Pressure-Induced Perturbation on the Active Site of β-Amylase Monitored from the Sulfhydryl Reaction

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ABSTRACT: We investigated the pressure effect on the conformation of β-amylase by monitoring the chemical reaction of the unpaired cysteine. Sweet potato β-amylase is composed of four identical subunits, each of which contains six cysteine residues. These residues are inert to 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in the native state due to steric hindrance. With the increase of the pressure from 0.1 to 400 MPa, the reactivity of one cysteine out of six residues was enhanced. We have identified that the reacted cysteine residue was Cys345 by the chemical cleavage at the reacted site. The reaction kinetics of Cys345 were pseudo-first-order, and the apparent rate constant was increased from 0.001 to 0.05 min^{-1} with the increase of pressure from 100 to 400 MPa. The activation volume of the reaction rate was calculated as -24 ± 2 mL/mol from the slope of the logarithmic plot of the pressure dependence of the rate constant. Hysteresis was not evident in the change of intrinsic fluorescence during the cycle of compression and decompression between 0.1 and 400 MPa, indicating that the tetramer does not dissociate under high pressure. This indicates that the enhancement of the reactivity of Cys345 was caused by the perturbation of local conformation under high pressure. The reaction of Cys345 was also enhanced by low concentrations of GuHCl, suggesting the significant role of hydration-driven fluctuation in the pressure-induced enhancement of the reactivity.

High pressure is a unique intensive parameter for perturbing the native conformations of proteins (*1*, *2*). There have been a number of studies of the pressure effect on protein dynamics by NMR (*3*−*6*), H−D exchange (*7*−*10*), phosphorescence (*11*, *12*), fluorescence (*13*), and MD simulation (*14*, *15*). These studies have shown two opposing effects of pressure on the protein dynamics. The dominant effect of applied pressure is to slow the dynamics as shown using apoazurin (*11*) and BPTI (*3*, *4*), whereas the dynamics of apomyoglobin (*10*), RNase T1, and phosphoglycerate kinase (*11*) are enhanced by an increase in pressure. These results have been explained by the fact that the pressure increase induces a volume reduction of the protein solution by two opposing factors: a decrease in the size of the internal cavities and an increase in the extent of hydration (*11*, *12*). The pressure-induced reduction of the size of the cavities will suppress the fluctuation, whereas progressive hydration under high pressure will enlarge it.

To understand more about the pressure effect on protein conformations, we introduced the chemical reaction of unpaired cysteine as a probe. The reactivity of the unpaired cysteine buried inside the protein is suppressed due to steric hindrance. When the applied pressure increases the level of conformational modulation, it will allow the reagent to react with the cysteine. We used this process to monitor the conformational change of sweet potato β-amylase under high pressure. The enzyme β-amylase hydrolyzes the α-1,4-glucosidic linkage of starch and glycogen with liberation of β-anomeric maltose from the nonreducing ends. This enzyme is distributed in the higher plants, and most of them are monomeric enzymes except sweet potato β-amylase which is composed of four identical 56 kDa subunits. Each subunit of sweet potato β-amylase has six half-cysteines which are inert to the sulfhydryl specific reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB),[1] due to steric hindrance (*16*). We found that the reactivity of Cys345 selectively increased when pressure increased from 0.1 to 400 MPa. The tetrameric structure of sweet potato β-amylase did not dissociate within this pressure range, indicating that the reaction of Cys345 is caused by pressure-induced perturbation of the local region.

EXPERIMENTAL PROCEDURES

Materials

Sweet potato β-amylase, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and 2-nitro-5-thiocyanobenzoic acid (NTCB) were obtained from Sigma Chemical Co. (St. Louis, MO). Sweet potato β-amylase was purified by S-300 gel filtration (Amersham Pharmacia Biotech). Other reagents were of the highest grade commercially available and were used without further purification. The solvent was 10 mM Tris at pH 7.5. Enzymatic activity was assayed as described by Somogyi (*17*).

Methods

Spectroscopic Measurements. Fluorescence spectra at elevated pressures were obtained using a high-pressure cell

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTE, dithioerythritol; DTT, dithiothreitol; GuHCl, guanidine hydrochloride; NTCB, 2-nitro-5-thiocyanobenzoic acid; SEC, size exclusion chromatography; TNB, 2-nitro-5-thiobenzoic acid.

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with three optical windows manufactured by Teramecs Co. (Kyoto, Japan), combined with a Shimadzu RF5000 spectrofluorimeter (Kyoto, Japan). The enzyme concentration was 0.5 μM. The average fluorescence wavelength was calculated as the center of mass of the spectrum as described previously (*10*). The light absorbance at elevated pressures was obtained using a high-pressure cell with two optical windows manufactured by Teramecs Co., combined with a Shimadzu Multispec-1500 spectrometer. The CD spectra were measured using a Jasco J-720 instrument (Tokyo, Japan), and the DLS was measured using an Otsuka DLS-7000 instrument (Osaka, Japan).

Chemical Reaction of the Cysteine. The sulfhydryl group was titrated as described by Ellman (*18*). Protein solutions with excess DTNB were incubated at a specified pressure and at 298 K in the presence of 2 mM EDTA. The absorbance of TNB at 412 nm was monitored at atmospheric pressure. Sweet potato β-amylase was cyanylated by incubating the protein with NTCB. *S*-Cyano protein was cleaved by incubation at pH 9.0 and 310 K for 24 h. The N-terminus of peptides blotted from gels was sequenced utilizing an Applied Biosystems (Foster City, CA) protein sequencer (model 476A) equipped with an on-line analyzer (model 610A) of phenylthiohydrantoin derivatives of amino acids.

HPLC Gel Filtration Measurements. The hydrodynamic radius of sweet potato β-amylase in the presence of GuHCl was evaluated by size exclusion chromatography on a Superdex 200HR column (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) using a Shimadzu SPD-6AD HPLC system.

RESULTS

Pressure Effect on the Reactivity of the Cysteine Residue. Figure 1A shows the ribbon structure of a subunit of sweet potato β-amylase with the positions of six cysteine residues using PDB entry 1FA2 (*19*). The three-dimensional CPK structure models in Figure 1B show that all the cysteine residues except Cys48 are buried inside the molecule. Since none of the cysteine residues of sweet potato β-amylase is reactive to DTNB in the native state, Cys48 is supposed to be at the subunit interface (the tertiary structure of sweet potato β-amylase is unknown). Glu187, which is shown as a red sphere in Figure 1, is a potent candidate for direct involvement in catalysis (*20*). Cys96 and Cys345 are located in the vicinity of Glu187, but are not involved in the catalytic step. The detailed structure of the active site of soybean β-amylase, which shares the same α/β-barrels as sweet potato β-amylase (*21*), has been resolved by examining the crystal structures of the substrate−inhibitor complexes (*22*, *23*). According to these results, Cys96 is located on the flexible loop segment which forms part of the active site (red in Figure 1A), and Cys345 is on the inhibitor binding site.

The effect of pressure on the reactivity of the cysteine residues was investigated by monitoring their reaction to DTNB. Figure 2A shows the time course of the reaction at 400 MPa obtained by in situ measurements of the absorbance of TNB at 412 nm. The logarithmic plot of the kinetics of the reaction (the inset) was approximately linear, indicating that the kinetics are pseudo-first-order. The concentration of the titrated cysteine residue after the incubation at 400 MPa for 2 h was estimated from the absorbance measurement at atmospheric pressure. The calculated molar ratio of reacted cysteine per subunit was 1.0, that is, one cysteine out of the six residues reacted at 400 MPa. The rate constants obtained from logarithmic plots of the time course analyzed by the first-order kinetics increased from 0.001 to 0.05 min^{-1} as the pressure increased from 100 to 400 MPa (Figure 2B). The apparent value of the activation volume ($\Delta V^{\ddagger}$) of the reaction rate of Cys345 was calculated as -24 ± 2 mL/mol from the slope of the logarithmic plot of the pressure dependence of the rate constant in Figure 3.

The cysteine residue that reacted at elevated pressure was identified by chemical cleavage at the reaction site, followed by determination of the molecular mass of the generated peptide fragments. By incubation of sweet potato β-amylase with NTCB at 400 MPa, 1 mol of the cysteine residue per mole of subunit was cyanylated. After the decompression, the N-terminus of the cyanylated cysteines was cleaved by incubation at pH 9.0. Figure 4 shows the electrophoretic mobility of the peptide fragments on SDS−PAGE, along with six theoretical profiles of the peptide fragments generated from cleavage at each of the other cysteine residues. The result in this figure indicates that Cys345 reacted at elevated pressure. The 38 kDa band corresponds to the N-terminal fragment of residues 1−344, and the 17 kDa band is the C-terminal fragment of residues 345−498. Blotting and N-terminal protein sequencing of the 38 kDa fragment consistently gave the Ala-Pro-Iso-Pro sequence, which corresponds to the initial sequence of the N-terminus of sweet potato β-amylase. The N-terminus of the 17 kDa fragment could not be sequenced since the N-terminus of the cyanylated cysteine is cyclized during cleavage.

Stability of the Tetramer under High Pressure. We have investigated the mechanism of the selective enhancement of the reaction of Cys345 under high pressure. It has been shown that high pressure induces dissociation of some oligomeric proteins followed by slow conformational drift of the dissociated monomer (*24*). When the tetramer of sweet potato β-amylase dissociates under high pressure, Cys345 may be exposed to enhance its reactivity by the conformational drift. To examine the stability of the tetrameric conformation, the intrinsic fluoresence of sweet potato β-amylase was measured under high pressure. The pressure-induced dissociation curve of the oligomeric proteins often shows hysteresis since conformational drift of the dissociated monomer accompanies subsequent reassociation into weaker oligomerization. On the other hand, the plot in the circle in Figure 5 shows the reversible 3.5 nm red shift of the average wavelength of the intrinsic fluorescence of sweet potato β-amylase during the compression and decompression cycle in the pressure range of 0.1−400 MPa. The reversibility of the protein structure after the decompression was confirmed also by CD, enzymatic activity, and SEC analysis, suggesting that the tetramer of sweet potato β-amylase did not dissociate within this pressure range.

To confirm this conclusion, we measured the intrinsic fluorescence of sweet potato β-amylase; under this pressure condition, the tetrameric structure is destabilized by GuHCl. Figure 6 shows the effect of various concentrations of GuHCl on the conformation of sweet potato β-amylase monitored by SEC and CD. The position of the SEC elution indicated a shift toward a lower molecular mass, and the elution profile became broader at 1 M GuHCl, indicating that some tetramer

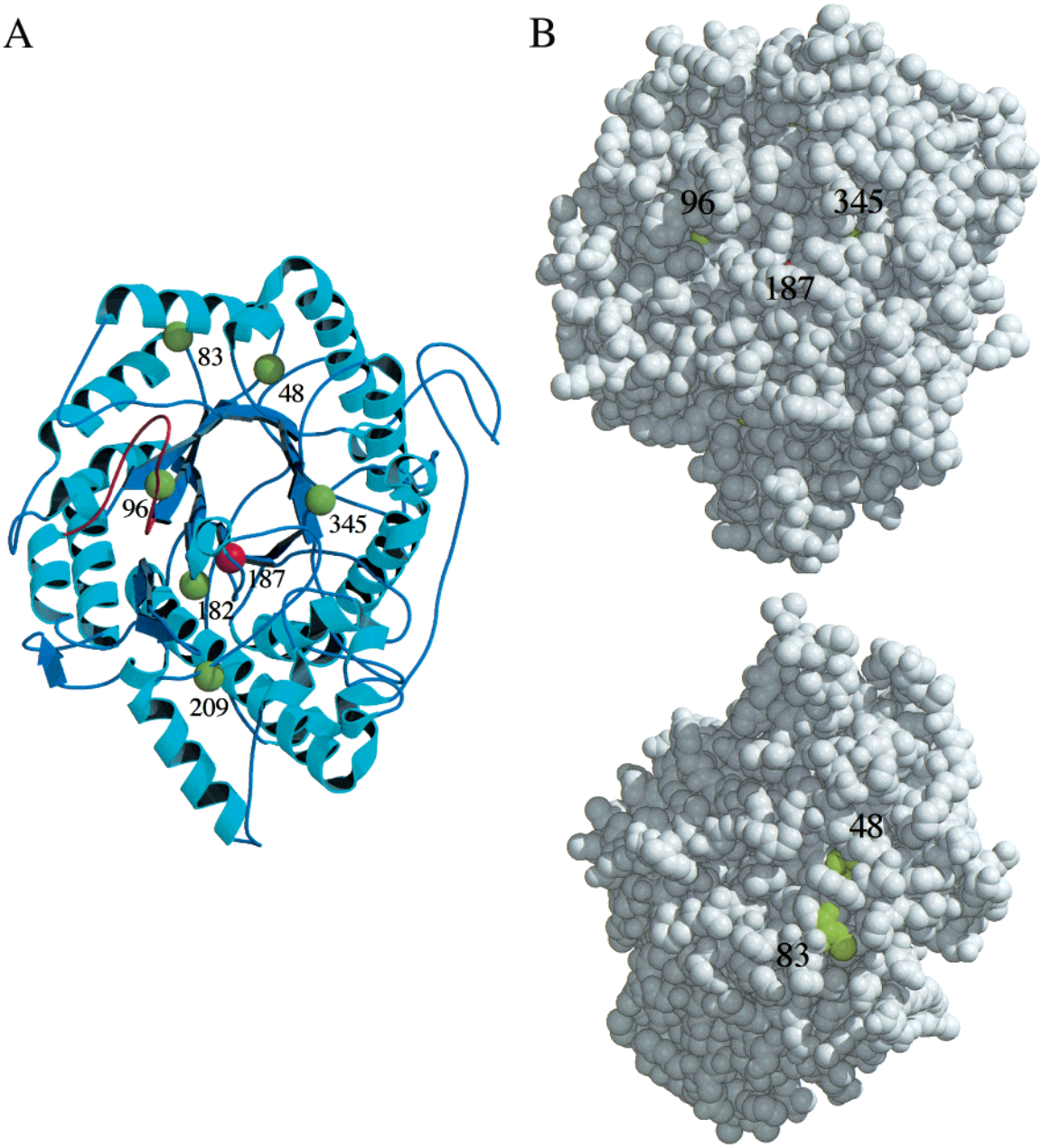

FIGURE 1: Schematic illustration of sweet potato β-amylase structure drawn using Molscript (*34*) and Raster 3D (*35*). (A) Ribbon model with positions of six cysteine residues represented by green spheres and that of Glu187 by a red sphere. (B) CPK model in which cysteine residues are colored green and Glu187 is red. The viewpoint for the upper panel is the same as that for panel A, and that for the lower panel is from the top. The three-dimensional structure of sweet potato β-amylase determined at 2.3 Å (*19*) is applied.

molecules dissociated. The CD spectrum at 1 M GuHCl slightly differed from that of the native state probably because of the conformational drift with the dissociation. The plot in the square in Figure 5 shows the change in intrinsic fluorescence of sweet potato β-amylase in the presence of 1 M GuHCl. During compression, the average wavelength was red shifted 6 nm when the pressure was increased from 0.1 to 300 MPa, and further shifted 7 nm when the pressure was increased from 300 to 400 MPa. On the other hand, the wavelength was gradually blue shifted 4 nm during the decompression from 400 to 0.1 MPa, indicating that the pressure-induced change was irreversible. The DLS measurement was performed on the pressure-treated protein solution at 1 M GuHCl and 400 MPa. The lifetime of the time correlation function was increased compared with that of the intact protein, indicating that the hydrodynamic radius was significantly increased (data not shown). These results indicate that the pressure treatment of sweet potato β-amylase at 1 M GuHCl induced the progressive dissociation of the tetramer followed by irreversible aggregation. A similar result has been reported for tetrameric transthyretin, which also underwent dissociation followed by irreversible aggregation under high pressure (*25*). Alternatively, the reversible change in fluorescence of sweet potato β-amylase at 0 M GuHCl indicates that the tetramer is stable in the pressure range of 0.1−400 MPa, and the enhancement of the reaction of Cys345 under high pressure cannot be explained by conformational drift.

Another possible explanation is that the reactivity of Cys345 is enhanced because its p*K* is reduced with the

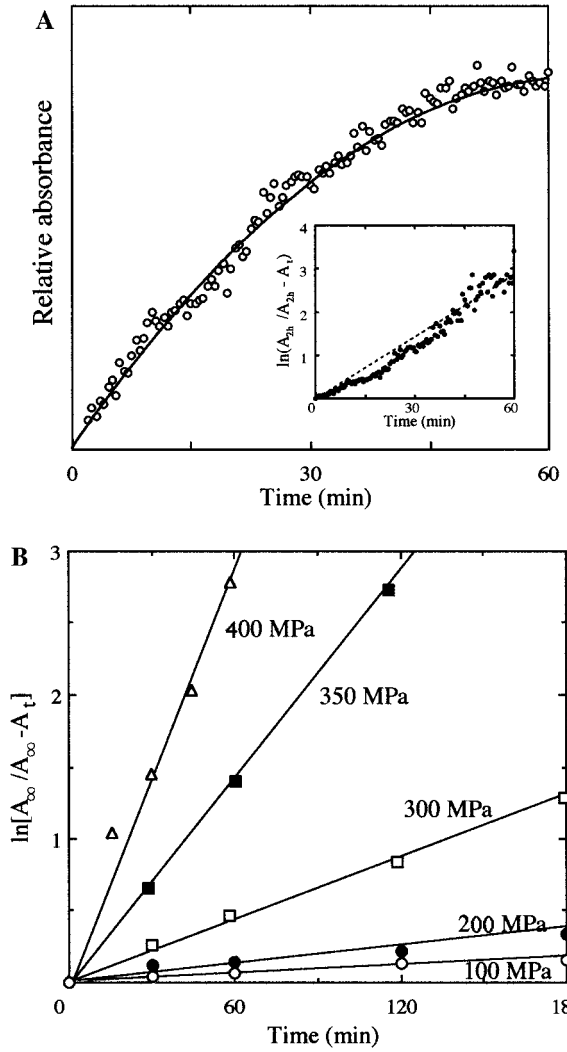

FIGURE 2: Time course of the reaction of cysteine residues of sweet potato β-amylase with DTNB at various pressures. (A) Increased absorbance at 412 nm of TNB liberated by the reaction of DTNB monitored by in situ measurement. The inset shows the logarithmic plot of the time course analyzed by first-order kinetics. (B) Logarithmic plots for the time course of the reaction at various pressures. The concentration of sweet potato β-amylase was 1 μM.

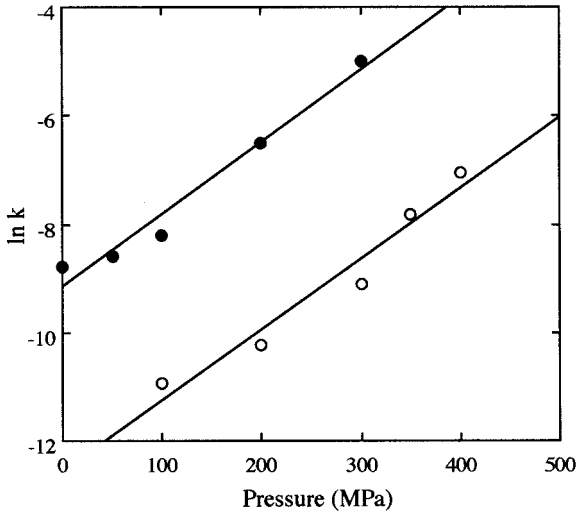

FIGURE 3: Logarithmic plot of the pressure dependence of the reaction rate of Cys345 in the absence and presence of 0.5 M GuHCl: (●) 0 M GuHCl and (○) 0.5 M GuHCl. $\Delta V^{\ddagger}$ values were calculated as -24 ± 2 mL/mol from the slope of these plots using the relationship, $\Delta V^{\ddagger} = -RT\mathrm{d}(\ln k)/\mathrm{d}P$.

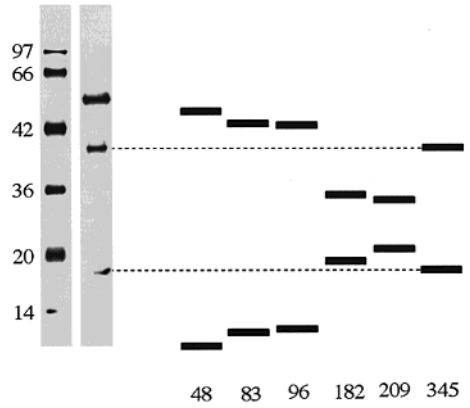

FIGURE 4: SDS−PAGE profile of the peptide fragment of sweet potato β-amylase after NTCB cleavage and theoretical profiles of peptide fragments generated from cleavage at one of six cysteine residues. Silver-stained 12.5% acrylamide gel.

increase in pressure. However, we have found that the reaction rate of Cys345 under high pressure was not influenced at different pHs, and the reduction of p*K* is not a reason for the selcetive reaction of this residue. Consequently, the reaction enhancement of Cys345 under high pressure is explained best by the perturbation of the local region around this residue.

Pressure-Induced Perturbation on the Local Region of Cys345. We investigated the detailed mechanism of the pressure-induced perturbation of the local region of sweet potato β-amylase by using the local unfolding model and the penetration model, which are introduced for the transient exposure of buried residue of folded protein in H−D exchange process (*26*). In the local unfolding model, segments of secondary structure undergo local, reversible unfolding, involving the cooperative breaking of the hydrogen bonds in the segment, the disruption of the secondary structure, and movement of the segment into bulk solvent. We used limited proteolysis to monitor the local unfolding of sweet potato β-amylase under high pressure (*10*, *27*). A solution of sweet potato β-amylase with subtilisin was incubated at pressures ranging from 0.1 to 400 MPa, and the product was analyzed by SDS−PAGE. The SDS−PAGE profile of the product treated at elevated pressures was the same as that at 0.1 MPa (data not shown), suggesting that sweet potato β-amylase was not locally unfolded under high pressure. However, this experimental result does not completely rule out the small-scale local unfolding that cannot be detected by the proteolysis.

In the penetration model, the chemical reagent diffuses into the protein matrix mediated by small, rapid fluctuations of interior atoms on the order of tenths to several angstroms. The motion of a single atom is not large enough to accommodate diffusion of reagent, but the summed effect of motions occasionally opens transient penetration pathways to Cys345. As shown in Figure 1B, Cys345 is half-buried and even a small increase in the amplitude of the fluctuation would allow the reagent to diffuse into the protein matrix. To examine the validity of the penetration model, we investigated the reaction of Cys345 in the presence of GuHCl, which increases the hydration to enhance the fluctuation. As shown in Figure 6, the elution profile of SEC and the CD spectrum at 0.5 M GuHCl are the same as those

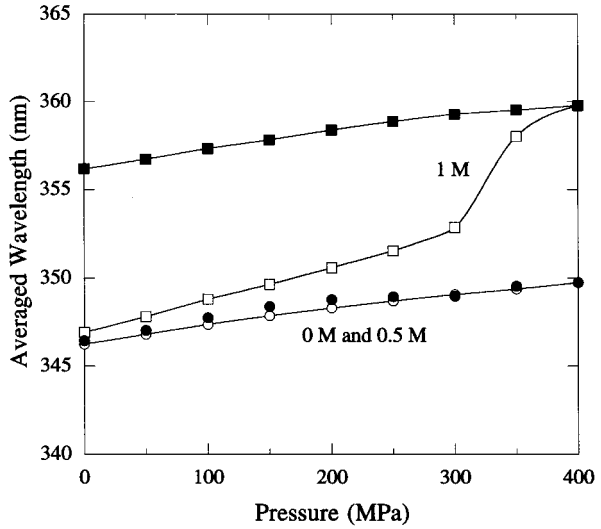

FIGURE 5: Averaged wavelength of intrinsic fluorescence of sweet potato β-amylase in pressure range from 0.1 to 400 MPa at 0, 0.5, and 1 M GuHCl: (white) compression, (black) decompression, (circles) 0 and 0.5 M, and (squares) 1 M. The concentration of sweet potato β-amylase was 0.5 μM in 10 mM Tris buffer (pH 7.5). The excitation wavelength was 295 nm. Oxidation of amylase sulfhydryl groups was prevented by adding 1 mM DTT.

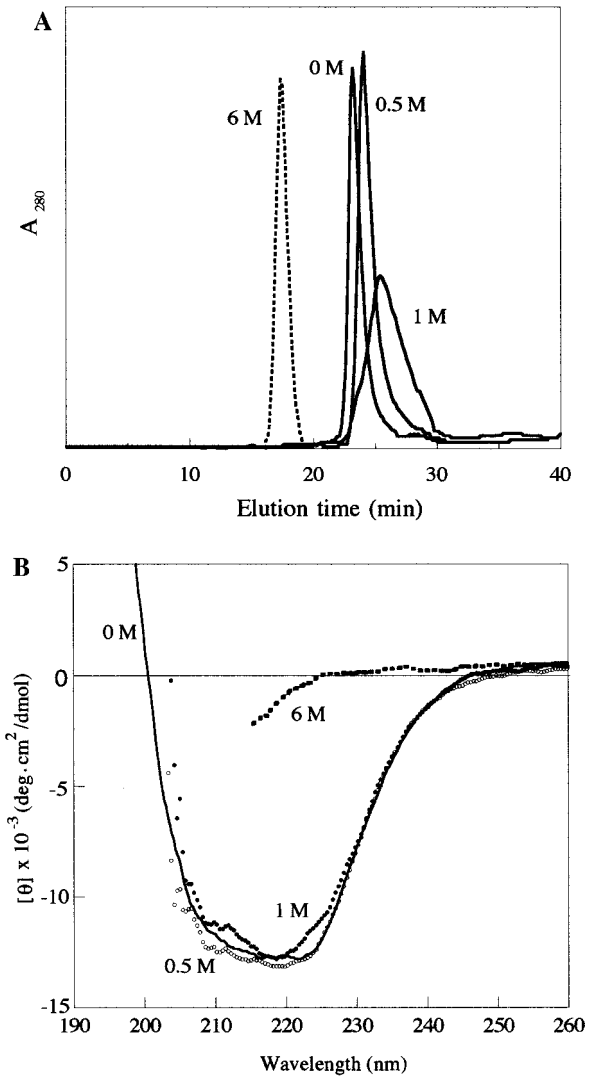

FIGURE 6: Effect of GuHCl on the structure of sweet potato β-amylase. (A) SEC−HPLC traces of sweet potato β-amylase in the presence of the indicated concentrations of GuHCl and 1 mM DTT. The sample injection volume was 20 μL. The protein solution monitored by absorbance at 280 nm. (B) CD spectra of sweet potato β-amylase at the indicated concentrations of GuHCl and 1 mM DTE. The sample concentration was 2 μM.

at 0 M GuHCl, indicating that the native structure was not influenced at 0.5 M GuHCl. Under this condition, the cysteine residue slowly reacted to DTNB, and one residue per mole of subunit was titrated after an 8 h incubation (Figure 7). The logarithmic plot of the kinetics of the reaction (the inset) was approximately linear, indicating that the kinetics are pseudo-first-order. The reacted residue was confirmed to be Cys345 by the SDS−PAGE profile of the peptide fragment generated by NTCB cleavage. The $\Delta V^{\ddagger}$ of the reaction at 0.5 M GuHCl from the logarithmic plot of the pressure dependence of the rate constant [Figure 3 (●)] was in the same range as that at 0 M GuHCl. Since the feature of the reaction of Cys345 under high pressure is similar to that at 0.5 M GuHCl, the pressure-induced reaction of Cys345 might be driven by the progressive hydration. To confirm the role of hydration on the reaction of Cys345 at elevated pressure, we examined its reactivity under high pressure in the presence of glycerol, which inhibits hydration (*28*). Only 0.09 mol of cysteine residue per mole of subunit reacted at 400 MPa in 70% glycerol for 2 h, indicating that the pressure-induced increase in the extent of the Cys345 reaction was suppressed by glycerol. These results demonstrated the significant role of hydration in the pressure-induced perturbation of sweet potato β-amylase, and selective reaction of Cys345 under high pressure is explained well by a hydration-driven enhancement of fluctuation in the context of the penetration model.

DISCUSSION

The conventional conformational drift model of oligomer dissociation assumes that the structure of undissociated oligomers is not influenced by applied pressure (*24*). However, our study showed that the pressure increase from 0.1 to 400 MPa perturbed the local structure of sweet potato β-amylase without dissociation of the tetramer. Similar results have been reported by Cioni and Strambini (*11*, *12*), who found that the pressure increase from 0.1 to 300 MPa affected the fluctuation of dimeric proteins prior to subunit dissociation by monitoring protein phosphorescence. Furthermore, the reactivity of the cysteine residue (*29*) and the enzyme activity (*30*, *31*) of some oligomeric proteins are influenced by increasing pressure without dissociating. Therefore, it might be necessary to consider the perturbation of the conformation in the predissociation pressure range for the quantitative analysis of pressure-induced dissociation of the oligomeric proteins.

The reaction kinetics of the Cys345 of sweet potato β-amylase are pseudo-first-order, and the apparent reaction rate was in the range of 0.001−0.05 min^{-1}. These values were much lower than that of the second-order kinetic constant of the thiol−disulfide exchange reaction between glutathione and cysteine that ranges from 450 to 610 M^{-1} min^{-1} at pH 7.4 (*32*). This is explained well by the fact that the reaction rate of Cys345 is limited by the slow process, which can be either local unfolding or fluctuation of the protein. Since pressure-induced local unfolding was not detected by proteolysis, we interpreted the enhancement of

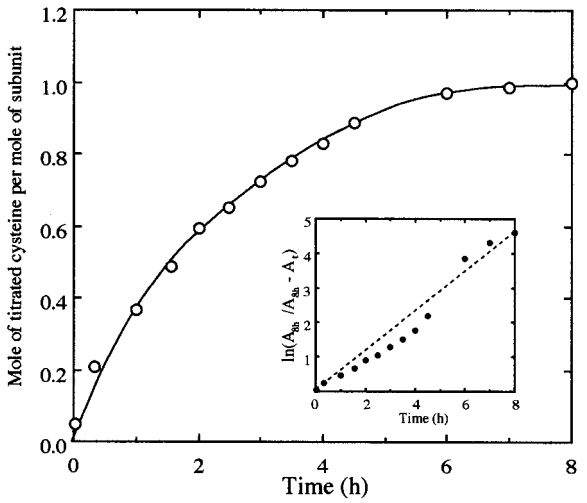

FIGURE 7: Time course of the reaction of cysteine residues of sweet potato β-amylase with DTNB in the presence of 0.5 M GuHCl monitored by the absorbance of TNB at 412 nm. The inset shows a logarithmic plot of the time course analyzed by first-order kinetics. The concentration of sweet potato β-amylase was 1 μM.

the reaction of Cys345 by the increase of fluctuation of protein in this study. The $\Delta V^{\ddagger}$ values of the protein fluctuation obtained from H−D exchange in apocytochrome b_{562} (*8*) and T4 lysozyme (*9*) ranged from −102 to −7 and −15 to 13 mL/mol, respectively, and $\Delta V^{\ddagger}$ values for apoazurin, alcohol dehydrogenate, and alkaline phosphatase were −20, 5, and 0 mL/mol, respectively, from acrylamide quenching of the phosphorescence (*12*). These positive and negative $\Delta V^{\ddagger}$ values of fluctuation are explained by the fact that the adaptations of protein structures to high pressure are determined by the reduction of internal cavities and the progressive hydration of polypeptide (*11*, *12*). The observed $\Delta V^{\ddagger}$ values of protein fluctuations are regarded as arising from positive cavity expansion and negative hydration, and the negative $\Delta V^{\ddagger}$ value of the local Cys345 region of sweet potato β-amylase indicates a significant role of hydration in the fluctuation. Such a role of hydration in the enhancement of fluctuation was also confirmed for the reaction of Cys345 in the presence of 0.5 M GuHCl and glycerol. The detailed structure of the active site of β-amylase with substrates and inhibitors (*22*, *23*) indicates that Cys345 is located on the inhibitor (α-cyclodextrin) binding site, of which the surface is composed of hydrophobic side chains. The progressive hydration at the local region of Cys345 under high pressure is consistent with the fact that hydrophobic hydration produces a negative volume change (*33*). On the other hand, the reaction of Cys96 with the hydrophobic substrate binding site was not enhanced by applied pressure, although this residue is also half-buried as shown in Figure 1B. The absence of Cys96 reactivity under high pressure might be explained by a negative contribution from the reduced cavity. The substrate complexes of this enzyme are associated with the interaction of the saccharide with the loop (red in Figure 1A) upon which Cys96 is located. Since this loop forms the cover of the active site in the enzyme−saccharide complex to construct the active site pocket, the cavity is located behind the loop in the absence of substrate. The tightening of this cavity with increasing pressure might slow the fluctuation and inhibit the reaction of Cys96. Studies of the pressure effect on the substrate and the inhibitor binding of this enzyme will prove or disprove the validity of this speculation.

In conclusion, we showed that pressure-induced perturbation of sweet potato β-amylase was monitored from reactions of cysteine residues. The selective reaction of Cys345 is explained well by the pressure-induced enhancement of the fluctuation of the local region. The reaction kinetics of cysteine under pressure provide information about the volumetric amplitude of perturbation, which can be widely applied to investigations of protein dynamics.

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