Foldability, Enzymatic Activity, and Interacting Ability of Barnase Mutants Obtained by Permutation of Secondary Structure Units

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ABSTRACT: Barnase, a well-characterized ribonuclease, has been decomposed into six modules (M1−M6) or secondary structure units (S1−S6). We have studied the foldability and activity of the barnase mutants obtained by permutation of the four internal modules (M2−M5) or secondary structure units (S2−S5) to investigate whether permutation of these building blocks is a useful way to create foldable and/or functional proteins. In this study, we found that one of the secondary structure unit mutants was expressed in *Escherichia coli* only when His102 was substituted by alanine, which is a catalytic residue of wild-type barnase. This mutant (S2354H102A) had ordered conformations, which unfolded cooperatively during urea-induced unfolding experiments. S2354H102A interacted with other barnase mutants to show a distinct RNase activity, although its own activity was quite weak. This interaction was specific, because S2354H102A interacted with only barnase mutants having His 102 and certain orders of the secondary structure units giving a distinct RNase activity. These results suggest that secondary structure units permuted in barnase mutants maintain their intrinsic "interacting ability" that is used for the folding of wild-type barnase, and the units can form certain conformations that complement those of the appropriate counterparts. Seven of 23 secondary structure unit mutants and only 2 of 23 module mutants had RNase activity. On the basis of the results of analyses of foldability and RNase activity of the mutants performed in this and previous studies, we conclude that secondary structure units are more suitable than modules as building blocks to create novel foldable and/or functional proteins in the case of barnase.

Globular proteins can be decomposed into different building blocks, such as modules, secondary structures, functional motifs, and so on. Block shuffling is therefore a possible strategy to create novel functional proteins. Recent technologies capable of combining several building blocks without homologous sequences for the construction of combinatorial protein libraries (*1*−*4*) would be used for selection experiments to obtain functional or foldable proteins from the global protein sequence space. However, it has not been established yet what kinds of peptide fragments are most appropriate as building blocks for the construction of novel functional or foldable proteins.

Modules, which are defined as peptides forming compact regions in globular domains, are considered to be structural and functional units of the globular proteins, and might have been used as building blocks in an early stage of protein evolution (*5*). Certain modules in the α- and β-subunits of hemoglobin have been successfully exchanged to create functional chimeric hemoglobins (*6*, *7*). Barnase, which is a well-characterized ribonuclease (*8*−*10*), was decomposed into six modules (M1−M6, Figure 1A), and three of the six modules (M2, M3, and M6) were found to have hydrolytic activity and binding ability with RNA as isolated peptides (*11*). Modules 1, 2, and 3 tended to form certain conformations, which were similar to their conformations in the intact barnase (*12*, *13*). These results support the above idea that modules are structural or functional units of globular proteins.

Barnase was also divided into six secondary structure units (S1−S6, Figure 1A), which were defined as extended regions in globular domains. We constructed 23 module mutants and 22 secondary structure unit mutants of barnase, by means of permutation of the internal four modules (M2−M5) or secondary structure units (S2−S5). The structural and functional properties of these mutants were analyzed to gain new insights from the "reconstructive approach", as opposed to the anatomical approach (*14*, *15*). In these studies, we have found that S2543 had both partially folded conformations and enzymatic activity. S4523 had RNase activity and took partially folded conformations only when the inhibitor GMP was present. S2435 and M2354 had RNase[1] activity,

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[1] Abbreviations: RNase, ribonuclease; 2′(3′)-GMP, guanosine 2′(3′)-monophosphate; 2′-AMP, adenosine 2′-monophosphate; 2′-CMP, cytidine 2′-monophosphate; 2′(3′)-UMP, uridine 2′(3′)-monophosphate; poly A, polyadenylic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CD, circular dichroism; eAp, 1,N^6-ethenoadenosine 5′-monophosphate. Designation of barnase mutants is based on the modules and secondary structure units in them: for example, M5324 represents a mutant containing a permutation of the four internal modules in which the six modules of barnase numbered from the N-terminal coding region are rearranged in the order 1, 5, 3, 2, 4, 6. Similarly, S4523 represents a mutant containing a permutation of the four internal secondary structure units in which the six structural fragments numbered from the N-terminal coding region are rearranged in the order 1, 4, 5, 2, 3, 6.

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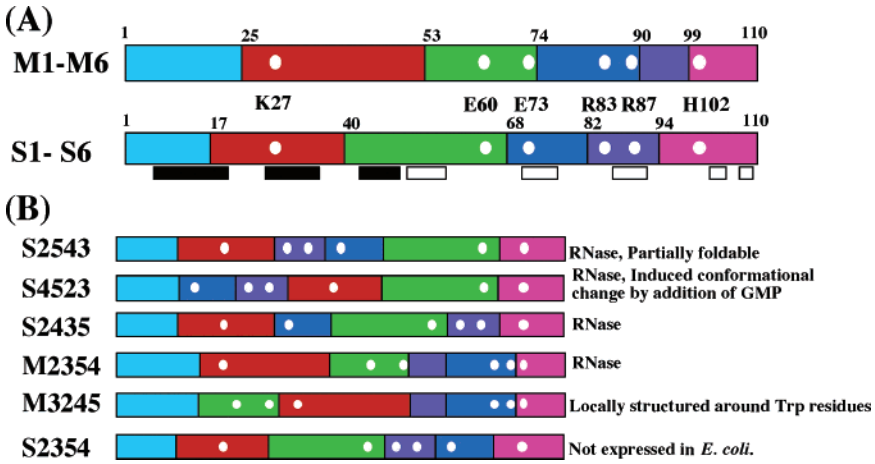

FIGURE 1: (A) Barnase was divided into six modules (M1−M6) and secondary structure units (S1−S6). Amino acid residues organizing an active site, K27, E60, E73, R83, R87, and H102, are shown by white circles. Black and white boxes indicate α-helices and β-sheets, respectively. (B) Summary of our previous results. Some barnase mutants and their features are indicated. Other mutants not indicated in this figure did not show foldability or enzymatic activity.

but did not have folded conformations. M3245 had locally structured regions around tryptophan residues, but did not have secondary structures or ordered backbone conformations (Figure 1B). These results suggest that the amino acid sequences derived from natural proteins have sufficient plasticity to be rearranged into different proteins, and secondary structure units are also good candidates as building blocks for the construction of novel foldable or functional proteins.

A question derived from the above studies has not been answered yet. The question is, why was the mutant S2354 not expressed in *Escherichia coli* under conditions in which all the other barnase mutants were well expressed? One possible explanation is that S2354 had distinct RNase activity, and the activity prevented the growth of the cells expressing the mutant gene. Indeed, wild-type barnase was expressed in *E. coli* when its inhibitor, barstar, was co-expressed (*16*). If this were the case, S2354 would have RNase activity and ordered conformations despite the interchange of S4 and S5.

In this study, we succeeded in expressing S2354H102A, a mutant of S2354 in which His102 is substituted by alanine (an amino acid present in the active center of wild-type barnase (*17*)), in *E. coli*. This mutant, S2354H102A, had secondary and tertiary structures, and unfolded in a cooperative manner during urea-induced unfolding experiments. Surprisingly, S2354H102A interacted with other barnase mutants to hydrolyze RNA. We found seven RNases among secondary structure unit mutants, but only two RNases among module mutants in an RNase assay performed at pH 7.5. These results are described below in detail.

EXPERIMENTAL PROCEDURES

Construction of the Mutant Genes. The procedures for the construction of barnase mutants obtained by permutation of modules or secondary structure units were described in our previous paper (*14*). Site-directed mutagenesis to construct mutants containing K27A, E60A, E73A, R83A, and R87A was carried out with overlap extension PCR using primers with altered codons for the mutation. The forward and reverse primers used were 5′-gat aat tac att aca *gca* tca gaa gca caa-3′ (K27Af), 5′-ttg tgc ttc tga *tgc* tgt aat gta att atc-3′ (K27Ar), 5′-atc ttc tca aac agg *gca* ggc aaa ctc-3′ (E60Af), 5′-gag ttt gcc *tgc* cct gtt tga gaa gat-3′ (E60Ar), 5′-ga aca tgg cgt *gca* gcg gat att aa-3′ (E73Af), 5′-tt aat atc cgc *tgc* acg cca tgt tc-3′ (E73r), 5′-aca tca ggc ttc *gca* aat tca gac c-3′ (R83Af), 5′-g gtc tga att *tgc* gaa gcc tga tgt-3′ (R83Ar), 5′-aga aat tca gac *gca* att ctt tac tca agc-3′ (R87Af), and 5′-gct tga gta aag aat *tgc* gtc tga att tct-3′ (R87Ar). Site-directed mutagenesis to construct mutants containing H102A was carried out with PCR, and the primer used was 5′-tct gat ttt tgt aaa ggt ctg ata *agc* gtc cgt-3′. All mutant genes were sequenced using a CEQ 2000 DNA analysis system (Beckman Coulter).

Expression, Purification, and Refolding of Mutants. BL21-(DE3) pLysS cells (*18*) were transformed with recombinant plasmids pPEP I encoding the T7 promoter and the mutant gene, and were cultured in TB (Terrific Broth) medium for 4 h at 37 °C. Isopropyl-β,D-thiogalactopyranoside (IPTG) was added to 1 mM, and cultures were continued for 4 h at 37 °C. The cells collected by centrifugation were suspended in 30 mL of lysis buffer (50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl), and were lyzed by sonication. The insoluble fraction collected by centrifugation was dissolved in the buffer (Hepes-NaOH, pH 7.5) containing 6 M urea, then centrifuged at 20600*g* for 15 min at 4 °C, and the supernatant was recovered. The supernatant was separated by perfusion chromatography (POROS HS/M column, Perspective Biosystem). Elution was performed with a linear gradient of 0−0.5 M ammonium acetate in Hepes-NaOH (pH 7.5) containing 6 M urea. The eluate was diluted to a protein concentration of 5 μM to avoid aggregation, dialyzed against 5 mM bis Tris-HCl (pH 6.0) at 4 °C, and then concentrated using a Centricon 3 (Amicon, Inc.). The protein molar concentration was determined from the UV absorption at 280 nm. The absorption coefficients of barnase mutants, 26 930 (in aqueous buffer), and 26 030 (in buffer containing 6 M urea), were calculated by the method of Gill and Hippel (1989) (*19*).

Structural Characterization. Far- and near-UV CD spectra of barnase mutants were measured at 5 °C on a Jasco spectropolarimeter, model J-600 using quartz cells of 1 and 10 mm. The intrinsic fluorescence of the barnase mutants was measured with a Shimadzu RF502 spectrofluorophotometer (excitation at 290 nm and emission at 300−400 nm)

at 5 °C. Urea-induced unfolding was monitored by measuring the change of CD intensity at 228 nm and the intrinsic fluorescence of tryptophan residues (340 nm) at 5 °C. The free energy, m-values, and midpoints of urea concentration for unfolding were calculated based on the two-state model as described previously (*14*). Unfolding experiments were performed at a protein concentration of 10 μM, and samples were equilibrated for 16 h at 5 °C. Nucleotides used for induced conformational change experiments were 2′ (*3*)-GMP (Yamasa), 2′-AMP (Sigma), 3′-CMP (Sigma), and 2′ (*3*)-UMP (Sigma).

Activity Staining. Activity staining was carried out on SDS−PAGE with a separate gel containing poly A. After SDS−PAGE, the gel was washed for 2 h in 50 mM Tris-HCl buffer (pH 7.5) to remove SDS. The gel was further left for 12 h in the same buffer to hydrolyze poly A, and then stained with the buffer containing 0.2% toluidine blue O (Merck). The procedures described above were performed at 4 °C.

Gel Filtration by HPLC. Gel filtration experiments were performed using a Superdex 75 column (Amersham Biosciences) placed on ice. Elution was done with 5 mM bis Tris-HCl (pH 6.0) containing 150 mM NaCl. The column was calibrated using ribonuclease (13.7 kDa), chymotrypsinogen A (25.0 kDa), ovalbumin (43.0 kDa), and albumin (67.0 kDa) as molecular markers.

RNase Titration Assay. The hydrolysis was started by adding RNA (800 ng) to enzyme solution, and the reaction was performed for 3 min on ice. The reactants were incubated for 10 min at 65 °C, and then subjected to agarose gel electrophoresis. The residual RNA was stained with ethidium bromide, and quantified using molecular imager FX (Bio-Rad). The enzyme solution contained 50 mM Tris-HCl (pH 7.4), 14 pmol of S2354H102A, and 0−17.5 pmol of S2543. The substrate RNA (140 base) was obtained by in vitro transcription (Ribo Max, Promega) by using a DNA template encoding a part of Bad protein.

RESULTS

Expression of S2354H102A. In the previous study, S2354 was not expressed in *E. coli* under conditions in which all the other barnase mutants were expressed (*14*). In this study, we tried to coexpress S2354 with barstar, which is an inhibitor of wild-type barnase, because wild-type barnase can be expressed in *E. coli* when barstar is coexpressed (*16*). However, we could not detect the expression of S2354 under conditions in which wild-type barnase was expressed. Next, we constructed S2354 in which His-102 was substituted by alanine, an active center residue of wild-type barnase (*17*). This mutant, S2354H102A, was successfully expressed in *E. coli*, like the other barnase mutants. The final yield of purified S2354H102A was 30 mg per liter of culture. In the case of S2543, the substitution of His102 by alanine decreased the RNase activity, but did not change the backbone conformations monitored in terms of the far-UV CD spectrum (data not shown). We analyzed the structural properties of S2354H102A to elucidate the foldability of S2354.

Structural Properties of S2354H102A. Figure 2 A shows far-UV CD spectra of S2354H102A in aqueous buffer (bold line) or 3 M urea solution (thin line). The spectral shape of

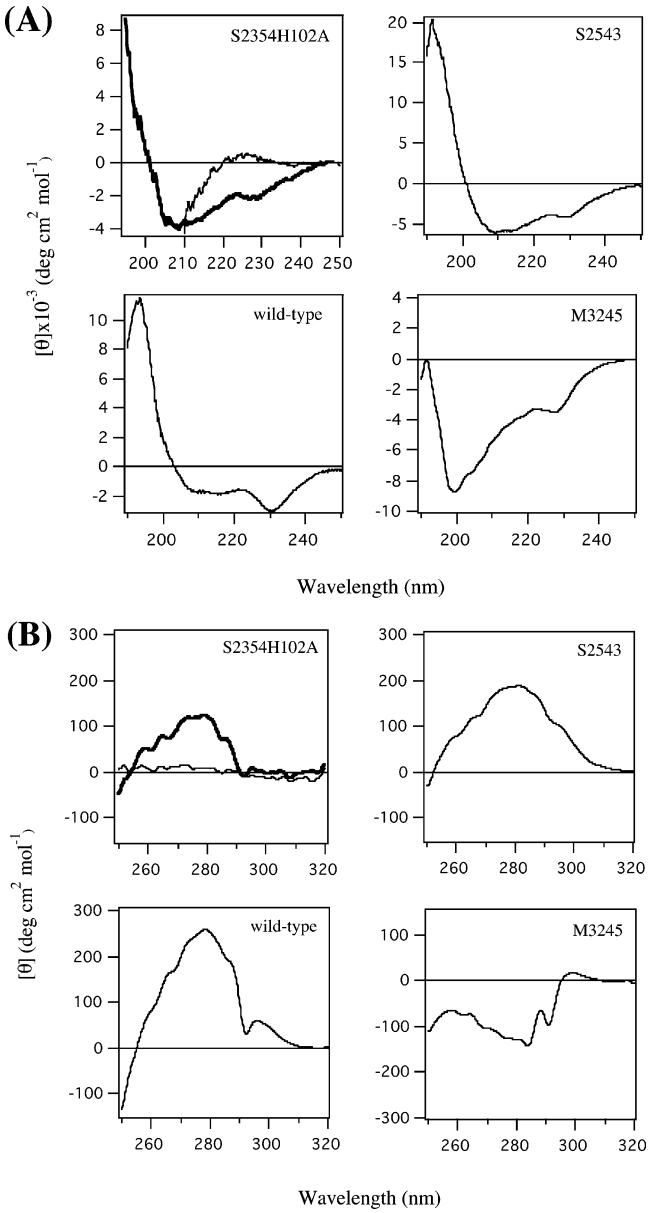

FIGURE 2: (A) Far-UV CD spectra of S2354H102A, wild-type barnase, S2543, and M3245. The spectra of S2354H102A in 5 mM bis Tris-HCl (pH 6.0) and in the buffer containing 3 M urea at 5 °C are indicated by bold and thin lines, respectively. The spectra were obtained at a protein concentration of 1 μM. (B) Near-UV CD spectra of S2354H102A, wild-type barnase, S2543, and M3245. The spectra of S2354H102A in 5 mM bis Tris-HCl (pH 6.0) and in the buffer containing 3 M urea at 5 °C are indicated by bold and thin lines, respectively. The spectra were obtained at a protein concentration of 10 μM.

the mutant in 3 M urea was typical of an unfolded protein, indicating that S2354H102A has an unordered conformation in urea solution. The spectrum of the mutant in aqueous buffer was quite different from that of the mutant in the unfolded state, showing a minimum around 230 nm and a strong maximum below 200 nm. The previous study showed that strong minima around 230 nm were also found in the spectra of M3254, S2543, and wild-type barnase, but not other barnase mutants constructed by permutation of modules or secondary structure units (*14*, Figure 2A). In the case of wild-type barnase, this minimum is due to Trp-94 (*20*), so it is possible that at least one of the three tryptophan residues in S2354H102A is in an ordered conformation. Because the

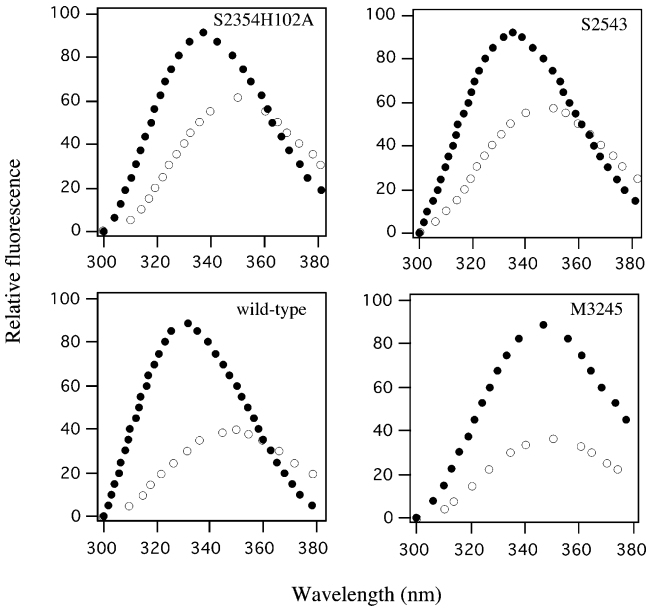

FIGURE 3: Tryptophan fluorescence spectra of S2354H102A, wild-type barnase, S2543, and M3245 in 5 mM bis Tris-HCl (pH 6.0, filled circles) and in the buffer containing 3 M (mutants, white circles) or 6 M (wild-type, white circles) urea at 5 °C. The spectra were obtained at a protein concentration of 1 μM.

high intensity below 200 nm indicates the formation of secondary structures such as α-helices or β-sheets, S2354H102A has ordered backbone conformations, like wild-type barnase and S2543.

Figure 2B shows near-UV CD spectra of S2354H102A in aqueous buffer (bold line) or 3 M urea solution (thin line). This mutant in urea solution showed no distinct positive or negative band, suggesting that there is no asymmetric environment around any of the aromatic residues in this mutant under these conditions. In contrast, this mutant in aqueous buffer showed positive Cotton effects, indicating the presence of asymmetric environments around aromatic residues. Thus, S2354H102A appears to have folded regions in aqueous solution. The previous study showed that distinct positive or negative Cotton effects were also found in the spectra of M3245, S2543, and wild-type barnase, but not other barnase mutants (*14*, Figure 2B).

Fluorescence spectra of the mutant proteins were measured as an indicator of the environment of tryptophan residues. Our previous study showed that the emission maxima of the tryptophan residues in wild-type barnase and S2543 were 332 and 335 nm, respectively, and the emission maxima of most of the other mutants, including M3245, were over 340 nm (*14*). Figure 3 shows the fluorescence spectra of S2354H102A, wild-type barnase, S2543, and M3245 in aqueous buffer or urea solution. The emission maxima of the tryptophan residues of S2354H102A in aqueous buffer and 3 M urea solution were at 337 and 350 nm, respectively. This result indicates that one or more tryptophan residues of the mutant in aqueous buffer are buried in hydrophobic environments, and the residues in urea solution are exposed to a polar hydrophilic environment. Thus, this mutant appears to have ordered structures around at least one of the three tryptophan residues in aqueous buffer.

Urea-induced unfolding experiments were performed to investigate whether S2354H102A unfolds cooperatively, like natural globular proteins. The CD intensity and intrinsic fluorescence of tryptophan residues of the mutant were monitored to detect changes of backbone conformations and environments surrounding tryptophan residues. As shown in our previous paper, the CD and fluorescence intensities of mutants without foldability increased monotonically with urea concentration. On the other hand, the CD intensity and fluorescence of S2354H102A increased and decreased cooperatively with increase of urea molarity (Figure 4A,B). Thus, ordered regions in the mutant seem to be stabilized by long-range interactions. However, the precise values of the thermodynamic parameters describing these transitions could not be obtained, because the pretransition baselines were extremely short. Therefore, we obtained these values tentatively based on the assumption that c_f (the slope of the linear dependence on urea concentration of intensity in the folded state) is nearly 0 (= 0.01). The extrapolated values of free energy of unfolding in water (ΔG^{H_2O}) based on CD and fluorescence measurements were 2.5 ± 0.7 and 3.8 ± 1.0 kcal mol^{-1}, respectively. The m-values were 2.9 ± 0.7 kcal mol^{-1} M^{-1} (CD) and 3.8 ± 0.9 kcal mol^{-1} M^{-1} (fluorescence). The urea concentrations at the midpoint for unfolding were 0.86 M (CD) and 1.0 M (fluorescence), respectively.

Previous studies demonstrated that S4523, one of the active mutants without stable folding, changed conformationally to form secondary and tertiary structures in the presence of 2′-(3′)-GMP, which is an inhibitor of wild-type barnase and S4523 (*15*, *21*, *22*). In this study, far- and near-UV CD spectra of S2354H102A were measured in the presence of 2′(3′)-GMP. The far-UV spectrum of this mutant in the presence of 2′(3′)-GMP differed slightly from those of the mutant in the presence of other nucleotides (2′-AMP, 2′-CTP, and 2′(3′)-UMP) or in the absence of 2′(3′)-GMP. The CD intensity at 210 nm increased, and the minimum in the vicinity of 230 nm became clearer (Figure 5A). Figure 5B shows the near-UV CD spectrum of S2354H102A measured in the presence of 2′(3′)-GMP. The spectral shape was clearly different from that measured in the absence of the nucleotide (Figure 2B). Thus, 2′(3′)-GMP induced conformational changes of S2354H102A. The presence of 2′(3′)-GMP did not change the far- or near-UV CD spectra of the other barnase mutant (*15*). Thus, the spectral changes described above were not artifacts.

RNase Activity of S2354H102A. RNase activities of barnase mutants were assayed by activity staining using poly A embedded in SDS−polyacrylamide gel at 4 °C. In our previous study, RNase activities of barnase mutants were measured at pH 6.0, because structural properties of wild-type barnase and mutants had been investigated at pH 6.0. In this study, RNase activities of all mutants were investigated at pH 7.5, where wild-type barnase is more active than at lower pHs (*17*). As shown in Figure 6A seven secondary structure unit mutants and two module mutants gave distinct bands where poly A had been cleaved and eliminated from the gel. These mutants thus have RNase activity at pH 7.5. Five mutants, S3452, S3542, S5423, S3245, and M2534, without activity at pH 6.0, showed activity at pH 7.5. On the other hand, S2543, with activity at pH 6.0, did not show activity at pH 7.5. The activities of S4523, S2435, and M2354 could be observed at both pH 6.0 and 7.5. S2354H102A also showed weak RNase activity. The amount of the mutants used for this assay was 10 pmol. On the other

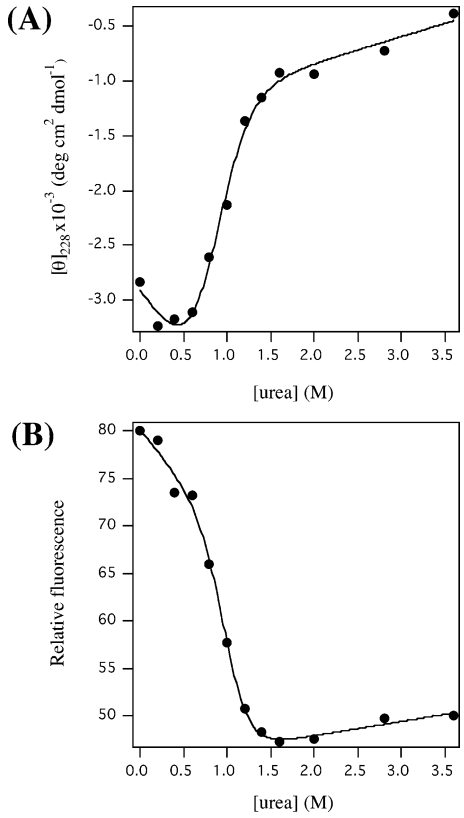

FIGURE 4: Urea-induced unfolding transitions of S2354H102A monitored by measuring CD at 228 nm (A) and fluorescence at 340 nm (excitation at 290 nm, (B)). The protein concentration used was 10 μM. The measurements were performed at 5 °C.

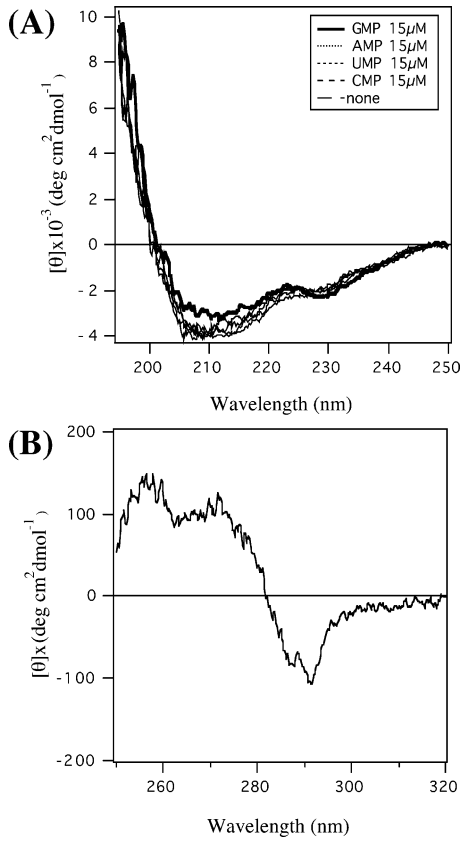

FIGURE 5: 2′(3)-GMP induced conformational changes of S2354H102A monitored in terms of the CD spectra. (A) Far-UV CD spectra of S2354H102A (3 μM) in the presence of various nucleotides (15 μM 2′ (3′)-GMP, 2′-AMP, 2′-CMP, and 2′(3′)-UMP). (B) Near-UV CD spectrum of S2354H102A (5 μM) with 2′(3)-GMP (15 μM). All CD spectra were obtained at 5 °C.

hand, 10 fmol of wild-type barnase gave a band with similar intensity, indicating that the RNase activities of the mutants were approximately 1/1000 of that of wild-type barnase. These data are consistent with our previous results (*15*). M2543 was subjected to activity staining as a negative control, and did not show any band (Figure 6A).

Interactions of S2354H102 with other Barnase Mutants to Hydrolyze RNA. It is noteworthy that distinct RNase activity, which is indicated by an arrow in Figure 6A, was observed at the boundary between S5423 and S2354His102A. We expected that this distinct RNase activity was a consequence of the interaction of these two mutants. To test this idea, S2354H102A was loaded on a SDS−polyacrylamide gel with other barnase mutants, and activity staining was performed. When the RNase activities of S2354H102A, S3542, and S5423 were assayed separately, they were weak. On the other hand, S2354H102A mixed with S3542 or S5423 showed distinct RNase activity, indicating that S2354H102A indeed interacted with these mutants to hydrolyze RNA (Figure 6B). All secondary structure unit mutants were tested similarly to see whether they interacted with S2354H102A to hydrolyze RNA. This assay revealed that 17 of 22 secondary structure unit mutants interacted with S2354H102A, while S2534, S3254, S3524, S5234, and S5324 did not. In other words, all barnase mutants having S2, S3, or S5 at the fifth block interacted with S2354H102A, but all barnase mutants having S4 at the fifth block did not interact with S2354H102A to express RNase activities. Representative data are shown in Figure 6C.

Gel filtration chromatography was performed to investigate the details of the interaction between S2354H102A and S2543. The results showed that S2354H102A was a mixture of monomer, dimer, and trimer (data not shown). S2354H102A and S2543 were co-injected into the column to examine the association of these mutants. However, no increase in the fraction of multimers was observed. The complex expressing RNase activity is presumably quite unstable, and few molecules may associate with each other in the buffer solution.

Figure 7A shows RNA substrates not cleaved during RNase titration assay. S2354H102A (lane 1) and S2543 (lane 7) alone showed weak RNase activity, as expected. S2354H102A mixed with increasing amounts of S2543 showed higher RNase activity (lanes 2−6), and the activity reached a plateau when these mutants were mixed in equal amounts, suggesting that they interact with 1:1 stoichiometry to hydrolyze RNA (Figure 7B).

To examine what residues of barnase mutants were involved in the interaction with S2354H102A, several amino acids of S4523 and S2543, whose structural and enzymatic properties have been investigated (*14*, *15*), were substituted by alanine using site-directed mutagenesis. The residues substituted corresponded to Lys-27, Glu-60, Glu-73, Arg-83, Arg-87, and His102 of wild-type barnase (Figure 1), which comprise the active site of wild-type barnase (*17*, *22*, *23*, *24*). RNase activities of these alanine mutants were assayed by activity staining, and the assay revealed that the activities of all the mutants other than E60A were weakened by the substitutions (Figure 8A). The RNase activity of each

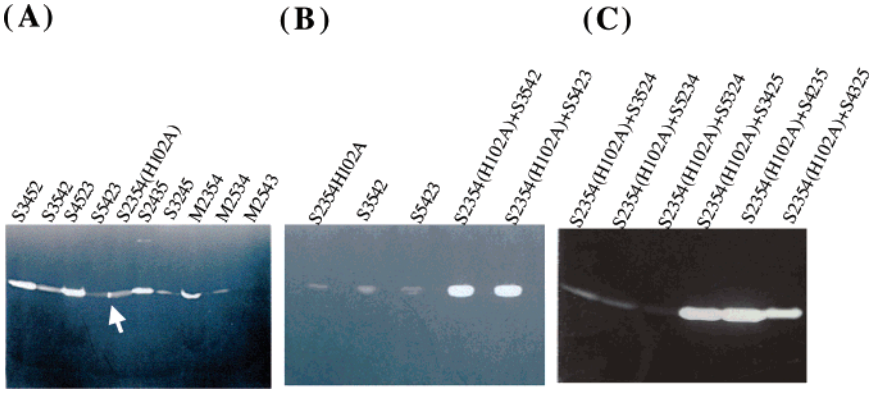

FIGURE 6: (A) Activity staining of barnase mutants. Distinct RNase activity was observed at the boundary of S5423 and S2354H102A, indicated by a white arrow. (B) RNase activities of S2354H102A, S3542, S5423, and their mixtures (S2354H102A+S3542, S2354H102A+S5423). (C) RNase activities of S2354H102A mixed with different barnase mutants. Each lane contains the same amount of barnase mutants (10 pmol). Activity staining was performed at 4 °C.

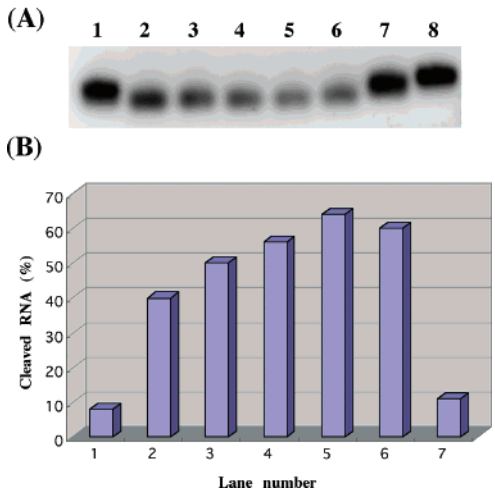

FIGURE 7: (A) Residual RNA during RNase titration assay. The amounts (pmol) of S2354H102A and S2543 in each lane were 14 and 0 in lane 1, 14 and 3.5 in lane 2, 14 and 7 in lane 3, 14 and 10.5 in lane 4, 14 and 14 in lane 5, 14 and 17.5 in lane 6, and 0 and 14 in lane 7. No mutant was in lane 8. (B) Cleaved RNA (%) during RNase titration assay. The cleaved RNA (%) was calculated according to the following equation. Cleaved RNA (%) in lane x = [(RNA in lane 8) − (RNA in lane x)]/(RNA in lane 8).

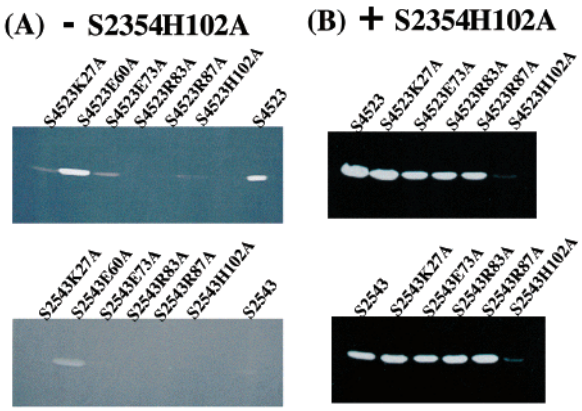

FIGURE 8: (A) RNase activities of alanine mutants of S4523 and S2543. (B) RNase activities of alanine mutants of S4523 and S2543 mixed with S2354H102A. Each lane contains the same amount of barnase mutants (10 pmol). Activity staining was performed at 4 °C.

alanine mutant mixed with S2354H102A was also examined (Figure 8B). Distinct RNase activities were found for all mixtures except that containing S4523H102A or S2543H102A. Thus, S2354H102A appears to use His102 of other barnase mutants to hydrolyze RNA.

DISCUSSION

To elucidate the foldability of S2354, His102 in the putative active center of this mutant was substituted by alanine, and S2354H102A was expressed in *E. coli*. Foldability of the expressed S2354H102A was analyzed in terms of far-UV CD, near-UV CD, and intrinsic fluorescence of tryptophan residues. Although the stability of the mutant was quite low relative to that of wild-type barnase, the mutant has secondary and tertiary structures, which unfold in a cooperative manner. S2354H102A can be categorized into the group of partially folded proteins, like S2543, because the hydrophobic dye 8-anilino-1-naphthalenesulfonic acid (ANS) bound to the mutant to give enhanced fluorescence (data not shown). In addition to secondary and tertiary structures, S2354H102A has an organized active site that can recognize a small molecule such as 2′(3′)-GMP, resulting in induced conformational changes. These lines of evidence strongly suggest that S2354 has distinct RNase activity and folded regions. This inference is not extraordinary, because the regions permuted in S2354 are the smallest among all the secondary structure unit mutants, and both S4 and S5, which are interchanged, contain a β-strand (Figure 1). It is known that mutants of dihydrofolate reductase obtained by permutation of two β-strands also have both foldability and enzymatic activity (*25*).

S2354H102A showed distinct RNase activity by using His102 of other barnase mutants. Although the exact mechanisms of the interactions are not clear, it seems likely that these interactions are based on the complementation between secondary structure units. S2354H102A interacted with all barnase mutants having S2, S3, or S5 at the fifth block, but not with any barnase mutant having S4 at the fifth block (Figure 9). In the folding of wild-type barnase, S4 interacts with S2, S3, and S5 (Figure 9A), and therefore a part of these interactions should be involved in the intermolecular interactions between S4 of S2354H102A and S2, S3, or S5 of other barnase mutants (Figure 9B,C,D), allowing His 102 of other barnase mutants to contact a part of the putative active site of S2354H102A. On the other hand, because there is no S4−S4 interaction, S2354H102A did not interact with barnase mutants having S4 at the fifth block to

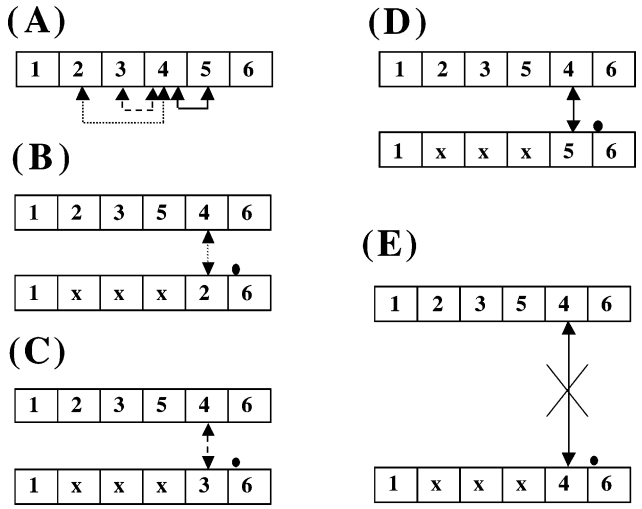

FIGURE 9: Interactions between secondary structure units. (A) Each secondary structure unit contacts each other unit in the globular conformation of wild-type barnase. Only interactions between S4 and S2 (dotted line), S3 (broken line), and S5 (solid line) are indicated. (B, C, and D) Interactions between S2354H102A and barnase mutants having S2, 3, and 5 at the fifth block are indicated. These intermolecular interactions would allow His102 of other barnase mutants to contact a part of the putative active site of S2354H102A to express RNase activity. (E) S4−S4 interaction would not occur, and S2354H102 cannot use His 102 of barnase mutants having S4 at the fifth block. The black circle indicates His 102 of barnase mutants, and "x" indicates secondary structure units other than S2 (B), S3 (C), S5 (D), and S4 (E).

express RNase activity (Figure 9E). It is likely that secondary structure units in barnase mutants have the potential to form certain conformations to complement the appropriate counterparts. It is known that some natural proteins, including barnase, form oligomers via a mechanism called three-dimensional domain swapping in a partially folded state (*26*, *27*). However, the heteromultimer of S2354H102A could not be observed in gel filtration chromatography. Thus, the interaction or complementation of S2354H102A with other barnase mutants was weak.

In the previous study, we found a partially folded protein with secondary and tertiary structures (S2543) only among secondary structure unit mutants. We inferred that secondary structure units, rather than modules, are suitable as building blocks to create partially folded proteins, because peptides forming secondary structures often form different stable conformations (*28*−*30*). This study provides further support for this view, because we again found a partially folded protein (S2354H102A), which was also a secondary structure unit mutant. In addition, we found eight RNases among 23 secondary structure unit mutants (S3452, S3542, S4523, S5423, S2354H102A, S2435, S3245, and S2543), but only two RNases among 23 module mutants (M2354 and M2534). On the basis of these results, it is reasonable to conclude that secondary structure units are more suitable than modules as building blocks to create novel foldable and/or functional proteins, at least in the case of barnase.

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