Protein Anatomy: Functional Roles of Barnase Module*

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Globular proteins are composed of several modules that are contiguous polypeptide segments of compact
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lated with the intron positions of genes that encode proteins. The modules may thus have a one-to-one correspondence with exons in primordial genes. They may also be vestiges of polypeptide segments that initially appeared as primordial proteins in prebiological evolution. Clarification as to whether modules disconnected from one another have functional potentiality may validate these possibilities. Thus, in this study, each module of a protein was synthesized and assessed for functional potentiality. For this purpose, barnase, a bacterial ribonuclease, was decomposed into six modules (M1-M6), which were examined to determine whether they have an affinity for RNA and RNase activity. M2, M3, and M6, all of which form a shallow but wide cavity for RNA binding in native barnase, were found to bind to RNA and to possess RNase activity. However, M1 and M5, which support the other modules from the back side, and M4 did not bind to RNA and had no RNase activity. Protein modules with catalytic functions are described in this paper for the first time. That some modules of barnase possess catalytic activity indicates that protein modules may possibly have functioned as primitive catalysts in prebiological evolution.

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**MATERIALS AND METHODS**

The tertiary structure of barnase was decomposed into six modules (M1-M6) by compactness criterion introduced by distance calculation between Cα atoms (3). Module boundaries at residues 24, 52, 73, 88, and 98 were assigned quantitatively as the local minima of centripetal profiles calculated based on the distance between Cα atoms (2). Compactness of the six modules (M1, residues 1-24; M2, residues 25-52; M3, residues 53-73; M4, residues 74-88; M5, residues 89-98; and M6, residues 99-110) had been synthesized. The resulting peptides (each with an NH2-terminal amino group and a COOH-terminal carboxyl group) were cleaved from the resin with hydrogen fluoride by the Low-High procedure (13) and purified by successive chromatography on a gel filtration column (Sephadex G-50F or G-25F), ion-exchange column (CM52 or DE52, Whatman), reversed-phase column (Shim-pack ODS-PREP(H), Shimadzu), and desalting column (Sephadex G-10). The purity of each peptide thus obtained was confirmed by TLC, analytical high pressure liquid chromatography, and amino acid analysis; and peptide sequences were determined as follows.

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The nucleotide sequence was determined by comparison of the degradation patterns of E. coli 5 S rRNA with those of partial alkaline digests on enzymatic sequencing gels. These bands can easily be recognized by the characteristic spacing they produce in the ladders.

Electrophoresis of barnase modules was carried out on SDS-polyacrylamide gel (16.5% T, 6% C; 13.5 x 15 x 0.1 cm) according to the method of Schagger and Von Jagow (14), except that yeast RNA (10 mg/15 ml) was added to the separation gel, and the gel was treated with 12.5% glutaraldehyde before staining with Coomassie Brilliant Blue.

Activity staining (15) was performed as follows. To remove SDS, after SDS-polyacrylamide gel electrophoresis, the gel was washed for 30 min at room temperature in 250 ml of 8 mM Tris-HCl, pH 8.0, containing 20% 2-propanol, followed by washing for 15 min in 150 ml of 10 mM Tris-HCl, pH 8.0. The gel was further incubated to cleave RNA for 2 h at 37 °C in 150 ml of 10 mM Tris-HCl, pH 8.0. The gel was washed for 30 min at room temperature in 200 ml of 10 mM Tris-HCl, pH 7.5, containing 0.2% toluidine blue O (Merek), and washed in 10 mM Tris-HCl, pH 7.5, to detect the activity bands.

Module-RNA binding was examined by membrane filter assay. A module (10 μM) was added to 0.1 M HEPES/NaOH, pH 7.5, containing 0–10 A600 units of E. coli MRE600 5 S RNA, incubated for 30 min at 4 °C, and then filtered through a Millipore Ultrafree C3LCC00 membrane filter (exclusion limit, M, 5000). An unbound module was recovered in the filtrate. No 5 S RNA was cleaved by modules under the conditions described above. The amount of an unbound module in the filtrate was determined by fluorometric assay of proteins (16). The amount of a bound module to 5 S RNA was calculated after subtracting that of an unbound module from the total amount of a module.

RESULTS AND DISCUSSION

The RNase activity of modules of barnase toward E. coli 5 S rRNA was assessed by polyacrylamide gel electrophoresis under denaturing conditions. A sample of 5 S rRNA was incubated with 140 μM barnase modules in 0.1 M Tris-HCl, pH 7.5, at 37 °C for 15 h. As shown in Fig. 1 (lanes 2, 3, and 6), 5 S rRNA was very prominent in the controls (lane 7) and was cleaved, and polynucleotide cleavage products were formed. It is thus evident that M2, M3, and M6 catalyze the cleavage of 5 S rRNA, whereas M1, M4, and M5 possess no detectable RNase activity (lanes 1, 4, and 5). Native barnase also catalyzed the cleavage of 5 S rRNA. The cleavage patterns of M2, M3, and M6 differed remarkably from that of native barnase. M3 showed the highest RNase activity of the three modules, followed by M2. Although M2, M3, and M6 are ribonucleolytic enzymes, their activity toward 5 S rRNA was considerably less than that of native barnase. Decomposition of barnase into modules lowered catalysis by 4 orders of magnitude. The RNase activity of M2, M3, and M6 was higher at 55 °C and pH 9.5 than at 37 °C and pH 7.5 (data not shown). No amino acid mixture corresponding to the amino acid composition of M2, M3, or M6 catalyzed the cleavage of 5 S rRNA under the identical conditions.

To determine whether the RNase activity associated with modules was due to contamination of enzymes with higher RNase activity, high molecular weight (M, >10,000) contaminants with RNase activity in the synthetic modules were removed by autoclaving and ultrafiltration. M2, M3, and M6 retained significant RNase activity after autoclaving at 120 °C for 10 min or after ultrafiltration with a Millipore Ultrafree C3LCC00 membrane filter (exclusion limit, M, 5000).

To exclude further the possibility of contamination of RNases, RNase activity was examined by cleavage of RNA embedded in SDS-polyacrylamide gels. Synthetic modules of barnase (M2, M3, and M6) were found to show significant RNase activity, whereas none could be detected for M1, M4, or M5 (Fig. 2b). The activity staining bands coincided well with the corresponding protein staining bands (Fig. 2a). This (together with the finding that RNase activity was retained following autoclaving and ultrafiltration) confirms that M2, M3, and M6 actually possess real RNase activity. M2 formed a dimer and a trimer also with RNase activity (Fig. 2, a and b).

The membrane filter assay for binding of modules of barnase with RNA has indicated that some modules possess RNA binding activity. M2, M3, and M6 bound to E. coli 5 S RNA; but M1, M4, and M5 lacked RNA binding activity (Fig. 3).
This is consistent with the experimental results for RNA cleavage. The RNA binding activity of modules of barnase was also measured with E. coli tRNA. M2, M3, and M6 were capable of binding to tRNA under conditions identical to those for 5 S rRNA, while M1, M4, and M5 were incapable of doing so (data not shown).

Glu-73, Arg-87, and His-102 were found to be the catalytic sites of barnase (17). M3 and M6, containing Glu-73 and His-102, respectively, showed RNase activity. Although M4 has two catalytic sites (Glu-73 and Arg-87), it possessed no RNase activity. M2, with no catalytic sites, showed catalytic function. It was quite recently suggested that the positive-charge side chains, Lys-27, Arg-59, and His-102 present on M2, M3, and M6, respectively, are clustered in the active site of barnase and that they are necessary for catalysis and binding of the negatively charged RNA substrate (18).

The use of excess basic amino acid residues (Arg and Lys), rather than acidic ones (Asp and Glu), in the modules may provide some indication of catalytic activity of the modules. M2 and both M3 and M6 have 1 and 2 extra basic amino acid residues, respectively; but M1 and M4 have 2 and 1 extra acidic amino acid residue, and M5 has the same number of acidic and basic residues. Basic amino acid residues have an important role in phosphate binding, and possibly the RNase activity of M2, M3, and M6 may be explained on the basis of the basic amino acid residues.

To estimate further the importance of the net charge on the barnase modules to RNA binding and RNase activities, we chose 22 control peptides possessing net charges of −3 to +5. Table I shows the sequences of the barnase modules and control peptides that are synthesized and that correspond to the short segments (15–26-mer) of different proteins. The control peptides with net charges of more than +3 bound to 5 S rRNA and catalyzed the cleavage of the RNA quite well, whereas this could not be detected for the control peptides possessing negative net charges. These data suggest that the net charges on the peptides are important in RNA binding and RNase activity. In the control peptides possessing net charges of 0 to +2, some had both RNA binding and RNase activities or only RNA binding activity, and others had no significant affinity for RNA and no RNase activity. This finding suggests that the peptides possessing net charges of 0 to +2, at least in the context of these peptides, are not sufficient for RNA binding or RNase activity. Since the net charges on M2, M3, and M6 are within the range of 0 to +2, there may be a different situation in the module itself.

Circular dichroism spectroscopy was used to estimate the level of secondary structure of the barnase modules and control peptides. The use of excess basic amino acid residues (Arg and Lys), rather than acidic ones (Asp and Glu), in the modules may provide some indication of catalytic activity of the modules. M2 and both M3 and M6 have 1 and 2 extra basic amino acid residues, respectively; but M1 and M4 have 2 and 1 extra acidic amino acid residue, and M5 has the same number of acidic and basic residues. Basic amino acid residues have an important role in phosphate binding, and possibly the RNase activity of M2, M3, and M6 may be explained on the basis of the basic amino acid residues.

### Table 1

| Barnase modules | Sequence | No. of amino acids | Net charge | RNA binding* | RNase activity* |
|-----------------|----------|--------------------|------------|--------------|----------------|
| M1              | AQtTNTGFDGVADYLTQHYKLPDNY | 24 | −2 |  |  |
| M2              | YITKSEQALGKVASKGNLADVAPGKSIG | 29 | +1 | ++ | ++ |
| M3              | GGGFNSRREGKLPKSGRTWRE | 22 | +2 | ++ | ++ |
| M4              | EADINYRTSFBNDR | 16 | −1 |  |  |
| M5              | ILYSSDLWLYK | 11 | 0 |  |  |
| M6              | KTTTDHYQTFTKIR | 13 | +2 | + | + |
| Control peptides* | | | | | |
| PKC1            | NQREFKGFSGFYGEDLMP | 17 | −3 | − | − |
| RGR2            | ATSTINGDSENNENSSNGNN-NH2 | 20 | 3 |  | + |
| PKC2            | VNPWTPITISPNNPDES-NH2 | 15 | −3 | − | − |
| BRA5            | IRDNPDVIKALGEA | 15 | 1 |  |  |
| TAUS            | DHGAEVYKSPVVG | 15 | −1 | − |  |
| CYT             | ISAVHAAABINEARG | 17 | −1 | − |  |
| BRA4            | AEGFSYTDANKNKGIT | 16 | 0 |  |  |
| BRA3            | QGQLPMFMHELK | 15 | 0 |  |  |
| BRA2            | PGLKESPLQIGAAGPLK | 17 | +1 |  |  |
| BRA1            | LSQDSKEKAPITLPLLPG-NH2 | 17 | +1 | − | − |
| MGP             | SSLNQERDLRYGG-NH2 | 15 | 1 |  |  |
| RBP             | PCTRSPAGGWWQAPQGPAAPLFF | 26 | +1 | ND |  |
| CDC2            | GKMALKHPTPDLDNQIKKM | 20 | +1 | + | ++ |
| CKD2            | AALAHPPGQVDTVPVPHLRL | 20 | +1 | ++ | ++ |
| RGR1            | ALKRNSEQQNGEQGQPV-NH2 | 18 | +2 | ++ | ++ |
| RGR3            | LDTKRIGLETPVANPS-NH2 | 18 | +2 | ++ | ++ |
| OVA1            | VMNEEKLKIVLPRM | 15 | 0 | ++ | ++ |
| TAU1            | VAVTKRDPPPSIAAAK | 15 | 0 | ++ | ++ |
| BRA1            | GYSIERVAYRPLGCRN-NH2 | 17 | +4 | ++ | ++ |
| PKC2            | AMFPFTNRGSIQKQIK | 17 | +4 | ++ | ++ |
| PKC3            | GEDKSIYRRSRWRKL | 17 | +5 | ++ | ++ |

* RNA binding was measured by the membrane filter assay method as described under “Materials and Methods.”

* RNA binding activity was measured by the activity staining method as described under “Materials and Methods.”

PKC1, residues 721–737 of murine brain protein kinase C-α (19); RGR1–RGR3, residues 23–40, 320–340, and 1065–1082 of a protein required for glucose repression of Saccharomyces cerevisiae, respectively (20); PKK, a rat brain protein (Y. Arimatsu et al., unpublished data); BRA1–BRA3, residues 87–123, 123–159, and 291–307 of a BraD protein, respectively, BRA4, residues 403–417 of a BraF protein and BRA5, residues 241–255 of a BraF protein, which are proteins for a Pseudomonas aeruginosa branched-chain amino acid transport system (22); TAU1, residues 1–17 of a bovine brain tau protein (23); TA2 and TAU3, residues 168–182 and 298–312 of a human tau protein, respectively (24); CYT, residues 43–58 of a pigeon cytochrome c (25); OVA1 and OVA2, residues 271–285 and 323–339 of chicken ovalbumin, respectively (26); MGP, residues 137–151 of chicken myoglobin (27); RBP, residues 1–28 of a rat brain-related protein (T. Hoshide et al., unpublished data); CDC2, residues 279–289 of mouse CDC2 (29); CDC2, residues 279–299 of human p34 protein kinase (30); PKC2, residues 137–153 of rat brain protein kinase C-β (31); PKC3, residues 109–125 of protein kinase C-f (32).

* Not determined (because of lack of a primary amino group at the NH₂-terminus).
control peptides. It was clear from the spectra that the barnase modules (M2, M3, and M6) as well as the control peptides take predominantly a random coil in aqueous solution (data not shown). These data suggest that the differences in RNA binding and RNase activity for the different peptides are probably not due to differences in secondary structure.

Native barnase cleaved a phosphodiester bond of GpN in the loop and hinge regions (Fig. 4a). This result indicates that barnase has a strong preference for guanosine in partial digestion of E. coli 5 S rRNA. Barnase is well known to cleave after only guanosine when catalyzing the hydrolysis of the dinucleotide substrate GpN (33). With longer substrates, however, it preferentially cleaves after guanosine; but it cleaves after other bases, yielding a mixture of mono- and dinucleotides in a total RNA digest (34). Barnase shows the preference order $A > G > C \approx U$ (35).

On the other hand, barnase module (M2, M3, and M6)-induced cleavages in the E. coli 5 S rRNA (Fig. 1) were mostly located in the loop and hinge regions, but their cleavage sites were different from those of native barnase (Fig. 4b). In the loop regions, one strong cleavage occurred at positions 103 and 104. This result indicates that flexible and dynamic regions appear as preferred target sites for barnase module-induced cleavage. The barnase modules preferentially cleaved a phosphodiester bond of YpA. Therefore, the barnase modules are quite different from native barnase, but they resemble pyrimidine-specific bovine pancreatic RNase A (36) in their preference for nucleoside.

The 3'-labeled cleavage products (Fig. 1) from E. coli 5 S rRNA with native barnase and barnase modules showed the same migration as the limited alkaline cleavage product of the same length (data not shown). This indicates that the cleavage products bear a 3'-phosphate terminus on the 3'-terminal fragment.

The presence of a basic amino acid residue in a position favorable for catalysis may be essential for the RNase activity of barnase modules because basic amino acid residues are frequently found in the active site of hydrolytic enzymes. It appears that phosphate binding of native barnase is affected mainly by positively charged residues including His-102, Lys-27, and Arg-87 (18).

M6 with a net charge of +2 showed lower RNA binding and RNase activities than M2 and M3 with net charges of +1 and +2, respectively. Some factors may be responsible for RNA binding, RNase activity, and specificity of barnase modules. Among the factors, imbalance of positively charged amino acid residues and peptide chain length in barnase modules together with their net charge may be much more important. Positively charged residues were delocalized on barnase modules and control peptides possessing RNA binding and RNase activity (Table I). A basic polypeptide such as poly(Leu-Lys) with alternating basic and hydrophobic residues cleaves oligoadenylates more efficiently than poly(Lys) (38). This suggests the importance of imbalance of positively charged residues and hydrophobicity for more efficient cleavage activity. High salt concentration (1 M NaCl) strongly inhibited the RNA binding of M2, M3, and M6 (data not shown). Thus, the interaction between a peptide and RNA appears to be primarily ionic in nature and secondarily nonelectrostatic. M2, M3, and M6 consist of 29, 32, and 13 amino acid residues,
charged species such as substrate, metal ions, cofactors, or other ligands.

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