Cumulative Effect of Amino Acid Replacements Results in Enhanced Thermostability of Potato Type L α -Glucan Phosphorylase

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The thermostability of potato type L α -glucan phosphorylase (EC 2.4.1.1) was enhanced by random and site-directed mutagenesis. We obtained three single-residue mutations—Phe39→Leu (F39L), Asn135→Ser (N135S), and Thr706→Ile (T706I)—by random mutagenesis. Although the wild-type enzyme was completely inactivated, these mutant enzymes retained their activity even after heat treatment at 60°C for 2 h. Combinations of these mutations were introduced by site-directed mutagenesis. The simultaneous mutation of two (F39L/N135S, F39L/T706I, and N135S/T706I) or three (F39L/N135S/T706I) residues further increased the thermostability of the enzyme, indicating that the effect of the replacement of the residues was cumulative. The triple-mutant enzyme, F39L/N135S/T706I, retained 50% of its original activity after heat treatment at 65°C for 20 min. Further analysis indicated that enzymes with a F39L or T706I mutation were resistant to possible proteolytic degradation.

α -Glucan phosphorylase (EC 2.4.1.1) catalyzes the reversible phosphorolysis of α -1,4 glucan and is widely distributed in microorganisms, plants, and animals. All known α -glucan phosphorylases require pyridoxal 5'-phosphate (PLP) for activity and seem to share a similar catalytic mechanism (17). Although all α -glucan phosphorylases belong to a large highly homologous group that includes glycogen phosphorylases from bacteria, yeast, and animals, starch phosphorylase from plants, and maltodextrin phosphorylases of bacteria, these enzymes from distinct origins are known to differ in their substrate preference and their mode of regulation (14, 22).

The wide distribution of α -glucan phosphorylase suggests that this enzyme plays an important role in the cellular metabolism of reserve polysaccharides: starch and glycogen. α -glucan phosphorylase is also potentially useful for the production of glucose 1-phosphate (G-1-P) and for the synthesis of linear amylose and other glucose polymers with various structures (6, 23). To obtain α -glucan phosphorylase suitable for industrial applications, we previously reported the properties and primary structures of thermostable α -glucan phosphorylases from *Bacillus stearothermophilus* (19) and *Thermus aquaticus* (18). In these studies, we found that bacterial α -glucan phosphorylases appeared to have higher K_m values of malto-oligosaccharides such as maltotetraose as the primer for glucan synthesis compared to plant α -glucan phosphorylases. To engineer useful α -glucan phosphorylase for various applications, the potato type L α -glucan phosphorylase gene was subjected to random mutagenesis by error-prone PCR, and thermostable variant enzymes were screened. Based on the results of random mutagenesis, we focused on specific amino acid replacements and obtained double and triple mutants by site-directed mutagenesis. We describe here the enhanced thermostability of potato

type L α -glucan phosphorylase. Possible antiproteolytic degradation of the mutant enzymes is also described.

MATERIALS AND METHODS

Materials. Bacto tryptone and Bacto yeast extract were purchased from Difco Laboratories (Detroit, Mich.). Agar was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Maltotetraose was purchased from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). All other chemicals were purchased from Waco Pure Chemical Industries, Ltd. (Osaka, Japan). The plasmid pPGP1 containing a DNA fragment that encodes the structural gene of potato type L α -glucan phosphorylase was kindly provided by K. Tanizawa (Osaka University).

Construction of the plasmid for potato type L α -glucan phosphorylase. The plasmid pPGP1 contained about 3-kb DNA fragment which covers entire coding the amino acids region of potato type L α -glucan phosphorylase mature protein. The 3-kb DNA fragment was ligated into pUC19 by using its unique SalI and SmaI restriction sites. A DNA fragment that corresponds to the structural gene of potato type L α -glucan phosphorylase was amplified by the PCR using pPGP1 as a template with primer A (5'-TTCGGATCCTCACCTTGAGTGAGAAAA TTCAC-3') and primer B (5'-TTCGGATCCTTTTCATTTCACITCCCCCTC-3'). Primer A and B corresponded to the amino-terminal region of potato type L α -glucan phosphorylase mature protein and the carboxyl-terminal region of the 3-kb DNA fragment of the plasmid pPGP1, respectively, and were designed to introduce new BamHI sites. The amplified fragment was inserted into BamHI sites of pET3d expression vector (Stratagene) to construct a plasmid designated pET-PGP113.

Error-prone PCR for random mutagenesis. A random mutation was introduced into the potato type L α -glucan phosphorylase gene by error-prone PCR, using a Diversify PCR random mutagenesis kit (BD Biosciences Clontech). Error-prone PCR was carried out using plasmid pET-PGP113 (1 ng) as a template and primers A and B (10 μ M each), which allowed us to introduce random mutations throughout the entire α -glucan phosphorylase gene. The reaction conditions were the same as that of the standard method except for containing 320 μ M MnSO₄ and 40 μ M extra dGTP, which was cycled in a thermal cycler for 1 cycle of 94°C for 30 s, 25 cycles of 94°C for 30 s and 68°C for 2 min, and 1 cycle of 68°C for 2 min. These conditions generated an error frequency of approximately one amino acid substitution per gene. The PCR product was digested by BamHI and ligated with BamHI-digested pET3d plasmid to produce a plasmid library.

Screening for mutant clones with thermostable α -glucan phosphorylases. *Escherichia coli* BL21(DE3) was transformed with the mutated plasmid library and then plated on LB agar (1% Bacto tryptone, 0.5% Bacto yeast extract, 1% NaCl, and 1.5% agar [pH 7.4]) plates containing 50 μ g of ampicillin/ml. The plates were incubated at 30°C for 42 h, and the colonies were transferred to nylon membrane filters, Hybond-N⁺ (Amersham Biosciences). The original plates were reincubated overnight at 37°C and then stored at 4°C as master plates. The

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filters were dried and incubated in 20 mM sodium citrate buffer (pH 6.7) at 60°C for 10 min. The filters were placed on 0.7% agar plates containing 100 mM sodium citrate buffer (pH 6.7), 50 mM G-1-P (Sigma-Aldrich), and 0.05% soluble starch and then incubated at 50°C for 2 h. The filters were peeled from the agar plates and stained with iodine solution (0.1% KI, 0.01% I₂, 0.01% HCl). Blue spots on the filter indicated the presence of active α -glucan phosphorylases. Possible thermostable α -glucan phosphorylases that retained activity after heat treatment at 60°C for 10 min were first screened as described above. Colonies corresponding to the blue spots were isolated from the master plates.

Site-directed mutagenesis. α -Glucan phosphorylase genes that contained two or three mutations were constructed by introducing a second or third mutation to genes containing a single mutation by site-directed mutagenesis. Site-directed mutagenesis was carried out by using a QuikChange XL site-directed mutagenesis kit (Stratagene). Mutagenic primers for F39L were 5'-CAAATACCATTGC AGAAGTACACACCTGTATTCTCTCC-3' and 5'-GGAGAGAATACAGGTG TGAGTTCTGCATGGTATTG-3', and those for N135S were 5'-CCAGATG CTGCTCTTGGAAAGTGGGGGTTGGGACGG-3' and 5'-CCGTCCCAAAAC CCCCACCTTCCAAGAGCAGCATCTGG-3'.

Production and purification of wild-type and mutant α -glucan phosphorylases. *E. coli* BL21(DE3) cells carrying each plasmid were grown for several hours in 200 ml of LB medium (1% Bacto tryptone, 0.5% Bacto yeast extract, 1% NaCl [pH 7.4]) containing 50 μ g of ampicillin/ml at 37°C. IPTG (isopropyl- β -D-thiogalactopyranoside; 0.1 mM) and pyridoxine hydrochloride (1 mM) were added to the culture to induce α -glucan phosphorylase gene expression, and cultivation was continued at 22°C for an additional 18 h. The cells were harvested and washed with 20 mM sodium citrate buffer (pH 6.7; buffer A). The cells were then suspended in 10 ml of buffer A and disrupted by sonication (Insonator model 200 M; Kubota Corp., Tokyo, Japan) at 4°C for 10 min. The cell debris was removed by centrifugation (12,000 $\times$ g, 10 min). The supernatant was filtered through a 0.45- μ m-pore-size membrane, loaded onto a Q-Sepharose Fast Flow column (10 by 30 mm; Amersham Biosciences), and washed with buffer A containing 100 mM NaCl. α -Glucan phosphorylase was eluted with buffer A containing 300 mM NaCl. The collected solution was mixed with ammonium sulfate in buffer A to give a final concentration of 1.44 M ammonium sulfate. The solution was loaded onto a Phenyl-TOYOPEARL 650 M column (10 by 30 mm; TOSHO, Co., Ltd., Tokyo, Japan) that had been equilibrated with 1.44 M ammonium sulfate in buffer A. After the column was washed with 0.9 M ammonium sulfate in buffer A, α -glucan phosphorylase was eluted with 0.36 M ammonium sulfate in buffer A. The active fraction was loaded onto a Resource Q column (1 ml; Amersham Biosciences), and α -glucan phosphorylase was eluted with a linear gradient of 100 to 400 mM NaCl. Active fractions were dialyzed against three changes of 4 liters of buffer A at 4°C for a total of 20 h. A dialysis tube (2 ml; pore size, 50 Å; Sanko Junyaku Co., Ltd., Tokyo, Japan) was used.

Assay of enzyme activity. The activity of α -glucan phosphorylase was measured in the direction of glucan synthesis. The production of inorganic phosphate from G-1-P in the presence of glucan primer was measured by the method described by Saheki et al. (16) with some minor modification. A reaction mixture (200 μ l) containing 1% (wt/vol) soluble starch, 12.5 mM G-1-P, and 100 mM sodium acetate buffer (pH 5.5) was incubated at 37°C for 15 min. The reaction was stopped by the addition of 800 μ l of molybdate reagent (15 mM ammonium molybdate, 100 mM zinc acetate [pH 5.0]), and 200 μ l of ascorbic acid reagent (10% [wt/vol]; pH 5.0) was then added to the mixture. This mixture was incubated at 30°C for 15 min, and the absorbance was measured at 850 nm. One unit of α -glucan phosphorylase activity was defined as the amount of enzyme that produced 1 μ mol of phosphate per min under this assay condition.

Tertiary structural model of potato type L α -glucan phosphorylase. A tertiary structural model of potato type L α -glucan phosphorylase was constructed by the Swiss PDB viewer (8), using *E. coli* α -glucan phosphorylase as a template (13). The data were obtained from the protein data bank (RCSB PDB website [http://www.pdb.org/]) (2).

Other procedures. Polyacrylamide gel electrophoresis (native PAGE) was performed on a 5 to 20% gradient gel, and the gel was stained with Coomassie brilliant blue or iodine solution for detecting α -glucan phosphorylase activity. Iodine solution was made from 100 μ l of iodine stock solution (0.26 g of I₂ and 2.6 g of KI in 10 ml of distilled water) mixed with 100 μ l of 1 M HCl and diluted to 26 ml with distilled water. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 5 to 20% gradient gel as described elsewhere (23). Protein concentrations in solution were determined by using the method of Bradford (Bio-Rad, Hercules, Calif.), with bovine gamma globulin as a standard.

The PLP was quantified according to the method of Wada and Snell (20). The circular dichroism (CD) experiments were carried out by using a JASCO J-820 spectropolarimeter (Japan Spectroscopic Co. Ltd., Tokyo, Japan) with a scan

TABLE 1. Specific activity of wild-type and mutants of potato type L α -glucan phosphorylase^a

Enzyme	Sp act (U/mg)
Wild type	20.6
F39L	15.2
N135S	17.3
T706I	23.6
F39L/N135S	14.9
F39L/T706I	20.8
N135S/T706I	17.4
F39L/N135S/T706I	17.3

^a The assays of α -glucan phosphorylase activity were performed in duplicate with less than 5% difference between the two readings.

rate of 10 nm/min at 20°C, and data were collected at 0.2-nm intervals in 0.1-cm quartz cells for the far-UV region (260 to 185 nm). Four spectra were averaged for each sample and corrected for the buffer baseline. The protein solutions were dialyzed against three changes of 10 mM acetate buffer (pH 6.0) at 4°C for a total of 20 h, and sample concentrations were corrected to 0.35 mg/ml.

RESULTS

Screening of α -glucan phosphorylases with enhanced thermal stability. The potato type L α -glucan phosphorylase gene was mutated by error-prone PCR and the mixture of DNA fragments, including various mutated gene, was introduced into the BamHI site of pET3d vector. *E. coli* BL21(DE3) cells were transformed with a mixture of the plasmids and plated onto an agar plate to form 100 to 150 colonies per plate. Nucleotide sequence analysis of the α -glucan phosphorylase genes in the plasmids from several independent colonies indicated that two to three nucleotide substitutions were introduced into each α -glucan phosphorylase gene. Colonies on the plate were transferred to a nylon membrane, subjected to heat treatment, and examined for remaining α -glucan phosphorylase activity. About 25,000 colonies were subjected to this screening, and three positive colonies were obtained. These three colonies were isolated from the master plate, and the plasmids were isolated and fully sequenced. Each α -glucan phosphorylase gene contained a single nucleotide substitution compared to the wild-type enzyme gene. These three mutant genes encoded three variant enzymes with single amino acid substitution: Phe39 to Leu (F39L), Asp135 to Ser (N135S), and Thr706 to Ile (T706I).

Wild-type and the three mutated α -glucan phosphorylase genes were expressed in *E. coli* BL21(DE3), and the variant enzymes were purified. The purified enzymes produced a single clear band on native-PAGE with an apparent molecular mass of 210 kDa (Fig. 4A). The specific activity of the variant enzyme with F39L or N135S was slightly reduced (74 or 84% of wild-type enzyme, respectively), whereas that of the variant enzyme with T706I was 115% of that of the wild-type enzyme (Table 1). To investigate the effect of each mutation on thermal stability, the purified enzymes were incubated at 60°C for 2 h, and their remaining activities were measured (Fig. 1A). Wild-type α -glucan phosphorylase was inactivated very rapidly and lost almost all of its activity after incubation at 60°C for 20 min. However, the three variant enzymes showed significantly increased thermal stability in that they retained more than 50% of its initial activity after incubation at 60°C for 20 min. The contribution of each amino acid substitution to thermal stabil-

FIG. 1. Thermostability of wild-type and mutated α -glucan phosphorylases. The enzymes were incubated at 60°C (A) and 65°C (B) in 20 mM sodium citrate buffer (pH 6.7), and the remaining activity was measured as described in Materials and Methods. The initial activity is denoted as 100%. The assays of α -glucan phosphorylase activity were performed in duplicate with <10% difference between the two readings. Symbols: $\circ$, wild type; $\square$, F39L; $\triangle$, N135S; $\diamond$, T706I; $\blacksquare$, F39L/N135S; $\blacktriangle$, F39L/T706I; $\blacklozenge$, N135S/T706I; $\bullet$, F39L/N135S/T706I.

ity appeared to be in the following order: N135S > T706I > F39L.

Construction of double- and triple-mutant enzymes. It is widely known that the thermostability of an enzyme can be increased by a combination of amino acid replacements that contribute to increase the thermostability of the enzyme (10). α -Glucan phosphorylase genes with two mutations (F39L/N135S, F39L/T706I, and N135S/T706I) and three mutations (F39L/N135S/T706I) were constructed, expressed in *E. coli* BL21(DE3), and purified to homogeneity. The specific activities of these four variant enzymes were not significantly different from those of the wild-type and the variant enzymes with a single amino acid replacement (Table 1). All of the α -glucan phosphorylase variants with two amino acid substitutions showed higher thermal stability than those with only a single

TABLE 2. Kinetic parameters of wild-type and mutant potato type L α -glucan phosphorylases in the synthesis reaction^a

Enzyme	Maltotetraose		G-1-P	
	K_m^b (mM)	K_{cat} (sec ⁻¹)	K_m^c (mM)	K_{cat} (sec ⁻¹)
Wild type	2.11	3.72×10^{-3}	0.57	1.00×10^{-3}
F39L	2.00	1.75×10^{-3}	0.96	2.31×10^{-3}
N135S	1.96	2.04×10^{-3}	0.59	1.93×10^{-3}
T706I	1.87	2.93×10^{-3}	0.33	2.55×10^{-3}
F39L/N135S	2.00	3.56×10^{-3}	0.62	4.21×10^{-3}
F39L/T706I	1.54	2.70×10^{-3}	0.76	4.95×10^{-3}
N135S/T706I	2.47	4.38×10^{-3}	0.56	4.06×10^{-3}
F39L/N135S/T706I	2.57	3.66×10^{-3}	0.48	3.64×10^{-3}

^a Determined at 37°C in 200 mM acetate buffer (pH 5.5). The assays of α -glucan phosphorylase activity were performed in duplicate with less than 5% difference between the two readings. The K_m values are based on a molecular mass of the α -glucan phosphorylase dimer of 210 kDa.

^b Determined at 12.5 mM G-1-P.

^c Determined at 25 mM maltotetraose.

amino acid substitution. The enzymes with two mutations (F39L/N135S, F39L/T706I, and N135S/T706I) retained more than 85% of their initial activity after incubation at 60°C for 60 min (Fig. 1A). The α -glucan phosphorylase variant with all three amino acid substitutions (F39L/N135S/T706I) showed the highest level of thermal stability among all of the other variants constructed. The enzyme with three mutations (F39L/N135S/T706I) retained more than 95% of its activity after incubation at 60°C for 60 min (Fig. 1A). This triple-mutant enzyme retained its enzymatic function after treatment at 65°C for 30 min, whereas all other variants were completely inactivated (Fig. 1B). Together, these results demonstrate that each individual mutation (F39L, N135S, and T706I) cumulatively contributed to enhance the thermostability of α -glucan phosphorylase.

Effect of amino acid replacement(s) on other enzyme properties of α -glucan phosphorylase. To investigate the effect of each amino acid replacement(s) on other properties of α -glucan phosphorylase, the kinetic parameters of variant enzymes were studied and compared to those of the wild-type enzyme. K_m values for maltotetraose and G-1-P and K_{cat} were measured in the glucan synthetic reaction and are summarized in Table 2. The K_m values of all of the variant enzymes examined for either maltotetraose or G-1-P were not significantly different from those of the wild-type, suggesting that these three amino acids (Phe39, Asn135, and Thr706) are not directly involved in substrate recognition. Despite the significant increase in the thermal stability of the variant enzymes, they all showed their highest activity at 40°C, which was exactly the same as the results with the wild-type enzyme (Fig. 2). No differences were found between the variants and wild-type with regard to the optimum pH (Fig. 3A). On the other hand, some variant enzymes appeared to be more stable than wild-type α -glucan phosphorylase under alkaline conditions, as shown in Fig. 3B.

Stability against storage. α -Glucan phosphorylase from potato or sweet potato undergoes proteolytic degradation in vitro (4). Brisson et al. (3) indicated that the proteolysis of potato type L α -glucan phosphorylase occurs in vivo and is not an artifact of the isolation procedure. To investigate the stability of variant enzymes against storage, seven variants and the

FIG. 2. Effect of temperature on activity of wild-type and mutated α -glucan phosphorylases. Enzyme activity was assayed in 0.1 M sodium acetate buffer (pH 5.5) at various temperatures. The highest activity was denoted as 100%. The assays of α -glucan phosphorylase activity were performed in duplicate with less than 2% difference between the two readings. Symbols: ○, wild-type; □, F39L; △, N135S; ◇, T706I; ■, F39L/N135S; ▲, F39L/T706I; ◆, N135S/T706I; ●, F39L/N135S/T706I.

wild-type enzymes were stored at 4°C for 5 months and then subjected to native PAGE and SDS-PAGE. All of the purified enzymes initially showed a single band (210 kDa) on a native gel. However, after storage at 4°C for 5 months, wild-type and N135S mutant enzymes produced a new band with a molecular mass of ca. 150 kDa, while the other variants still showed their original molecular mass (210 kDa), as shown in Fig. 4A. The native gel was then subjected to activity staining to investigate whether or not the degraded polypeptides still retained their activity. As shown in Fig. 4B, the 150-kDa polypeptides showed levels of the α -glucan phosphorylase activity that were similar to that of the 210-kDa polypeptide, implying that the 150-kDa polypeptides were produced by limited degradation of the 210-kDa polypeptide without any loss of activity. The same samples were also subjected to SDS-PAGE to understand their subunit structures (Fig. 4C). Wild-type and N135S enzymes produced two major bands (ca. 45 kDa), whereas all of the other mutants produced a single band with a molecular mass of 105 kDa, which corresponded well to the value for the α -glucan phosphorylase subunit. Similar results were obtained when purified enzymes were incubated at 37°C for 10 days (data not shown). These results indicate that the possible proteolytic degradation of potato type L α -glucan phosphorylase was suppressed by either the F39L or T706I amino acid substitutions but not by N135S.

PLP contents and CD experiments. The PLP content of the wild-type enzyme was 1.1 ± 0.2 mol/subunit of protein. The PLP contents of the various mutants were same as that of the wild-type enzyme. The far UV CD spectra of the wild-type and the various mutant enzymes were very similar (data not shown). When analyzed over the wavelength range 260 to 185 nm by using method of Yang et al. (24), the secondary-structure content of the wild-type enzyme was found to be 19%

FIG. 3. Effect of pH on activity (A) and stability (B) of wild-type and mutated α -glucan phosphorylases. (A) The activity was measured as described in Materials and Methods in 0.1 M sodium acetate (pH 3.5 to 6.0), sodium citrate (pH 5.5 to 6.7), Tris-HCl (pH 7.0 to 9.5), or glycine sodium (pH 9.0–11.0) buffer. The highest activity was denoted as 100%. (B) Enzymes were treated at 37°C for 30 min in 50 mM sodium acetate (pH 3.5 to 6.0), sodium citrate (pH 5.5 to 6.7), Tris-HCl (pH 7.0 to 9.5), or glycine sodium (pH 9.0 to 11.0) buffer, and the remaining activity was measured in 0.1 M sodium acetate buffer (pH 5.5). The initial activity is denoted as 100%. The assays of α -glucan phosphorylase activity were performed in duplicate with less than 2% difference between the two readings. Symbols: ○, wild-type; □, F39L; △, N135S; ◇, T706I; ■, F39L/N135S; ▲, F39L/T706I; ◆, N135S/T706I; ●, F39L/N135S/T706I.

α -helix, 52% β -sheet, 7% β -turn, and 22% random coil. The secondary-structure contents of the various mutants were same as that of the wild-type enzyme.

DISCUSSION

Three amino acid substitutions (F39L, N135S, and T706I) contributed to enhance the thermal stability of potato type L α -glucan phosphorylase. The CD data suggested that the over-

FIG. 4. Native PAGE (A and B) and SDS-PAGE (C) of purified potato type L α -glucan phosphorylases. The protein samples (0.6 μ g) were loaded onto a gel. After electrophoresis, the gel was stained with Coomassie brilliant blue (A and C) and with iodine solution after incubation in 50 mM G-1-P with 0.05% soluble starch and 100 mM acetate buffer (pH 5.5) for 3 h at 40°C (B). Lanes 1a and 1b are the wild-type enzyme samples just after purified and stored at 4°C for 5 months, respectively. Lanes 2, 3, 4, 5, 6, 7, and 8 are the variants with F39L, N135S, T706I, F39L/N135S, F39L/T706I, N135S/T706I, and F39L/N135S/T706I mutations stored at 4°C for 5 months, respectively. Numbers on the left are the estimated molecular masses of marker protein.

all folding of the wild-type and mutant enzymes were very similar in the secondary structure. Since the tertiary structural data of this enzyme have not been reported, it is not possible to identify the positions of these three amino acid residues in the three-dimensional structure of this enzyme. However, structural data are available for α -glucan phosphorylases from rabbit (12) and human muscle (15) and for α -glucan phosphorylase from *E. coli* (13). The alignment of 14 α -glucan phosphorylase sequences from animals, plants, and microorganisms suggested that the amino acid residues involved in substrate or cofactor binding in rabbit muscle α -glucan phosphorylase were all conserved in other α -glucan phosphorylases (9). To obtain structural information about these three amino acids (Phe39, Asn135, and Thr706), a structural model of potato type L α -glucan phosphorylase was constructed with SWISS-PDB viewer (8) using α -glucan phosphorylase from *E. coli* (13) as a template (Fig. 5). α -Glucan phosphorylase from *E. coli* was chosen because this enzyme shows the highest identity (40%) and regulatory properties similar to those of potato type L α -glucan phosphorylase. The activity of α -glucan phosphory-

lases from animals is controlled by allosteric regulation, while α -glucan phosphorylases from higher plants and α -glucan phosphorylase from bacteria are not subjected to such control (5, 21). A model of the dimer structure of potato type L α -glucan phosphorylase is shown in Fig. 5, where the three amino acids that are involved in enhancing the thermal stability (Phe39, Asn135, and Thr706) are shown in red. To visualize the position of the active site of this enzyme, the side chain of 15 amino acid residues involved in PLP binding is shown in green.

Asn135 is completely conserved in the alignment of the 14 α -glucan phosphorylases (9) and corresponds to Asn133 of rabbit muscle α -glucan phosphorylase, which is involved in PLP binding in this enzyme. In the model of potato type L α -glucan phosphorylase, Asn135 is located around the active cleft together with the other amino acid residues colored in green, which suggests that Asn135 is also involved in PLP binding in potato type L α -glucan phosphorylase. We indicated that N135S mutation significantly increased the thermal stability of this enzyme (Fig. 1). Since the PLP contents of the wild-type and the various mutant enzymes were very similar, the N135S mutation did not affect PLP binding as a cofactor. A similar result was also reported in α -glucan phosphorylase from *E. coli*. Asn133 of the enzyme is known to interact with amino acids involved in substrate binding, and the replacement of Asn133 by Ala enhanced the thermal stability of the enzyme (7). Since Asn133 of α -glucan phosphorylase from *E. coli* corresponds to Asn135 in potato type L α -glucan phosphorylase, Asn133 might be a hotspot for enhancing the thermal stability of α -glucan phosphorylases from various sources. Phe39 is also located in a region that is highly conserved among various plant α -glucan phosphorylases. In the model of potato type L α -glucan phosphorylase, Phe39 is in an α -helix that is located at the surface of the α -glucan phosphorylase monomer and interacts with the other subunit in the dimer structure. Phe39 might be involved in the formation of the dimer structure of potato type L α -glucan phosphorylase. A substitution of Phe into Leu, with a smaller side chain, might stabilize the α -helix and thus improve the thermostability. Thr706 is also in the α -helix located at the surface of the α -glucan phosphorylase in this model. A substitution of Thr by a hydrophobic amino acid, Ile, may strengthen the α -helix and provide better thermal stability for the enzyme, as has been reported in other enzymes (1).

Introduction of the F39L or T706I mutation not only affected the thermal stability of potato type L α -glucan phosphorylase but also affected the stability of the enzyme upon storage. It has been reported that type L α -glucan phosphorylase from potato and sweet potato are easily degraded when they are stored. Mori et al. (11) reported that purified potato type L α -glucan phosphorylase was digested within the 78-amino acid insertion sequence (amino acid number 414 to 491) and broken down to several fragments with a molecular mass of ca. 50 kDa. Chen et al. (4) also reported that the presence of these amino acid sequences was recognized by a site-specific protease and that these sequences were destined to participate in the proteolytic process in the insertion sequence in the sweet potato α -glucan phosphorylase that corresponds to the potato insertion sequence, where the intact α -glucan phosphorylase with a molecular mass of 110 kDa was broken down into two

FIG. 5. Ribbon drawing of the tertiary structure model of potato type L α -glucan phosphorylase dimer. Each subunit is shown in a different color (blue and yellow). The model contains only 796 of the 916 residues of potato type L α -glucan phosphorylase mature protein; the deleted 118 residues (residues 1 to 24, 211, 212, 216, 217, 250, 315, 325, 326, 408 to 415, 421 to 454, 457 to 484, 488 to 491, 497 to 503, 583, and 913 to 916) were not mapped onto the structure. The main chain and side chains for the mutated amino acid residues (Phe39, Asn135, and Thr706) are shown in red, and the mutated residues that disappeared are indicated by an arrow (Thr706 of blue subunit). Since residues from 408 to 491 were deleted in this model, the 87 amino acids residues (residues 407 to 492) that included the region of the 78-residue insertion (residues 414 to 491) are shown in dark blue. The side chains of PLP cofactor binding residues, except for the mutated amino acid residue (Asn135), in potato α -glucan phosphorylase are shown in green (Phe92, Gly136, Arg140, Trp568, Val645, Lys646, Arg647, Tyr730, Asn731, Val732, Ala735, Ser759, Asn760, and Lys762).

polypeptides with a molecular mass of ca. 50 kDa. In our experiment, wild-type α -glucan phosphorylase and the N135S variant were also broken down to several smaller polypeptides (data not shown) and finally into two polypeptides with similar molecular masses of ca. 45 kDa (Fig. 4), which indicates that degradation occurred within the insertion sequence, as reported by Mori et al. (11). In the present study, we found that α -glucan phosphorylase with a F39L or T706I mutation was not broken down under the same storage conditions and retained a clear single band with a molecular mass of 105 kDa (Fig. 4, lanes 2 and 4 to 8). These results suggest that the introduction of a F39L or T706I mutation protects potato type L α -glucan phosphorylase from degradation, which is most likely to occur in the insertion sequence. The position of the insertion sequence (amino acids 414 to 491) and its interaction with Phe39 or Thr706 are of great interest. Unfortunately, most of the amino acid residues for the insertion sequence (residues 408 to 415, 421 to 454, 457 to 484, and 488 to 491) were missing in the modeled structure (Fig. 5), since this sequence is only present in plant type L α -glucan phosphorylase and is absent in the *E. coli* α -glucan phosphorylase, which was used as a template to construct this model. However, we could estimate the approximate position of the insertion sequence from the positions of nearby amino acids. In Fig. 5, amino acid residues 407 to 492 are shown in dark blue and indicate that the insertion sequence is located on the surface of this protein. Mori et al. (11) predicted that the 78-residue insertion sequence located on the surface of the molecule has a flexible structure. Therefore, it is possible that Phe39 and Thr706 might be located at an interactive distance to the insertion

sequence in potato type L α -glucan phosphorylase, since the insertion sequence consists of eight times more amino acid residues than the *E. coli* α -glucan phosphorylase.

These results suggest that mutations F39L and T706I probably cause a change in the conformation of potato type L α -glucan phosphorylase that stabilizes the protein from thermal denaturation, as well as proteolytic degradation.

In summary, we successfully enhanced the thermostability of potato type L α -glucan phosphorylase by random and site-directed mutagenesis. Some variants were also stable toward proteolytic degradation, which readily occurred when the wild-type enzyme was placed at 4°C. These enhanced properties may be very important not only for the use of this enzyme for industrial purposes but also for the production and purification of this enzyme on an industrial scale.

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