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N- and C-terminal Fragments of a Globular Protein Constructed by Elongation of Modules as a Units Associated for Functional Complementation

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We have been interested in partially folded proteins with marginal stability and activity, because they have a potential to be mature proteins by artificial evolution. A module is defined as a contiguous peptide chain forming a compact region in a globular protein. Modules may be used as building blocks to create partially folded proteins. Barnase, a ribonuclease consisting of 110 amino acids, has been divided into six modules (M1–M6), four peptide fragments, M12 (1–52), M123 (1–73), M1234 (1–88) and M12345 (1–98), have been constructed by progressive elongation of the modules from the N-terminus. Only M12345 (1–98) had a partially folded conformation, but it lacked detectable RNase activity. A mixture of M12345 (1–98) with M56 (89–110) showed weak but distinct RNase activity. Unfolded M12345 (1–96) was constructed by removal of two residues from the C-terminus of M12345 (1–98). The mixture of M12345 (1–96) with M56 (89–110) also showed RNase activity. Further, the interaction endowed M12345 (1–96) with conformational stability. We propose that N- and C-terminal fragments obtained by successive elongation of modules would interact to be a complex with marginal stability and activity, which would be used for creating a mature complex by artificial evolution.

Key words: artificial evolution, exon shuffling, functional complementation, module, protein folding.

Abbreviations: ANS, 8-anilino-1-naphthalensulfonic acid; CD, circular dichroism; UV, ultraviolet.

Several techniques capable of constructing combinatorial protein library by mixing peptide fragments without homologous sequences have been developed in the past decade (*1–8*), and novel functional proteins were successfully obtained from such libraries (*4*, *5*, *7*, *8*). We have proposed that secondary structure units would be appropriate as building blocks, because functional and/or partially folded proteins have been obtained by permutation of the units of barnase (*9*). We are interested in the biophysical properties of such proteins, because partially folded proteins with weak biological activity might be representative of evolutionary intermediates, which would have the potential to evolve into the mature functional proteins by artificial evolution (*10*). Recently, we found that one of the barnase mutants with a partially folded conformation was activated by functional complementation with its interacting partners (*11*, *12*).

The exon theory of genes was led from the discovery of the exon/intron structure of genes (*13*, *14*). The theory hypothesized that pre-existing smaller units encoded by ancient exons had been combined to create novel proteins using introns as hotspots for the recombinations (*15*, *16*). If the exons encoded structured units with a function, exon shuffling had created foldable and functional proteins efficiently. Go found a protein structure unit termed 'module', which consists of contiguous peptide chain forming a compact region in a globular protein (*17*). Further, the module boundaries were found to correspond to exon/intron boundaries in several globular proteins (*18*, *19*). Therefore, module shuffling would simulate one of the mechanisms of protein evolution. Indeed, some modules in haemoglobin and β-xylanases have been exchanged to create functional chimeric proteins *in vitro* (*20–23*). It is plausible that, monomeric or multimeric polypeptides consisting of modules with marginal stability and activity would be present in earlier stages of protein evolution. Molecules with these properties constructed in a test tube would be used as materials to create mature functional proteins by artificial evolution.

Barnase, a ribonuclease produced by *Bacillus amyloliquefaciens*, is a monomer protein consisting of 110 amino acid residues (*24*). It has three α-helices in the first 45 residues and five anti-parallel β-strands in the last 65 residues (*25–27*). The folding reaction of the protein has been extensively studied (*28*), and is thought that the N-terminal helical part and the C-terminal part containing transiently generated β-strands associate rapidly to form a fully folded and functional structure (*29–31*).

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Barnase has three tryptophan residues (Trp-35, 71 and 94), and their contributions to the circular dichroism (CD) and fluorescence spectra have been studied (*32*, *33*). The tertiary structure of barnase has been divided into six modules (M1–M6) (*34*). In aqueous buffer containing trifluoroethanol (TFE), local secondary structures of dissected M2 and M3 were observed at similar positions to those in intact barnase (*35*). Molecular dynamics simulation showed that five of the dissected modules (M1–M5) tended to retain native-like conformations in water (*36*). Three modules (M2, M3 and M6) were found to have RNase activity as isolated peptides (*37*). These results imply that modules were structural and/or functional units of ancient proteins (*38*), and existing modules in modern proteins would be used as building blocks to create molecules with marginal stability and activity.

In the present study, we constructed four peptide fragments [M12 (1–52), M123 (1–73), M1234 (1–88) and M12345 (1–98)] by progressive elongation of the barnase modules from the N-terminus (Fig. 1). We found that only M12345 (1–98) had a partially folded conformation, but it did not show detectable RNase activity. Although a mixture of M12345 (1–98) with M6 (99–110), which contains the active residue (His-102) of barnase, did not show any RNase activity [our previous study showed that three of six barnase modules (M2, M3 and M6) had RNase activity, as revealed by RNase assay using activity staining Yanagawa *et al*. (*37*). In the present study, hydrolytic assay was performed by measuring the increment of fluorescence intensity of poly eAp. Activity of M6 was not detected under these conditions], a mixture of M12345 (1–98) with M56 (89–110) showed weak but definite RNase activity under identical conditions. To confirm the interaction, we constructed truncated fragments, M12345 (1–97) and M12345 (1–96), and found that M12345 (1–96) was conformationally unstable. When M12345 (1–96) was mixed with M56 (89–110), the mixture also showed RNase activity. Further, this interaction was found to render M12345 (1–96) conformationally stable, providing direct evidence for the interaction between the two fragments. Here, we present our findings on the foldability and interacting ability of the peptide fragments, which were analysed by means of several biophysical methods and hydrolytic assay.

MATERIALS AND METHODS

Construction of the Peptide Fragments of Barnase—Expression plasmids containing structural genes of the peptide fragments M12 (1–52), M123 (1–73), M1234 (1–88), M12345 (1–98), M12345 (1–96) and M12345 (1–97) were obtained by standard procedures. BL21 (DE3) pLysS cells were transformed with the plasmids, and used for overexpression of the peptide fragments. M12 (1–52), M123 (1–73) and M1234 (1–88) obtained from soluble fractions were gel-filtered (Bio-Gel P-30, Bio–Rad, Japan), dialysed against 10 mM ammonium acetate buffer (pH 4.5 or 8.0), and purified by ion-exchange column chromatography (CM53 or DE52, Whatman, USA). M12345 (1–96), M12345 (1–97) and M12345 (1–98) were obtained as inclusion bodies, and then purified as described in our previous paper (*9*). Peptide fragments M6 (99–110) and M56 (89–110) were synthesized by a solid-phase method on a peptide synthesizer and purified as described in our previous paper (*38*). The molar concentration was determined from the UV absorption at 280 nm. The absorption coefficients were calculated by the method of Gill and Hippel (*39*).

CD Spectroscopy—Far- and near-UV CD spectra of the peptide fragments in 5 mM bis–Tris–HCl buffer (pH 6.0) were measured at 5°C on a Jasco spectropolarimeter, Japan, model J-820 fitted with a thermostatted cell holder. Protein concentrations were 5 μM (far-UV CD) and 20 μM (near-UV CD). Urea-induced unfolding was monitored by measuring the change of CD intensity at 231 nm at 5°C. Protein concentration used for unfolding experiments was 10 μM. The free energy and midpoint of urea concentration for unfolding were calculated based on the two-state model as described previously (*9*). For the complementation study, far-UV CD spectra of solutions containing 5 μM of N- and C-terminal fragments were recorded.

Fluorescence Spectroscopy—The fluorescence spectra of the peptide fragments in 5 mM bis–Tris–HCl buffer (pH 6.0) were measured on a Jasco FP-777W spectrofluorophotometer (excitation at 290 nm) fitted with a thermostatted cell holder. Urea-induced unfolding was monitored by measuring the change of the intrinsic fluorescence of tryptophan residues (340 nm) at 5°C. Protein concentration was 1 μM. The free energy and midpoint of urea concentration for unfolding were calculated based on the two-state model as described previously (*9*). Fluorescence spectra of 8-anilinonaphthalene 1-sulphonate (ANS, 85 μM) in the absence and presence of 3.9 μM M12345 (1–98) were measured at 5°C (excitation wavelength, 380 nm). The pH titration was performed by adding peptide solution [50 μl of 4 μM peptide fragments in 5 mM bis–Tris–HCl buffer (pH 6.0)] to 950 μl of the buffer. Buffers were all at the same ionic strength ($I = 0.05$); bis–Tris–HCl at pH 5.5–7.0, and Tris–HCl at pH 7.0–9.0. For the complementation study, fluorescence intensities and maximum wavelength of solutions containing 1 μM of N- and C-terminal fragments were recorded.

Gel Filtration by HPLC—Gel filtration experiments were performed on a Superdex 75 column (Amersham Biosciences, USA) placed in an ice bath. 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. Protein concentration was 10 μM.

Hydrolysis of Polymers of 1,N^6-ethenoadenosine 5′-monophosphate (poly eAp)—The hydrolysis was started by adding enzyme solution [50 μl of 4 μM peptide fragments in 5 mM bis–Tris–HCl (pH 6.0)] to a buffer solution (950 μl) containing 0.2 mM poly eAp in a thermostatted cell holder. The increment of fluorescence was monitored with a spectrofluorophotometer (JASCO FP-777W). Buffers were all at the same ionic strength ($I = 0.05$); bis–Tris–HCl at pH 5.5–7.0, and Tris–HCl at pH 7.0–9.0. Excitation was done at 320 nm with emission measured at 410 nm. Poly eAp was synthesized as described in our previous paper (*10*).

We estimated apparent dissociation constant of the complex based on the RNase activity and some assumptions as follows.

Equilibrium state was assumed to be

$$[\text{complex}] \longleftrightarrow [\text{M12345}] + [\text{M56}] \quad (1)$$

Dissociation constant can be obtained by

$$K_d = \frac{[\text{M12345}][\text{M56}]}{[\text{complex}]} \quad (2)$$

If specific activity of the functional complex is similar to that of wild-type barnase, molar ratio of the complex would be 1/100 of each fragment, because RNase activity of the mixture was 1/100 of that of wild-type barnase. As the concentration of the each peptide fragment used for the RNase assay was 4 μM, then K_d was obtained to be ~400 μM.

RESULTS

Construction of N- and C-terminal Fragments of Barnase—Figure 1 shows all the constructs used in this study. Five of the six barnase modules were elongated progressively from the N-terminus for the construction of M12 (1–52), M123 (1–73), M1234 (1–88) and M12345 (1–98). The C-terminal end of M12345 (1–98) was truncated by one or two residues to construct M12345 (1–97) and M12345 (1–96). The peptide fragments corresponding to M6 (99–110) and M56 (89–110) were chemically synthesized.

Conformational Properties of M12345 (1–98)—Far-UV CD spectra of M12 (1–52), M123 (1–73) and M1234 (1–88) showed that these fragments did not have definite ordered conformations (data not shown). However, the far-UV CD spectrum of M12345 (1–98) showed that this fragment had ordered conformation (Fig. 2A, gray line). The spectrum also showed a strong minimum at around 230 nm, suggesting the presence of a structured region around Trp-94 (*33*). Figure 2B shows the near-UV CD spectra of M12345 (1–98) in aqueous buffer (gray line) and 7 M urea solution (dashed gray line). This fragment showed no distinct band in urea solution, but showed positive Cotton effects like those of wild-type barnase in aqueous buffer, indicating the presence of asymmetric environments around aromatic residues. Thus, M12345 (1–98) appears to have a folded region in aqueous solution.

The emission maxima in the fluorescence spectra of M12 (1–52), M123 (1–73) and M1234 (1–88) were at 350 nm, indicating that tryptophan residues of these fragments were exposed to the buffer. The emission maximum of the tryptophan residues of M12345 (1–98) in aqueous buffer was in the vicinity at 335 nm, but the maximum of the fragment in 7 M urea solution was at 350 nm (Fig. 2C). Thus, M12345 (1–98) appears to have ordered structure around at least one of the three tryptophan residues in aqueous buffer.

In the case of wild-type barnase, the fluorescence intensity increases cooperatively when the pH is increased. This increase is thought to reflect deprotonation of His-18, which is present in the vicinity of Trp-94, and has a pK_a value of 7.6–7.8 at 25°C (*32*, *40*). We observed such a cooperative transition with the pK_a value of 8.0 in M12345 (1–98) when the fluorescence intensity was measured at 5°C, but not at above 10°C (Fig. 2D). Thus, the environment around His-18 and

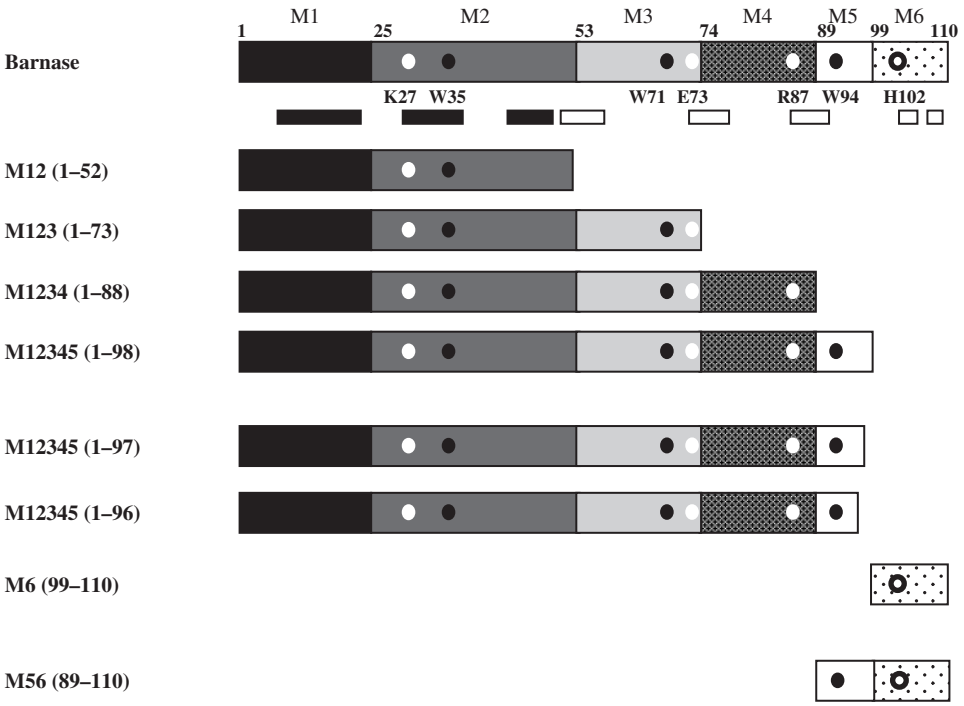

Fig. 1. **The dissection of barnase into six modules and eight peptide fragments used in this study is shown.** White circles indicate active residues, K27, E73, R87 and H102 (*33*). Black circles indicate W35, W71 and W94. Black and white boxes indicate α-helices and β-strands, respectively.

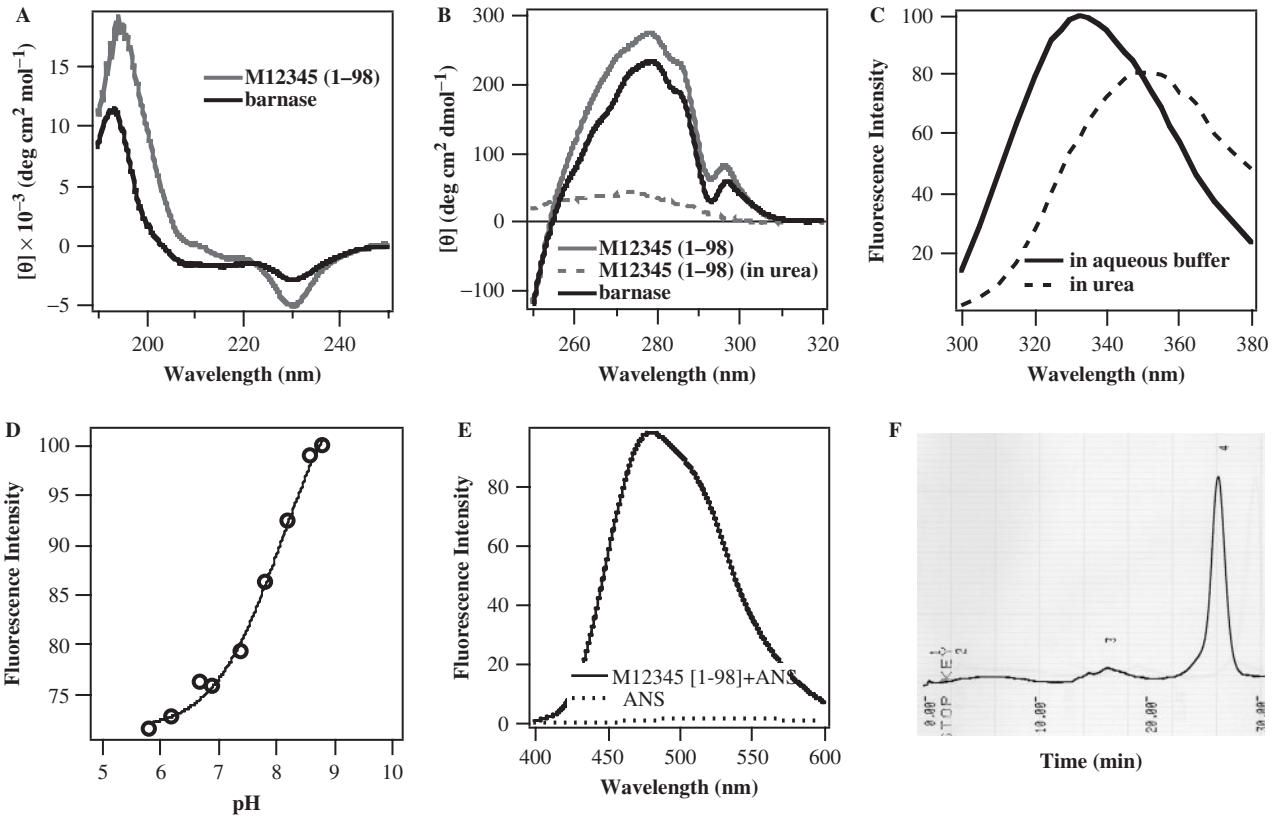

Fig. 2. **Biophysical characterization of M12345 (1–98).** (A) Far-UV CD spectra of wild-type barnase and M12345 (1–98) measured at 5°C. (B) Near-UV CD spectra of wild-type barnase and M12345 (1–98) measured at 5°C. (C) Fluorescence spectra of M12345 (1–98) in aqueous buffer or 7 M urea solution measured at 5°C. (D) The pH titration of M12345 (1–98) monitored in terms of fluorescence intensity at 340 nm, at 5°C. (E) Fluorescence spectra of ANS in the presence and absence of M12345 (1–98) measured at 5°C. (F) Elution profile of gel filtration chromatography of M12345 (1–98).

Trp-94 in M12345 (1–98) appears to be similar to that of wild-type barnase, but is not entirely native-like.

The fluorescence spectra of ANS in the presence and absence of M12345 (1–98) were investigated (Fig. 2E). The spectrum was also measured in the presence of wild-type barnase. Although wild-type barnase did not enhance ANS fluorescence as reported previously (*41*), the M12345 (1–98) increased the fluorescence intensity of ANS, indicating that hydrophobic clusters exposed to the solvent are present in it (*41*). Thus, M12345 (1–98) can be categorized as a partially folded protein.

The oligomeric state of M12345 (1–98) was investigated by gel filtration chromatography, using compact globular proteins as standards. The fragment was eluted mainly as a single peak, whose elution volume corresponded to the monomer (Fig. 2F).

The CD and fluorescence intensities of M12345 (1–98) were monitored during urea-induced unfolding experiments (Fig. 3A). The intensities changed cooperatively in a similar manner to those of natural globular proteins, indicating that ordered regions in M12345 (1–98) are stabilized by long-range interactions. The extrapolated values of free energy of unfolding in water (ΔG^{H_2O}) based on CD and fluorescence measurements were 2.6 ± 0.7 and 2.3 ± 0.8 kcal/mol, respectively. The urea concentrations at the midpoint for the unfolding were 1.4 M (CD) and 1.2 M (fluorescence), respectively.

Thermal unfolding of M12345 (1–98) was examined by measuring fluorescence intensity and maximum wavelength (Fig. 3B and C). Cooperative unfolding was observed, as in natural globular proteins, and the melting temperatures were 22.4 ± 0.87 (fluorescence intensity) and 24.1 ± 0.66 (wavelength maximum). As described above, the environment around His-18 and Trp-94 was changed at above 10°C. Thus, the conformation around His-18 and Trp-94 in M12345 (1–98) is fragile compared with the structures revealed by monitoring changes of fluorescence intensity and maximum wavelength at higher temperatures.

Hydrolytic Activity of the Fragments—Hydrolytic activity of the fragments can be measured by following the increment of fluorescence intensity of poly eAp (*42*). No hydrolytic activity of M12345 (1–98) was detected in the pH range of 5.8–8.8 (data not shown). This is possibly because the fragment lacks the active residue of wild-type barnase, i.e. His-102, present in M6 (*43*). The hydrolytic activity of a mixture containing an equal amount of M12345 (1–98) and M6 (99–110) was measured, but no activity was detected under these experimental conditions (Fig. 4A). These fragments apparently cannot bind to form a complex that expresses hydrolytic activity. Some peptide fragments with overlapping segments can associate and express activity (*44–46*). For example, Hartley (*47*) showed that

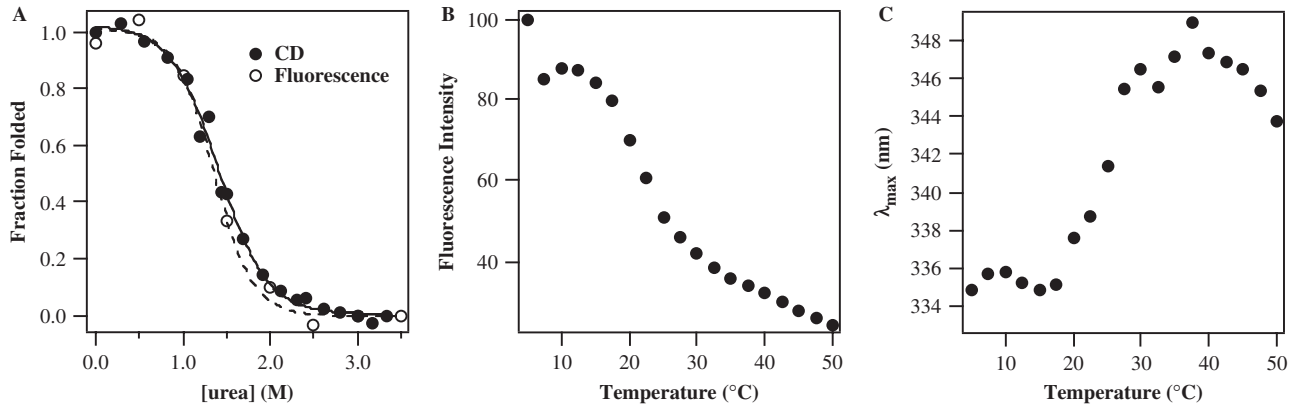

Fig. 3. **Unfolding of M12345 (1–98).** (A) Urea-induced unfolding of M12345 (1–98) monitored by measuring CD (231 nm) and fluorescence (340 nm) at 5°C. Thermal unfolding of M12345 (1–98) monitored by measuring fluorescence intensity (340 nm, B) and maximum wavelength (C).

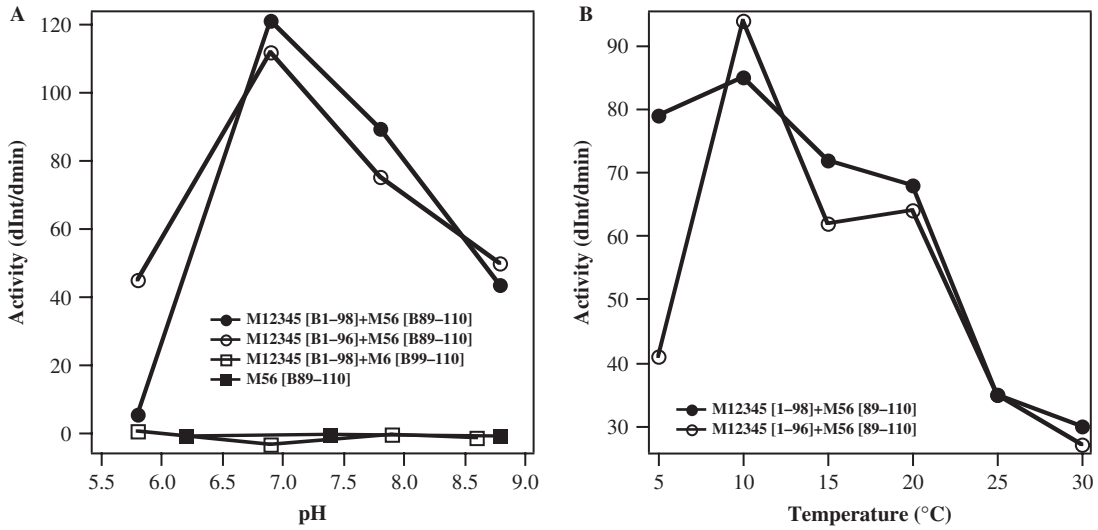

Fig. 4. **The pH (A) and temperature (B) dependency of the hydrolytic activity of M12345 (1–98) and M12345 (1–96) in the presence of M56 (89–110).**

a fragment of barnase, 1–102, associated with 88–110, 95–110, or 99–108 to express RNase activity. Thus, we investigated the hydrolytic activity of a mixture of equal amounts of M12345 (1–98) and M56 (89–110), and found that the mixture had distinct hydrolytic activity (Fig. 4A). M56 (89–110) alone did not show any activity (Fig. 4A) under these experimental conditions. Figure 4B shows the effects of temperature on the hydrolytic activity of the mixture. The optimum temperature was at 10°C, and the activity was lost rapidly above the optimum temperature, as is the case with natural enzymes. The activity of the mixture under optimum conditions was 1% of that of wild-type barnase. Based on the relative activity and some assumptions, we estimated apparent dissociation constant of the functional complex to be ~400 μM (see MATERIALS AND METHODS section). The optimum temperature of wild-type barnase activity is 47°C (*10*).

Interaction of M12345 (1–96) and M56 (89–110)—We examined the interaction between M12345 (1–98) and M56 (89–110) by biophysical methods. The conformational change of M12345 (1–98) induced by binding of M56 (89–110) was detected by CD spectroscopy, but the spectral change was not very clear (data not shown), presumably because M12345 (1–98) already had some ordered and stable conformation. We removed one or two amino acid residues from the C-terminus of M12345 (1–98) to afford M12345 (1–97) and M12345 (1–96) (Fig. 1). The far-UV CD spectrum of M12345 (1–97) was quite similar to that of M12345 (1–98) (Fig. 5A). On the other hand, the spectral shape of M12345 (1–96) was very different from those of M12345 (1–97) and M12345 (1–98), and the strong positive bands <200 nm and the minimum at 230 nm were considerably weakened (Fig. 5A). Although secondary structures remained in M12345 (1–96), some ordered regions in M12345 (1–98) and M12345 (1–97) were apparently destabilized in M12345 (1–96) by the removal of the Tyr-97 residue. The fragment M12345 (1–96) still showed hydrolytic activity in the presence of the M56 (89–110) (Fig. 4).

We used M12345 (1–96) to detect conformational changes induced by the binding of M56 (89–110).

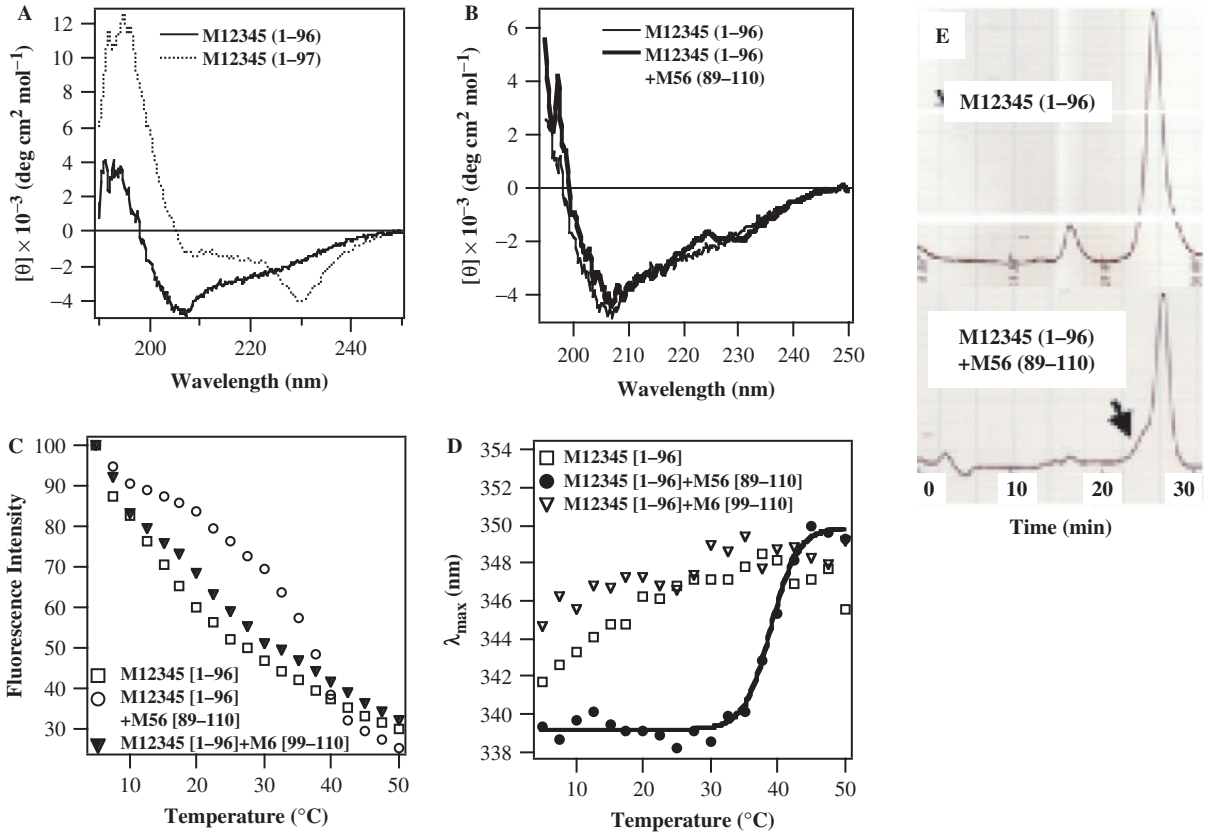

Fig. 5. **Interaction of M12345 (1–96) and M56 (89–110) monitored by biophysical methods.** (A) Far-UV CD spectra of M12345 (1–97) and M12345 (1–96) measured at 5°C. (B) Far-UV CD spectra of M12345 (1–96) in the presence and absence of M56 (89–110) measured at 5°C. (C) Thermal unfolding of M12345 (1–96) monitored by measuring fluorescence intensity at 340 nm in the presence and absence of M56 (89–110) and M6 (99–110). (D) Thermal unfolding of M12345 (1–96) monitored by measuring the wavelength of maximum fluorescence in the presence and absence of M56 (89–110) and M6 (99–110). (E) Gel filtration experiments of M12345 (1–96) in the presence and absence of M56 (89–110).

The far-UV CD spectra of M12345 (1–96) in the absence and presence of M56 (89–110) were compared, and we found that the spectrum measured in the presence of M56 (89–110) showed a distinct minimum at 230 nm, indicating that conformational change, especially around Trp-94, was induced by their interaction (Fig. 5B). The fluorescence intensity and maximum wavelength of M12345 (1–96) were also measured in the presence and absence of M56 (89–110) at various temperatures (Fig. 5C and D). The maximum wavelength of M12345 (1–96) in the presence of M56 (89–110) was slightly shifted toward the blue region compared with that of M12345 (1–96) alone at 5°C, suggesting the formation of hydrophobic environments around tryptophan residue(s) (Fig. 5D). Furthermore, thermal transitions of M12345 (1–96) in the absence and presence of M56 (89–110) were quite different. In short, cooperative transitions of M12345 (1–96) were observed only in the presence of M56 (89–110) (Fig. 5C and D). The mixture of M12345 (1–96) and M6 (99–110) did not show such cooperative transitions (Fig. 5C and D). The melting temperature of the cooperative transition was at 37°C, which is considerably higher than that of the thermal denaturation monitored in terms of hydrolytic activity (Fig. 4B). Therefore, the thermally induced conformational changes monitored with fluorescence might not directly reflect the conformational change around the active site of the complex, but interaction between M12345 (1–96) and M56 (89–110) was clearly observed.

Gel filtration of M12345 (1–96) was performed in the absence and presence of M56 (89–110) (Fig. 5E). When M12345 (1–96) alone was injected into the column, it was eluted mainly as a monomer with a minor fraction of a tetramer. The elution profile upon co-injection of M12345 (1–96) and M56 (89–110) showed an elution peak with a small shoulder (indicated by an arrow). The difference of molecular weight between the complex [M12345 (1–96) + M56 (89–110)] and M12345 (1–96) is 2702 Da, which means that the complex is eluted 1.3 min earlier than M12345 (1–96). Therefore, the shoulder of the peak should be the complex of M12345 (1–96) and M56 (89–110). The reason why the tetramer seen in the elution profile of M12345 (1–96) alone disappeared in the mixture has not been explored yet.

The fluorescence intensity of the complex was investigated at different pH values at 5°C, but a cooperative transition such as that shown in Fig. 2D was not observed.

DISCUSSION

In the present study, we constructed four peptide fragments by progressive elongation of the barnase modules from the N-terminus (Fig. 1). The conformational characterization suggested that M12345 (1–98) had an ordered backbone conformation and aromatic residues buried in hydrophobic environments (Fig. 2A–C). The fragment was present as monomer state as wild-type barnase (Fig. 2F), but had hydrophobic clusters exposed to the solution (Fig. 2E). Thus, M12345 (1–98) appeared to have a partially folded conformation. These results are consistent with the previous report by Neira *et al*. (*48*), who showed that B105 (which comprises the 105 residues from N-terminal end of barnase) had secondary and tertiary structures, but B95 had a disordered conformation with residual secondary structures. Further we constructed M12345 (1–97) and M12345 (1–96), and characterized their backbone conformation. Far-UV CD spectra suggested that the backbone conformation of M12345 (1–97) is quite similar to that of M12345 (1–98), but M12345 (1–96) has only residual secondary structures (Fig. 5A). Thus, Tyr-97 which is involved in formation of hydrophobic $core_3$ in the wild-type barnase (*49*), appears to have a significant role to maintain an ordered conformation of M12345 (1–97) and M12345 (1–98).

The present study shows that M12345 (1–98) does not require M6 (99–110) to form partially folded conformation. Takahashi *et al.* (*50*, *51*) reported a similar result; they constructed mini-barnase (M13456) by deleting M2 from intact barnase, and showed that mini-barnase has some conformational properties that are similar to those of wild-type barnase. Mini-barnase has hydrophobic cores, and unfolds cooperatively with a melting temperature of about 37°C. Conformation of mini-barnase is stable compared with that of M12345 (1–98). This may be due to the fact that M2 forms a small number of hydrogen bonds and hydrophobic contacts with the remaining protein than does M6 (*50*, *51*). Although permutation of modules often breaks the folded conformation of globular proteins (*9*), deleting a module from globular proteins would be a good way to create smaller folded or partially folded proteins as suggested by Takahashi *et al*. (*50*, *51*).

Several pairs of N- and C-terminal fragments of barnase [(B1-22+B23-110), (B1-36+B37-110), (B1-56+B57-110), (B1-68+B69-110), and (B1-79 + 80-110)] can associate each other without overlapped segments to form a native-like structure (*29*, *30*, *51*). In addition, B1-102 can interact with peptide fragments with overlapped segment (B88-110, B95-110 and B99-108) to express RNase activity (*47*). It is proposed that the presence of native-like residual structure in the fragments is required for the formation of functional complex (*52*). Our results showed that M12345 (1–98) and M6 (99–110) could not form such a functional complex (Fig. 4A). This would be due to the reason that a peptide fragment corresponding to M6 does not have enough amounts of residual structure to complement with M12345 (1–98) that has a partially folded conformation. Molecular dynamics simulation study would support this explanation, because the simulation study showed that

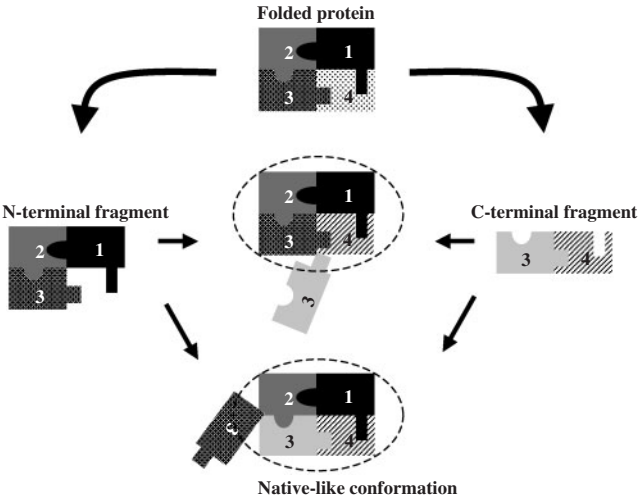

Fig. 6. **Hypothetical model of the interaction between N- and C-terminal fragments of globular proteins.** Both fragments interact to be a complex with marginal stability and activity. A complex would form native like conformation (indicated by dashed circles) by a mechanism similar to functional complementation or 3D domain swapping-like mechanism (*53*).

an isolated fragment corresponding M6 was unstable in comparison with other five peptides corresponding to M1–M5 (*36*). Further, although experiments exploring conformational properties of the six peptides corresponding to barnase modules have been performed extensively (*35*, *38*), there are no reports that find a native-like residual structure in M6 peptide.

We showed that M12345 (1–98) and M56 (89–110) interact with each other to express weak but distinct RNase activity (Fig. 4A). Several lines of evidence for the complementation were obtained from functional and structural analysis of M12345 (1–96). In the presence of M56 (89–110), intrinsically inactive M12345 (1–96) was activated (Fig. 4A). Furthermore, CD and fluorescence measurements showed that conformation around Trp-94 was organized, and thermal stability was increased (Fig. 5B–D). These results would be due to the reason that enough amounts of residual structure which was not detected by CD measurements would be present transiently in longer C-terminal fragment, M56 (89–110). Results obtained in the present study suggest that N- and C-terminal fragments of globular proteins obtained by successive elongation of modules would interact to be a complex with marginal stability and activity (Fig. 6). These molecules would be used as materials to create functional complex by artificial evolution.

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CONFLICT OF INTEREST

None declared.

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