with 20 mL of TBS buffer (20 mM Tris, 500 mM NaCl, pH 7.5) containing 600 mg of gelatin (Bio-Rad) at room temperature for 1 h. The blocked membrane was incubated in 10 mL of TTBS buffer (20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.5) containing 100 μg of the mouse anti-Sfamy antibody and 100 mg of gelatin at room temperature for 2 h. Then the membrane was washed with TTBS buffer for 5 min and incubated in 10 mL of TTBS buffer containing 100 mg of gelatin and 5 μCi of ^{125}I-protein A (Amersham) at room temperature for 1 h. The membrane was rewashed first with 100 mL of TTBS and then with 100 mL of TBS buffer for 5 min and dried on a filter paper. The autoradiogram of the membrane was taken with X-ray film (Fuji) at −80 °C overnight.

HPLC Analysis of the Products from p-Nitrophenyl α-D-Maltopentaoside (G_5-PNP). The enzyme reactions were carried out in the solution (30 μL) containing the enzyme and G_5-PNP in 50 mM acetate buffer (pH 5.25) at 37 °C. The substrate concentration was 6.7×10^{-3} M, and the enzyme concentrations were 1×10^{-7}, 5×10^{-7}, and 5×10^{-7} M for the native, K210R, and K210N enzymes, respectively. Aliquots (10 μL) of the reaction mixtures were taken out at appropriate time intervals, and their reactions were stopped with 3 μL of glacial acetic acid. Reactants and products were analyzed with a high-performance liquid chromatograph LC-6A (Shimazu) equipped with TSK-GEL oligo-PW column (Toso, i.d. 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 differencial 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.

Analysis of the Product from the Reducing End-Labeled Maltotetraose (G_4).* Radioactive analysis was adopted to determine the bond-cleavage pattern and the kinetic parameter (k_{cat}/K_m, where k_{cat} and K_m mean the molecular activity and the Michaelis constant, respectively) for the enzymatic hydrolyses of a pure sugar substrate, maltotetraose, in low concentration. The labeled maltotetraose, G_4*, which has a labeled glucose residue at its reducing end, was prepared according to the reported procedure (Kobayashi et al., 1983). The enzymatic reaction was performed in a solution (420 μL) containing the substrate (G_4*) and the enzyme in 25 mM acetate buffer (pH 5.25) at 25 °C. The concentration of the substrate was 1.9×10^{-5} M, and those of the native, K210R, and K210N enzymes were 1.8×10^{-8}, 4.2×10^{-8}, and 3.6×10^{-7} M, respectively. Aliquots (100 μL) of the reaction mixtures were taken out at appropriate time intervals, and their reactions were stopped with 50 μL of glacial acetic acid. The reaction mixtures were boiled for 5 min and lyophilized. The reaction products were separated by paper chromatography, and their radioactivities were counted with a liquid scintillation counter (Suganuma et al., 1978). All labeled products were identified using authentic labeled maltooligosaccharides.

When the condition $[E]_0 \ll [S] \ll K_m$ is satisfied (where $[E]_0$ and $[S]$ represent the concentrations of the enzyme and substrate, respectively), the enzymatic hydrolysis can be regarded as a first-order reaction with a first-order rate constant (k) equal to $(k_{cat}/K_m)[E]_0$. Thus, the kinetic parameter (k_{cat}/K_m) can be calculated from the slope (k) of a plot of ln ($[S]_0/[S]$) against reaction time (where $[S]_0$ and $[S]$ represent the substrate concentrations at zero time and after a reaction time t, respectively) (Matsui et al., 1991a).

Estimation of the 8th Subsite Affinity of the K210R and K210N Enzymes. On the basis of the subsite theory (Hiromi, 1970), Suganuma et al. devised a simple method to evaluate the subsite affinity by utilizing a series of end-labeled substrates. This method is based on the kinetic parameters (k_{cat}/K_m) and the bond-cleavage patterns for oligomeric substrates determined at sufficiently low substrate concentrations where transglycosylation and condensation can be ignored (Suganuma et al., 1978). According to this method, we have already determined and reported the subsite affinities of the native Sfamy (Matsui et al., 1991a). Assuming that all subsite affinities and k_{int} values (the intrinsic rate constant for the hydrolysis) of the K210R and K210N enzymes were identical with those of the native enzyme except for the 8th subsite affinities, we can estimate the 8th subsite affinities of the mutant enzymes by using the k_{cat}/K_m values and the bond-cleavage patterns for the mutant enzyme hydrolyses of the end-labeled maltotetraose (G_4*) and by those obtained previously for the native enzyme hydrolysis of the end-labeled maltotriose (G_3*) (Matsui et al., 1991a).

Measurement of the Kinetic Parameters for the Hydrolysis of Amylose A and a Maltooligosaccharide Derivative. The enzymatic activities of the native and mutant enzymes for amylose A (MW = ca. 2900, the average degree of polymerization is 18; Nakarai Chemicals) were compared with those for a both end-blocked maltooligosaccharide, 3-ketobutylic chloronitrophenyl maltopentaose (3KB-G_5-CNP). The hydrolysis of amylose A in 50 mM acetate buffer (pH 5.25) at 25 °C was followed by measuring the reducing power of the hydrolysate by the Somogy–Nelson method (Hiromi et al., 1963), and the reaction of 3KB-G_5-CNP at pH 7.0 and 37 °C was followed by measuring an absorbance change at 400 nm by a spectrometer UV-260 (Hitachi) after coloration of the products with a assay kit, Diacolor (Ono Pharmaceutical Co.) (Makise, 1984). 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 both substrates were calculated from the plots of v against the substrate concentrations by using the Michaelis–Menten equation and the least squares method (Sakada & Hiromi, 1976).

Results

Production and Purification of the Native and Mutant Sfamy's. The transformants KK4:pSA5N, KK4:pSA5K210R, and KK4:pSA5K210N were cultured in the YPD medium at 30 °C for 6 days. The native, K210R, and K210N Sfamy genes were expressed using Sfamy's original promoter and terminator, and the products were secreted into the medium. The K210R and K210N enzymes were secreted more abundantly than the native enzyme from *S. cerevisiae* cells. After the purification processes, the isolated native and mutant enzymes each showed a single band at the same molecular weight position on SDS–PAGE as indicated in Figure 1A. Western immunoblotting analysis showed that the mutants enzymes were immunologically indistinguishable from the native enzyme as displayed in Figure 1B.

Determination and Comparison of Bond-Cleavage Patterns of G_5-PNP Based on HPLC Analysis of the Products in the Native, K210R, and K210N Enzyme Reactions. The enzyme reactions were stopped at the 50% digestion point of G_5-PNP, and the reaction products were analyzed with high-performance liquid chromatography (HPLC). From the product analysis, the bond-cleavage patterns were determined, as summarized in Table I. The K210R enzyme cleaves the

Table I: Bond-Cleavage Patterns in the Hydrolysis of G_5-PNP by the K210R, K210N, and Native Enzymes[a]

K210R	G——	G——	G——	G——	G——	PNP
	0.08	0.08	0.43	0.41	0	
K210N	G——	G——	G——	G——	G——	PNP
	0.05	0.03	0.60	0.13	0.19	
Native	G——	G——	G——	G——	G——	PNP
	0.07	0.09	0.80	0.03	0	

[a] The enzyme reactions were stopped at the 50% digestion point of G_5-PNP, and the reaction products were analyzed with HPLC.

Table II: Comparison of the Kinetic Parameters (k_{cat}/K_m) in the Hydrolysis of Reducing End-Labeled G_4 by the K210R, K210N, and Native Enzymes

	k_{cat}/K_m[a] (s^{-1} M^{-1})	$[S]_0$ (M)	$[E]_0$ (M)
K210R	1.4×10^4 (50)	1.9×10^{-5}	4.2×10^{-8}
K210N	2.8×10^2 (1)	1.9×10^{-5}	3.6×10^{-7}
native	2.8×10^4 (100)	1.9×10^{-5}	1.8×10^{-8}

[a] Values in parentheses are in percent (%) and are expressed regarding the k_{cat}/K_m value of the native enzyme as 100%.

Table III: Comparison of the Bond-Cleavage Patterns in the Hydrolysis of Reducing End-Labeled G_4 by the K210R, K210N, and Native Enzymes[a]

K210R	G——	G——	G——	G*
	0	0.407	0.593	
K210N	G——	G——	G——	G*
	0.120	0.266	0.614	
Native	G——	G——	G——	G*
	0	0.745	0.255	

[a] The asterisk (*) represents a radioactive glucose residue.

substrate at two major points; the second and third glycosidic bonds from the reducing end are hydrolyzed with 41% and 43% frequencies, respectively. The K210N enzyme cleaves the substrate at one major and two minor points; the first, second, and third bonds are hydrolyzed with 19%, 13%, and 60% frequencies, respectively. On the other hand, the native enzyme hydrolyzes predominantly the third bond of the substrate with 80% frequency.

Comparison of the Bond-Cleavage Patterns and the Kinetic Parameters (k_{cat}/K_m) in the Hydrolysis of Reducing End-Labeled G_4 by the Native and Mutant Enzymes. The kinetic parameters (k_{cat}/K_m) of the native and mutant enzymes for the labeled maltotetraose, G_4*, determined from their digestion rates in low substrate concentration (19 μM) are summarized in Table II. The K210R and K210N enzymes hydrolyze G_4* 2 and 100 times more slowly than the native enzyme, respectively. The bond-cleavage patterns of the substrate by the native and mutant enzymes are also summarized in Table III. In the native enzyme reaction, end-labeled maltose (G_2*) is produced with 74% frequency, whereas the K210R and K210N enzyme reactions afford end-labeled glucose (G_1*) with 59% and 61% frequencies, respectively. The mutant enzymes cleave mainly the first glycosidic bond from the reducing end of G_4, while the native enzyme hydrolyzes the second bond from the

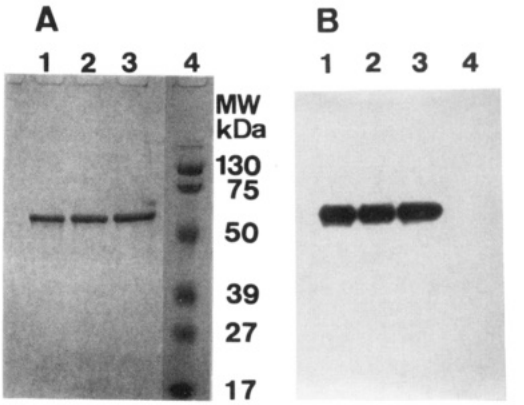

FIGURE 1: SDS–PAGE and Western blotting analysis of the K210R, K210N, and native enzymes. SDS–PAGE was performed on a 10% gel. The enzymes (2.7 μg) were dissolved in 5 μL of water, mixed with 5 μL of SDS buffer solution, and boiled for 5 min. The sample solutions were mixed with 4 μL of marker dye solution 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, 2, and 3 are the K210R, K210N, and native enzymes, respectively, and lane 4 is the molecular weight markers. (B) Western immunoblotting patterns: lanes 1, 2, 3, and 4 correspond to the lanes numbered in panel A.

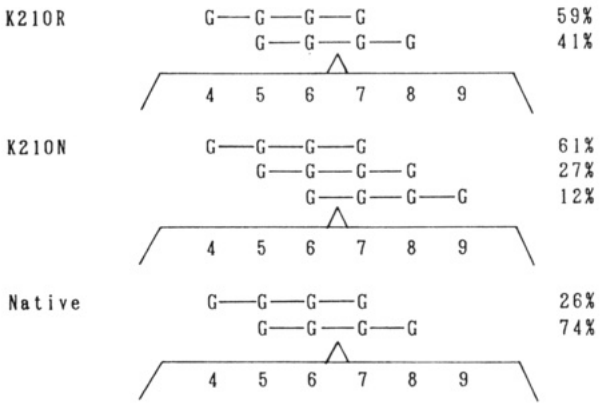

FIGURE 2: Schematic models and their proportions for the predominant productive ES complexes of the K210R, K210N, and native enzymes. The wedges represent the catalytic sites.

reducing end. The K210N enzyme has acquired an activity to the third bond cleavage of the substrate, giving end-labeled maltotriose (G_3*) with 12% frequency. The K210N enzyme shows more diversified cleavage points to the nonreducing end side than those of the native and K210R. On the basis of the bond-cleavage patterns, schematic models for the predominant productive ES complexes of the native, K210R, and K210N enzymes are proposed in Figure 2, where the catalytic site locates between the 6th and 7th subsites.

Kinetic Parameters for the Hydrolysis of Amylose A and 3-Ketobutylic Chloronitrophenyl Maltopentaose (3KB-G_5-CNP) by the Native and Mutant Enzymes. The kinetic parameters for the hydrolysis of amylose A ($\sim G_{18}$) and 3KB-

Table IV: Comparison of the Kinetic Parameters for the Hydrolysis of Amylose A and 3-Ketobutylic Chloronitrophenyl Maltopentaose (3KB-G_5-CNP) by the K210R, K210N, and Native Enzymes

substrate:	amylose A[a]			3KB-G_5-CNP[b]		
enzyme:	K210R	K210N	native	K210R	K210N	native
$[E]_0$ ($\times 10^{-9}$ M)	22.1	22.1	4.8	5.9	14.7	3.4
k_{cat} (s^{-1})	10.6	12.7	82.3	8.5	6.2	27.0
K_m ($\times 10^{-5}$ M)	3.3	11.8	11.1	10.1	37.1	8.7
k_{cat}/K_m[c] ($\times 10^5$ s^{-1} M^{-1})	3.2 (43)	1.1 (15)	7.4 (100)	0.8 (26)	0.2 (7)	3.1 (100)

[a] The hydrolysis reaction was carried out at pH 5.25 and 25 °C. [b] The hydrolysis reaction was carried out at pH 7.0 and 37 °C. [c] Values in parentheses are in percent (%) and are expressed regarding the k_{cat}/K_m value of the native enzyme as 100%.

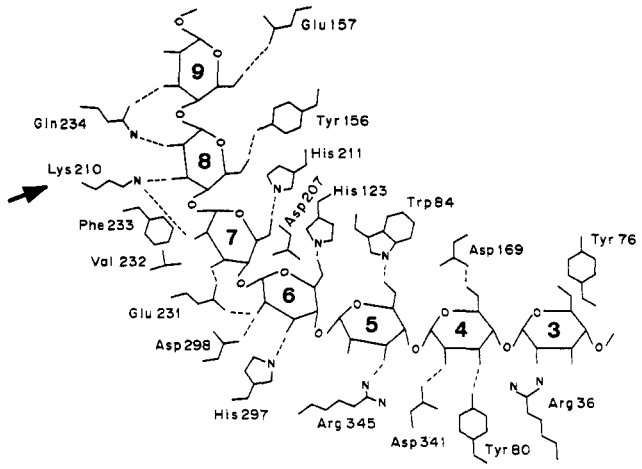

FIGURE 3: Substrate binding model of Sfamy. The arrow represents the 210th lysine residue replaced. The substrate is hydrolyzed between the 6th and 7th glucose residues. The possible catalytic residues are the 207th aspartic acid, 231th glutamic acid, and 298th aspartic acid.

G_5-CNP by the native and mutant enzymes are determined and listed in Table IV. The K210R and K210N enzymes have lower k_{cat} values for the both substrates than the native enzyme; their values for amylose A are 13% and 15% of that of the native enzyme, and those for 3KB-G_5-CNP are 31% and 23%, respectively. The k_{cat}/K_m values of the K210N enzyme for amylose A ($\sim G_{18}$), 3KB-G_5-CNP, and G_4* are 15%, 7%, and 1% of those of the native enzyme, respectively, while the k_{cat}/K_m values of the K210R enzyme are 43%, 26%, and 50%, respectively.

Discussion

In the preceding paper (Matsui et al., 1990), we revealed that Sfamy is structurally composed of main (M) and carboxy-terminal (C) domains whose amino acid sequences have 59% and 36% homologies with those of taka-amylase A (TAA). The steric structure of Sfamy was predicted theoretically with a biochemical expert system, BIOCES [E] (NEC), from the known structure of TAA (Matsuura et al., 1984) by utilizing the homology in their primary sequences. The results indicate that the M domain of Sfamy closely resembles that of TAA, but the C domain appears to be more compact than that of TAA because of deletions at four regions. Then we determined the subsite structure of Sfamy more minutuely than that of TAA by analyzing a wider range of substrate-binding sites; by employing longer oligosaccharide substrates, we could evaluate eleven subsite affinities for Sfamy, whereas nine subsite affinities were reported for TAA (Suganuma et al., 1978). The distribution and the strength of the subsite affinities are similar to those of TAA, being characteristic of the active site of endo cleavage-type enzymes (Hiromi et al., 1973). Furthermore, we proposed a substrate-binding model of Sfamy shown in Figure 3 by modifying that of TAA on the basis of the predicted steric structure and subsite structure of Sfamy. According to the model, the major subsite 8 might be composed of the 156th tyrosine (Y156), 210th lysine (K210), and 234th glutamine residues. Hydrolytic activity changes in point mutants at these residues should give information on their roles in the formation of the 8th subsite affinity. Thus, we constructed the Sfamy mutants altered at K210 and examined the influence of the mutation on the bond-cleavage patterns of various substrates.

p-Nitrophenyl α-D-maltopentaoside (G_5-PNP) was adopted as a substrate, and HPLC analysis of its enzymatic reaction products afforded the bond-cleavage patterns shown in Table I. Major cleavage by the K210R enzyme occurs at the second and third glycosidic bonds from the reducing end of the substrate, and that by the K210N enzyme takes place at the third bond together with minor scission of the second and first bonds, while the native enzyme shows preferential cleavage at the third bond. The mutant enzymes have digestion points diversified and shifted toward the reducing end side of the substrate in comparison with the native enzyme. This must be caused by a significant decrease in the 8th subsite affinities of the mutant enzymes.

The substrate, G_5-PNP, is a convenient substrate for product analysis, but effects due to the complex interaction of its PNP group with the amino acid residues contributing to the subsite affinities cannot be excluded. Therefore, we adopted a simple but labeled substrate, G_4*, to determine the kinetic parameters (k_{cat}/K_m) and the bond-cleavage patterns from which the 8th subsite affinities of the mutant enzymes were estimated. The k_{cat}/K_m values and the bond-cleavage patterns are summarized in Tables II and III. The k_{cat}/K_m values of the native and K210R enzymes for G_4* are 100 and 50 times as large as that of the K210N enzyme, respectively. This suggests the importance of a positively charged residue at the 210th position for the catalytic activity of Sfamy. The K210R and K210N enzymes cleave mainly the first glycosidic bond from the reducing end of G_4*, while the native enzyme hydrolyzes mainly its second bond. The replacement of K210 in Sfamy is found to shift the major cleavage point of the substrate toward the reducing end side by one glucose unit. From the data on the bond-cleavage position and frequency described in Table III, schematic models for the predominant productive ES complexes of the native, K210R, and K210N enzymes are deduced, as shown in Figure 2, where the catalytic site locates between the 6th and 7th subsites. As seen from Figure 2, the 8th subsite affinities of the mutant enzymes toward G_4* are clearly smaller than that of the native enzyme as well as toward G_5-PNP. Then the 8th subsite affinities of the mutant enzymes are estimated according to the method described in Materials and Methods. The 8th subsite affinities of the K210R and K210N enzymes are calculated to be +2.52 and −0.01 kcal/mol, respectively, whereas that of the native enzume is +3.32 kcal/mol as reported in the previous paper (Matsui et al., 1991a). These results suggest that the K210 residue really composes the 8th subsite and its positive charge plays an important role in generating the 8th subsite affinity, which strongly affects the bond-cleavage patterns of substrate. The kinetic parameters of the mutant enzymes for the substrates with different sugar chain lengths are compared with that of the native enzyme to examine the relationship between the subsite structure and the catalytic activity. As shown in Tables II and IV, the k_{cat}/K_m values of the K210N enzyme for amylose A, 3KB-G_5-CNP, and G_4* are 15%, 6.5%, and 1% of those of the native enzyme, respectively. The activity of the K210N decreases significantly with a decrease in the substrate chain length. On the other hand, the K210R does dot exhibit drastic reduction of the activity, since its k_{cat}/K_m values for these substrates are 43%, 26%, and 50% of those of the native enzyme, respectively. The K210N enzyme might be less suitable for the short substrates like G_4* than long substrates like amylose A ($\sim G_{18}$). The difference between the activities of the two mutants especially toward short substrates should be attributable to the remarkable decrease in one of the major subsite affinities of the K210N enzyme.

In a previous paper, we reported that one of the mutants, W84L, where the tryptophan residue located at the 5th subsite is replaced by a leucine residue (L), has a greatly increased

transglycosylation activity in comparison with the native enzyme (Matsui et al., 1991b). Hydrolysis and transglycosylation reactions can be understood comprehensively in terms of the reaction mechanism involving a carbonium ion intermediate (Imoto et al., 1972). These reactions take place when the carbonium ion intermediate is attacked nucleophilically by water and by the intact substrate, respectively. Since the W84L mutation increases transglycosylation activity and the K210R and K210N mutations shift the cleavage position of substrate toward their reducing end side, the doubly mutated enzymes, W84LK210R and W84LK210N, are expected to form the transglycosylation products different in size from those produced by the single-mutant, W84L enzyme. This is very interesting from the standpoint of the improvement of industrial enzymes. The construction of the doubly mutated genes and their expression in yeast cells are now in progress in our laboratory.

References

Bhella, R. S., & Altosaar, I. (1985) *Can. J. Microbiol. 31*, 149–152.

Burnette, W. N. (1981) *Anal. Biochem. 112*, 195–203.

Hill, J. E., Myers, A. M., Koerner, T. J., & Tzagoloff, A. (1986) *Yeast 2*, 163–167.

Hinnen, A., Hicks, J. B., & Fink, G. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1929–1932.

Hiromi, K. (1970) *Biochem. Biophys. Res. Commun. 40*, 1–6.

Hiromi, K., Takahashi, Y., & Ono, S. (1963) *Bull. Chem. Soc. Jpn. 36*, 563–566.

Hiromi, K., Nitta, Y., Numata, C., & Ono, S. (1973) *Biochim. Biophys. Acta 302*, 362–375.

Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C., & Rupley, J. A. (1972) in *The Enzyme* (Boyer, P. D., Ed.) Vol. 7, 665–868, Academic Press, New York.

Ishikawa, K., & Hirata, H. (1989) *Arch. Biochem. Biophys. 272*, 356–363.

Itoh, T., Yamashita, I., & Fukui, S. (1987) *FEBS Lett. 219*, 339–342.

Kobayashi, S., Kainuma, K., & French, D. (1983) *J. Jpn. Soc. Starch Sci. 30*, 62–68.

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488–492.

Laemmli, U. K. (1970) *Nature 227*, 680–682.

Makise, J. (1984) *Med. Technol. 12*, 149–156.

Matsui, I., Matsui, E., Ishikawa, K., Miyairi, S., & Honda, K. (1990) *Agric. Biol. Chem. 54*, 2009–2015.

Matsui, I., Ishikawa, K., Matsui, E., Miyairi, S., Fukui, S., & Honda, K. (1991a) *J. Biochem. 109*, 566–569.

Matsui, I., Ishikawa, K., Miyairi, S., Fukui, S., & Honda, K. (1991b) *Biochim. Biophys. Acta 1077*, 416–419.

Matsuura, Y., Kusunoki, M., Harada, W., & Kakudo, M. (1984) *J. Biochem. 95*, 697–702.

Sakada, M., & Hiromi, K. (1976) *J. Biochem. 80*, 547–555.

Suganuma, T., Matsuno, R., Ohnishi, M., & Hiromi, K. (1978) *J. Biochem. 84*, 293–316.

Yamashita, I., Itoh, T., & Fukui, S. (1985) *Agric. Biol. Chem. 49*, 3089–3091.

Yoshizumi, H., & Ashikari, T. (1989) *TIBTECH. 5*, 277–280.