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Protein Anatomy: Structure and Function of Peptide Fragments Corresponding to the Secondary Structure Units of Barnase

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Globular proteins can be decomposed into several modules or secondary structure units. It is useful to investigate the functions of such structural units in order to understand the folding units of proteins. In our previous work, barnase was divided into six peptide fragments corresponding to modules, and some of them were shown to have RNA-binding and RNase activity [Yanagawa, *et al.* (1993) *J. Biol. Chem.* 268, 5861–5865]. Barnase mutant proteins obtained by permutation of the structural units also had RNase activity [Tsuji, T. *et al.* (1999) *J. Mol. Biol.* 286, 1581–1596]. Here we investigated the structure and function of peptide fragments corresponding to secondary structure units of barnase. The results of circular dichroism spectroscopy indicated that some of the peptide fragments form helical structures in aqueous solutions containing over 30% 2,2,2-trifluoroethanol, and the S6 (94–110) peptide fragment is induced to form a β-sheet structure in the presence of RNA. The S6 peptide fragment forms aggregate complexes with RNA. Electron microscopic analysis showed that the aggregate complexes were comprised of filaments. These results indicate that not only modules but also secondary structure units dissected from a globular protein have functional and structure-forming capabilities.

Key words: filamentous structure, module, RNA, secondary structure units, secondary structure.

It is important to search for the smallest meaningful units of structure and function of globular proteins, for two main reasons. One is that such units have been viewed as fundamental building blocks for protein design, and the other is that many proteins fold at least partially *via* small structured intermediates (*1–8*). Some peptide fragments that lack tertiary interactions were shown to adopt native-like structures under certain conditions. For instance, the conformational preferences of peptide fragments corresponding to regions of a secondary structure of T4 lysozyme provided clues to identify potential initiation sites for folding (*7, 8*). Indeed, the functions of such proteins are directly related to their tertiary structures. Many proteins lack intrinsic globular structures under physiological conditions (*9*). For example, RNA-recognition regions of human immunodeficiency virus (HIV-1) Rev protein and bovine immunodeficiency virus (BIV) Tat protein are disordered in aqueous solution, but each region forms a stable α-helical or β-hairpin structure when these proteins bind to Rev response element and trans-acting response element, respectively (*10, 11*). Most reports have described the structure and function of only particular regions of proteins. However, it is also necessary to study the whole protein to understand the principles of protein design and the folding pathways of proteins.

Barnase is a small extracellular ribonuclease produced by the prokaryote *Bacillus amyloliquefaciens*. It is well-characterised as a monomeric α + β domain protein containing 110 amino acids (*12*). Its crystal (*13*) and solution structures (*14*) have been determined, and the structure contains two major α-helices in the first 45 residues and a five-stranded antiparallel β-sheet in the last 65 residues. The entire sequence has been dissected into a series of small overlapping fragments, and their the ability to form secondary structures in the absence of tertiary interactions has been tested (*15–17*). Recently, seven fragments of barnase, obtained by progressive elongation from its N-terminus, have been characterized. The largest fragment (residues 1–105) could fold compactly (*16*). The C-terminal fragments of this ribonuclease have also been characterized. A fragment that involves the whole structure of the protein except the first helix showed native-like near and far-UV circular dichroism (CD) spectra (*17*).

In eukaryotic cells, many genes have intron/exon structures. According to the exon theory of genes, it is predicted that the exons encode functional elements, folding peptides or structural units such as modules, and that the shuffling of exons could account for the evolution of novel proteins (*18, 19*). However, this hypothesis remains controversial (*20, 21*). A module is a compact structural unit in a globular protein (*22*). Module boundaries are closely correlated with the intron positions of genes. This indicates that modules as structural units in a protein correspond to exons as cod-

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Abbreviations: BIV, bovine immunodeficiency virus; CD, circular dichroism; HIV, human immunodeficiency virus; PHF(s), paired helical filament(s); TFE, 2,2,2-trifluoroethanol.

ing regions of the gene. Examining the functions of modules may lead to a better understanding of the meaning of exons. To test this hypothesis, the tertiary structure of barnase was decomposed into six modules (M1–M6) by means of a compactness criterion using centripetal and extension profiles (*23*). The peptide fragments corresponding to the modules of barnase were synthesized and their catalytic functions were examined (*1*). M2, M3, and M6 peptide fragments were found to bind to RNA and to possess RNase activity. M1 peptide fragment in aqueous solution had a predominantly random coil structure at first, but its structure was gradually converted into a mixture of helical and β-sheet structures (*24*). Recently, many barnase mutants have been constructed by permutation of modules or secondary structure units which are extended structure units in globular protein, and their foldability and RNase activity have been examined (*3*). Some mutants were found to possess partially foldable structures and RNase activity (*2*). More mutants having partially foldable structures and RNase activity were obtained by permutation of secondary structure units than by permutation of modules. In this study, peptide fragments corresponding to secondary structure units of barnase were synthesized and characterized to examine whether modules or secondary structure units are more meaningful units of structure and function of barnase.

MATERIALS AND METHODS

Chemicals—Chemicals used are 2,2,2-trifluoroethanol (TFE) (Nacalai Tesque), 5S rRNA (Roche Molecular Biochemicals), and congo red (Nacalai Tesque).

Peptide Synthesis—Peptide fragments corresponding to modules or secondary structure units of barnase were synthesized by a solid-phase method (*25*) using a peptide synthesizer (Biosearch Model 9500). The peptide fragments correspond to M1 (1–24), M2 (24–52), M3 (52–73), M4 (73–88), M5 (88–98), M6 (98–110), S1 (1–16), S2 (17–39), S3 (40–67), S4 (68–81), S5 (82–93), S6 (94–110), S6-1 (95–110), S6-2 (96–110), and S6-3 (97–110), respectively. The synthesized peptide fragments (each with an NH_2-terminal amino group and a COOH-terminal carboxyl group) were cleaved from the resin with hydrogen fluoride by the Low-High procedure (*26*), and purified by successive chromatography on a gel filtration column (Sephadex G-50F or G-25F), an ion-exchange column (CM52 or DE52, Whatman), a reverse-phase column [Shim-pack ODS-PREP (H), Shimadzu], and a desalting column (Sephadex G-10). The purity of each peptide fragment was confirmed by TLC and analytical high-pressure liquid chromatography.

Circular Dichroism Measurement—CD spectra were measured at 5°C by using a JASCO spectropolarimeter model J-600 and an AVIV Instruments circular dichroism spectrometer model 202. Far-UV CD spectra were obtained using a quartz cell with a 1-mm light-path.

Gel Assay—Gel assay was performed at room temperature. A mixture (20 μl) of a peptide fragment (30 μM) and 3 μM 5S rRNA containing 50 mM bis-Tris HCl (pH 6) was incubated for 30 min on ice. The resulting peptide fragment-RNA complexes were electrophoresed on 1.2% agarose (1× TAE gels) and stained with ethidium bromide (Sigma). To calculate the relative amounts of S6 peptide fragment-RNA complexes, 3 μM 5S rRNA was incubated on ice for 30 min with 0–55.5 μM S6 peptide fragment in 20 μl solution containing 5 mM bis-Tris HCl (pH 6). The resulting complexes were electrophoresed on 1.2% agarose (1× TAE gels) and stained with Vistra Green (Amersham Pharmacia Biotech.). The amounts of complexes were measured by using a FluorImager 595 (Molecular Dynamics).

Electron Microscopy—A sample solution containing aggregate complexes was placed on carbon-coated collodion grids, stained with 2% uranyl acetate, and examined under a JEOL 1200 EX electron microscope at 80 eV.

Quantification of Congo Red Binding—Congo red bound to the aggregate complexes was quantified in a mixture containing 2 μM S6 peptide fragment, 1 μM 5S rRNA, 5 μM congo red, 20 mM potassium phosphate (pH 7.4), and 0.15 M NaCl with spectrophotometer DU 640 (Beckman). The concentration of bound congo red (C_b) was calculated using the following equation:

$$C_b = ({}^{540}At/25{,}295) - ({}^{477}At/46{,}306)$$

where ${}^{477}At$ and ${}^{540}At$ are the absorbance in the presence of the aggregate complexes at 477 and 540 nm, respectively (*27*).

RESULTS AND DISCUSSION

Synthesis of Peptide Fragments—As shown in Fig. 1, barnase was divided into six modules (M1–M6) or six secondary structure units (S1–S6) (*1, 3*). We synthesized chemically the peptide fragments corresponding to secondary structure units (S1–S6), modules (M1–M6), and S6 derivatives (S6-1, S6-2, and S6-3) (Table I). In general, the boundaries of modules are positioned in the middle of the β-sheet structure, but those of secondary structure units are positioned in loop regions (Fig. 2).

Structure of Peptide Fragments—The far-UV CD spectra of S1–S6 peptide fragments showed them to be largely unstructured in aqueous solution. However, the S1, S2, and S3 peptide fragments showed two minima at 205 nm and 222 nm in 20–50% TFE solutions (Table II and Fig. 3), indi-

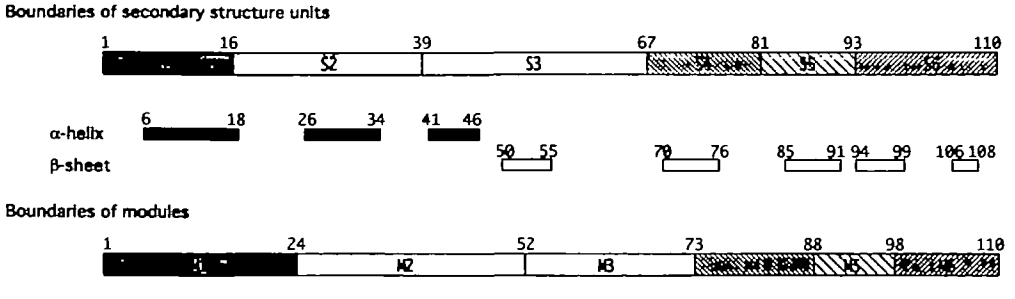


Fig. 1. **Peptide fragments corresponding to modules and secondary structure units of barnase.** Filled and open boxes represent α-helices and β-sheets, respectively.

cating that they take a mainly α-helical conformation in TFE solutions. Among them, the S2 peptide fragment took a definite helical structure even in 20% TFE solution. The S6 peptide fragment showed a broad minimum around 205 nm and a maximum around 195 nm in 30–50% TFE solutions, indicating that it contains both α-helical and β-sheet structures. The peptide fragments other than S1 showed no change in CD spectra after incubation at 4°C for several days. S1 peptide fragment precipitated after incubation at 4°C for several days in the presence or absence of TFE.

It is not surprising that peptide fragments are predominantly unstructured in aqueous solution as this tendency has been observed for most β-sheet–derived peptide fragments in aqueous solution (*1, 5, 7, 8, 16, 17, 24*). For example, a peptide fragment corresponding to the β-sheet region of T4 lysozyme (*7*) showed little tendency to adopt secondary structure in aqueous solution. TFE stabilizes α-helical structure only in peptide fragments or protein regions with an inherent helical propensity (*28*). Robson's protein secondary structure prediction analysis (*29*) showed that only S2 peptide fragment of the peptide fragments corresponding to the secondary structures of barnase has a propensity for helix formation from residues 13 to 21 in aqueous solution. The CD data were consistent with the secondary structure prediction of S2 peptide fragment.

TABLE I. **Chemically synthesized peptide fragments corresponding to secondary structure units and modules of barnase.**

Name	Sequence
S1	A QVI NTF DGVAD YLQT
S2	Y HKL PDNYI TKS EAQALGWV ASK
S3	G NLA DVA PGKSI GGDI FSNR EGKLPGKS
S3	G NLA DVA PGKSI GGDI FSNR EGKLPGKS
S4	G RTWREA DI NYT SG
S5	F RNS DRI LYSSD
S6	WLI Y KTT DHYQT FTKI R
M1	A QVI NTF DGVAD YLQTYHK L PDNY
M2	Y I TKSEA QALGWVAS KGNL A DVAPGKSI G
M3	G GDI FSNREGKL PGKS GRT WRE
M4	E ADI NYT SGFRNS DRI
M5	I LYS SDWLI YK
M6	K TTD HYQ TFTKI R
S6-1	L I YK TTD HYQTF TKI R
S6-2	I YKT TDHYQTFT KI R
S6-3	Y KTT DHY QTFTK I R

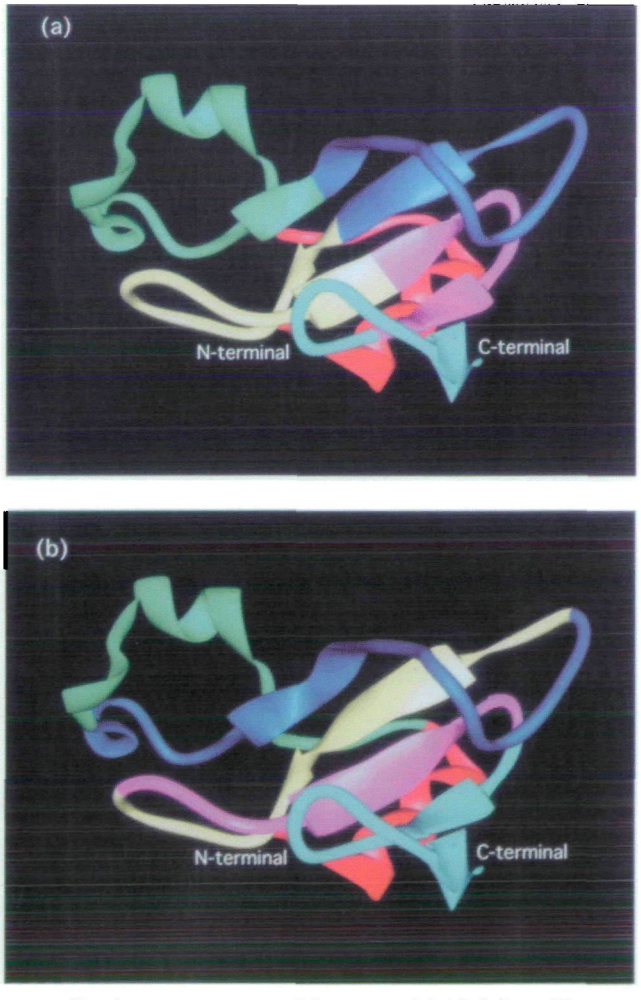


Fig. 2. **Tertiary structure of barnase.** (a) Modules of barnase shown by different colors. M1, M2, M3, M4, M5, and M6 correspond to red, green, blue, yellow, pink, and sky-blue regions, respectively. (b) Secondary structure units of barnase shown by different colors. S1, S2, S3, S4, S5, and S6 correspond to red, green, blue, yellow, pink, and sky-blue regions, respectively.

TABLE II. **Formation of secondary structures of peptide fragments in aqueous TFF solutions.**

Peptide fragments	TFF						
	0%	5%	10%	20%	30%	40%	50%
S1	R	R	R	R	α	α	α
S2	R	R	R	α	α	α	α
S3	R	R	R	R	α	α	α
S4	R	R	R	R	R	R	R
S5	R	R	R	R	R	R	R
S6	R	R	R	R	α + β	α + β	α + β

R, α and α + β indicate random coli, α-helix, and a mixture of α-helix and β-sheet, respectively.

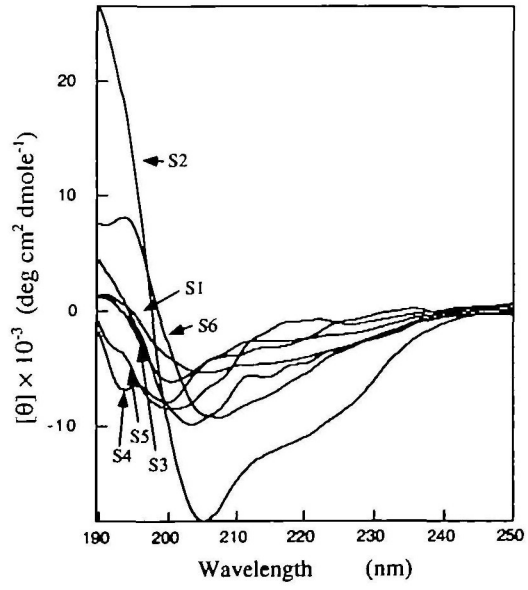


Fig. 3. **CD spectra of peptide fragments in TFE solutions.** The CD spectra of S1–S6 peptide fragments were measured in a solution containing 10–30 μM each peptide fragment, 5 mM bis-Tris HCl (pH 6) and 50% TFE.

RNA Binding of S6 Peptide Fragment—The interaction of peptide fragments corresponding to modules or secondary structure units with RNA was further examined by gel assay (Fig. 4). 5S rRNA mobilities and quantities in the presence of peptide fragments other than S6 were similar to those in the absence of the peptide fragments (Fig. 4, A and B). The quantity of 5S rRNA decreased in the presence of S6 peptide fragment, and 5S rRNA was stacked in the well (Fig. 4C). 5S rRNA decreased with increasing amounts of S6 peptide fragment, and the amounts of the resulting aggregate complex increased. When 3 μM 5S rRNA and 30 μM S6 peptide fragment were mixed, the formation of S6 peptide fragment–5S rRNA complex reached a plateau (Fig. 4D). These results indicate that S6 peptide fragment interacted with 5S rRNA and formed the aggregate complex. The quantity of the complex was dependent on the concentration of S6 peptide fragment.

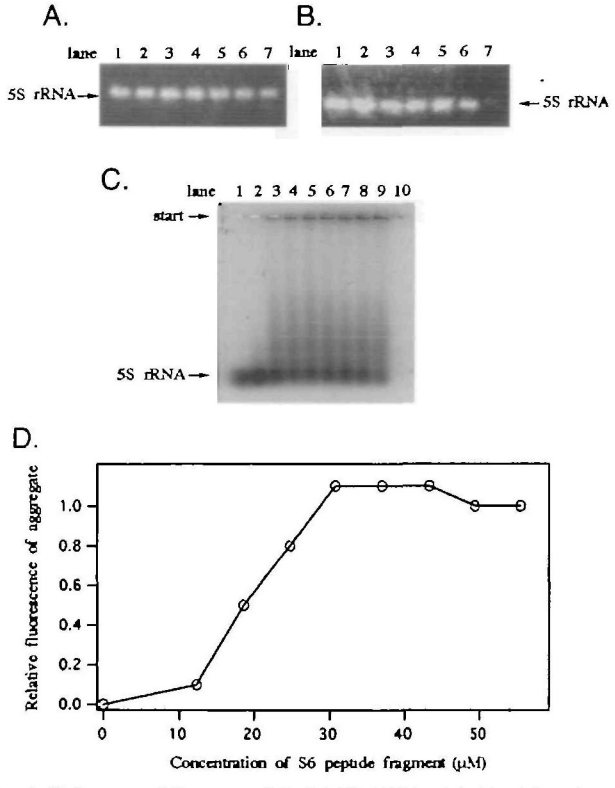


Fig. 4. **Gel assay.** Mixtures of 3 μM 5S rRNA with 30 μM each peptide fragment corresponding to modules (A) and secondary structure units (B) were incubated on ice for 30 min in a solution containing 5 mM bis-Tris HCl (pH 6). These mixtures were electrophoresed on 1.2% agarose gel and stained with ethidium bromide. (A) lane 1, no peptide fragment; lanes 2–7, M1–M6 peptide fragments, respectively. (B) lane 1, no peptide fragment; lanes 2–7, S1–S6 peptide fragments, respectively. (C) Dependence of the aggregate complex formation on the concentration of S6 peptide fragment. Mixtures of 3 μM 5S rRNA with 0–55.5 μM S6 peptide fragment were incubated on ice for 30 min in a solution containing 5 mM bis-Tris HCl (pH 6). These mixtures were electrophoresed on 1.2% agarose gel and stained with Vistra Green. Lanes 1–10 correspond to 0, 12.3, 18.5, 24.7, 30.8, 43.2, 49.3, and 55.5 μM S6 peptide fragment, respectively. (D) Graph of dependence of the aggregate complex formation on the concentration of S6 peptide fragment. The amounts of the aggregate complex at each concentration were compared with that at 49.3 μM S6 peptide fragment.

The CD spectra of S1–S5 peptide fragments in the presence of 5S rRNA were much the same as those in the absence of 5S rRNA. Thus, 5S rRNA did not affect the formation of the secondary structures of S1–S5 peptide fragments. As shown in Fig. 5, only S6 peptide fragment interacted with 5S rRNA and formed the β-sheet structure. The amount of the β-sheet structure was dependent on the relative concentration of S6 peptide fragment to 5S rRNA. The β-sheet structure formation definitely occurred at 5S rRNA/S6 peptide fragment ratios greater than 0.2, when the concentration of 5S rRNA was varied at a constant concentration of 10 μM S6 peptide fragment (Fig. 5).

The CD spectra of S6 peptide fragment were measured in the presence and absence of 5S rRNA at different pHs and temperatures. In the absence of 5S rRNA, the CD spectrum of S6 peptide fragment showed it to be unstructured. In the presence of 5S rRNA, the efficient β-sheet structure formation of S6 peptide fragment was observed at pH 5–8 (Fig. 6), and it was shown that the pH optimum for RNase activity of barnase is 7.5–8.5 (*30, 31*). These pH values largely overlap with in the optimum pH for the β-sheet structure formation of S6 peptide fragment in the presence of 5S rRNA. Further, the CD spectrum of S6 peptide fragment in the presence of 5S rRNA showed a predominantly

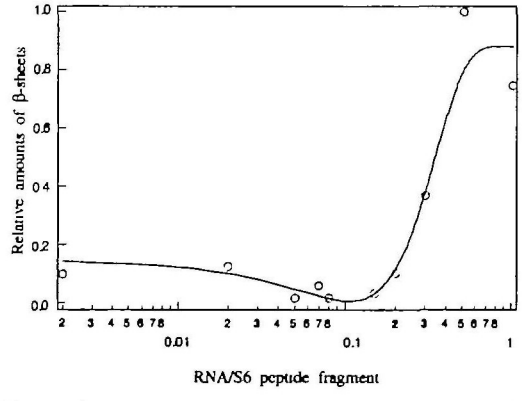


Fig. 5. **Dependence of β-sheet structure formation on the concentration of 5S rRNA.** To investigate the amounts of β-sheet structure in solutions containing 10 μM S6 peptide fragment, 5 mM bis-Tris HCl (pH 6), and 0.02, 0.2, 0.5, 0.7, 0.8, 1, 1.5, 2, 3, 5, or 10 mM 5S rRNA, the ellipticity at 218 nm was monitored. For convenience, values are normalized to the value at 5 μM 5S rRNA.

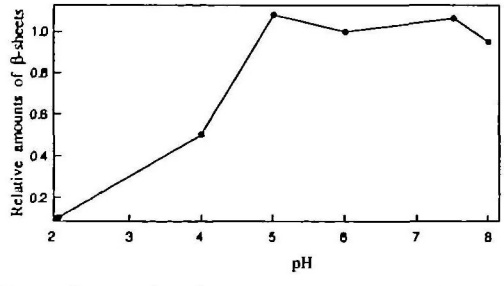


Fig. 6. **Dependence of β-sheet structure formation on pH.** To investigate the amounts of β-sheet structure in solutions containing 5 μM 5S rRNA and 10 μM S6 peptide fragment at 5°C and different pHs, the ellipticity at 218 nm was monitored. For convenience, values are normalized to the value at pH 6.

β-sheet conformation with a bell-shaped temperature optimum at around 42°C (Fig. 7). The optimum temperature of the β-sheet structure formation was close to that of RNase activity of native barnase (*6*), and the S6 region includes the active-site residue His102 and two β-sheets in native barnase (*14*). These results suggest that S6 is a very important functional unit for the formation of the secondary structure of the active-site of barnase.

The CD spectrum of M6 (98–110) peptide fragment showed it to be largely unstructured in the, presence and absence of 5S rRNA (data not shown). The CD spectrum of M5 (88–98) peptide fragment, however, showed a predominantly β-sheet conformation (data not shown). These results suggest that the four residues at the N-terminus of S6 peptide fragment are important for β-sheet structure formation. To search for sequences capable of forming β-sheet structure, three deletion mutants (S6-1, S6-2, and S6-3) of the S6 peptide fragment were chemically synthesized (Table I). In the presence of 5 μM 5S rRNA, the CD spectra of S6 and S6-1 peptide fragments showed a predominantly β-sheet conformation, while the CD spectra of S6-2 and S6-3 peptide fragments showed them to be largely unstructured (Fig. 8). In the absence of 5S rRNA, the CD spectra of all S6 deletion mutants and S6 peptide fragments showed them to be mainly unstructured. These results indicate that S6-1 peptide fragment contains the key residues for β-sheet conformation in the presence of 5S rRNA, and that a leucine residue which was deleted from S6-1 peptide fragment played a particularly important role in the β-sheet structure formation. It was previously shown that most of the sequence of barnase is necessary for folding (*16, 17*), but our results suggest that a shorter sequence of baranse is necessary for folding in the presence of RNA. Thus, not only within protein interactions, but also protein–substrate interactions may be important for protein folding.

Assembly of S6 Peptide Fragment—It is known that many peptide fragments and proteins can self-assemble and form amyloid fibrils (*32, 33*) with predominant β-sheet conformation. For instance, the microtubule-associated protein tau is a main component of the paired helical filaments (PHFs) of Alzheimer's disease. The previous work showed that the peptide fragment corresponding to M1 region of barnase had a predominantly random coil structure, which was gradually converted into mixed helical and β-sheet structures. This was mediated by aggregation, and the aggregate was shown to be comprised of filamentous helical structures (*24*). In this study, S6 peptide fragment did not form a filamentous structure in the absence of 5S rRNA, but it formed a definite filamentous structure in the presence of 5S rRNA (Fig. 9). The S6 peptide fragment–5S rRNA complex consisted of filamentous structures with some branches. When a solution containing this complex was incubated at 4°C for a month, the filaments grew longer. The amounts of β-sheet structure measured by CD spectroscopy also increased after longer incubation (data not shown). These results indicate that S6 peptide frag-

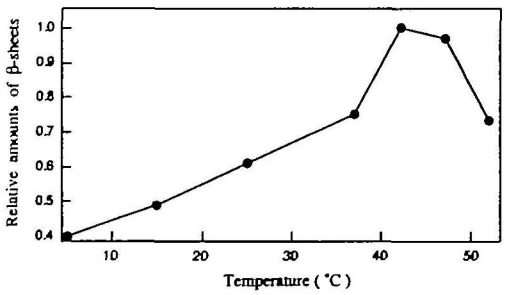


Fig. 7. **The temperature dependency of β-sheet structure formation.** To investigate the amounts of β-sheet structure formation in a solution containing 10 μM S6 peptide fragment, 5 μM 5S rRNA and 5 mM bis-Tris HCl (pH 6) at different temperatures, the ellipticity at 218 nm was monitored. For convenience, values are normalized to the maximum value.

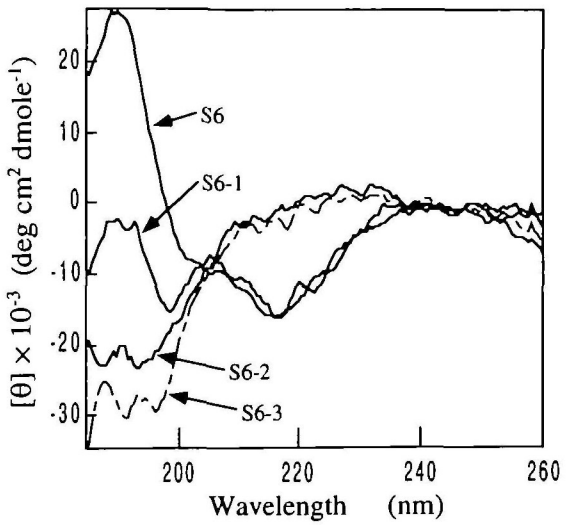
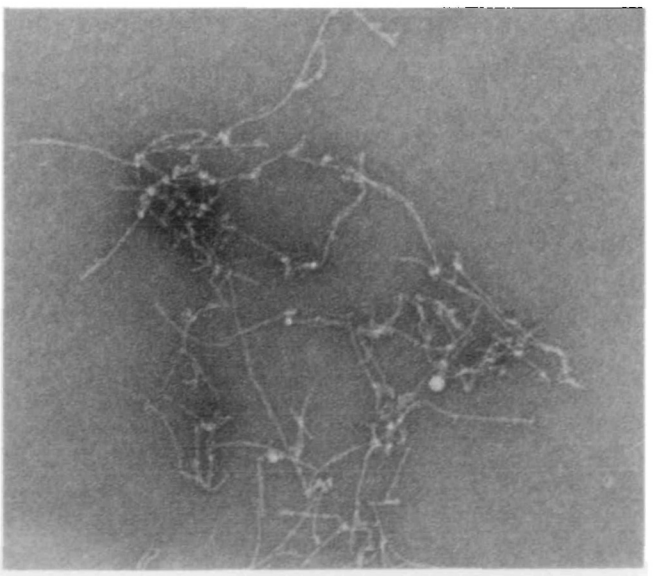


Fig. 8. **The CD spectra of deletion mutants of S6 peptide fragment.** The CD spectra of deletion mutants of S6 peptide fragment were measured in a solution containing 10 μM each peptide fragment, 5 μM bis-Tris HCl (pH 6), and 5 μM 5S rRNA.



300 nm

Fig. 9. **Electron micrographs of the filaments of S6 peptide fragment-5S rRNA complex.** Sample solutions were incubated at 4°C for a month. The preparation of negatively stained samples is described in "MATERIALS AND METHODS."

ment formed the β-sheet structure in the aggregate complex. Congo red is known to stain amyloid protein like tau protein, because it binds to the β-pleated sheet conformation, and the concentration of bound congo red can be calculated based on the absorbance (*27*). The concentration of congo red bound to S6 peptide fragment–5S rRNA complex was calculated to be 1.9 μM, and congo red did not bind to S6 peptide fragment (Fig. 10). The results of the congo red binding experiment are consistent with the electron microscopy and the CD spectroscopy results.

In the absence of 5S rRNA, the CD spectrum showed the M5 peptide fragment to have predominantly β-sheet conformation when its concentration was over 30 μM (data not shown). This result indicates that the M5 peptide fragment could self-assemble and form secondary structure in aqueous solution. To investigate whether S6 peptide fragment can also self-assemble and form secondary structure, its CD spectrum was measured at various concentrations. The CD spectrum showed it to be unstructured (data not shown), indicating that the concentration of S6 peptide fragment did not affect the formation of its secondary structure.

In this study, it was found that some peptide fragments corresponding to not only modules but also secondary structure units formed the secondary structures under certain conditions, and the structures were correlated with secondary structures of barnase. Our results also indicate that the secondary structure units may be small functional units. In particular, S6 peptide fragment bound to 5S rRNA and formed filaments with a β-pleated sheet conformation. When only two residues were deleted, the function of S6 peptide fragment was lost (Fig. 8), although S6, S6-1, S6-2, S6-3, and M6 peptide fragments possess the same net charges. Probably, the pathway of formation of S6 peptide fragment–5S rRNA complex consists of two steps: first S6 peptide fragment binds 5S rRNA through interactions of the net charges, and next the fragments assemble through hydrophobic interactions. S6-2, S6-3, and M6 might not be sufficiently hydrophobic for the second step to occur. Further, our results also support the hypothesis that various secondary structure units possessing distinct functions might have played a role in early biological evolutionary processes.

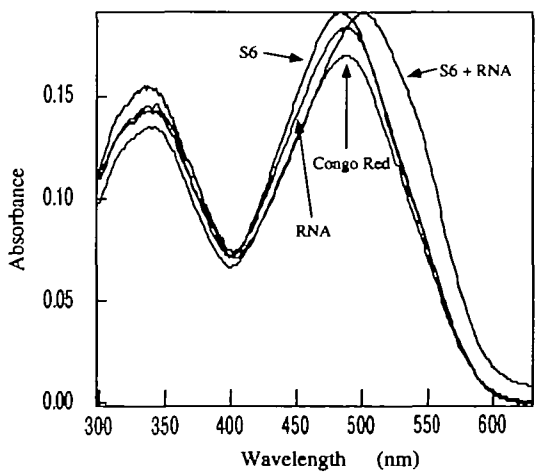


Fig. 10. **Absorbance spectra of congo red in the presence of S6 peptide fragment, 5S rRNA, and a mixture of S6 peptide fragment and 5S rRNA.** Absorbance spectra of a 5 μM congo red solution were measured in the presence of 2 μM S6 peptide fragment, 1 μM RNA or a mixture of S6 peptide fragment (2 μM) and 5S rRNA (1 μM).

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