Structure and Binding Mode of a Ribosome Recycling Factor (RRF) from Mesophilic Bacterium*

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Hiroaki Nakano†, Takuya Yoshida‡, Susumu Uchiyama‡, Masako Kawachi‡, Hitomi Matsuo‡, Takayuki Kato‡, Atsushi Ohshima‡, Yoshiharu Yamaichi‡, Takeshi Honda‡, Hiroaki Kato*, Yuriko Yamagata**, Tadayasu Ohkubo†, and Yuji Kobayashi‡‡

From the †Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan, the ‡Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan, the ‡Membrane Dynamics Research Group, RIKEN, Harima Institute at SPring-8, 1-1-1 Koto, Mikazuki-cho, Sayo, Hyogo 679-5148, Japan, and the **Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan

X-ray and NMR analyses on ribosome recycling factors (RRFs) from thermophilic bacteria showed that they display a tRNA-like L-shaped conformation consisting of two domains. Since then, it has been accepted that domain I, consisting of a three-helix bundle, corresponds to the anticodon arm of tRNA and domain II and a βαβ sandwich structure, corresponds to the acceptor arm. In this study, we obtained a RRF from a mesophilic bacterium, Vibrio parahaemolyticus, by gene cloning and carried out an x-ray analysis on it at 2.2 Å resolution. This RRF was shown to be active in an in vitro assay system using Escherichia coli polyosomes and elongation factor G (EF-G). In contrast, the above-mentioned RRFs from thermophilic bacteria were inactive in such a system. Analysis of the relative orientations between the two domains in the structures of various RRFs, including this RRF from mesophilic bacterium, revealed that domain II rotates about the long axis of the helix bundle of domain I. To elucidate the ribosome binding site of RRF, the peptide fragment (RRF-DI) corresponding to domain I of RRF was expressed and characterized. RRF-DI is bound to 70 S ribosome and the 30 S subunit with an affinity similar to that of wild-type RRF. But it does not bind to the 30 S subunit. These findings caused us to reinvestigate the concept of the mimicry of RRF to tRNA and to propose a new model where domain I corresponds to the acceptor arm of tRNA and domain II corresponds to the anticodon arm. This is just the reverse of a model that is now widely accepted. However, the new model is in better agreement with published biological findings.

At the final step in each cycle of protein biosynthesis, ribosome recycling factor (RRF) disassembles the post-termination complex, which is composed of 70 S ribosome, deacylated tRNA and mRNA in concert with elongation factor G (EF-G) in a GTP-dependent reaction (1–3). In Escherichia coli, the lack of RRF causes increased errors in translation (4) and unscheduled reinitiations (5, 6).

Presently there are four published reports regarding the three-dimensional structures of RRF molecules. They are crystal structures of RRFs from Thermotoga maritima (7), Escherichia coli (8), and Thermus thermophilus (9) and a solution structure of RRF from Aquifex aeolicus (10). All of these structures consist of two domains; domain I displays a three-helix bundle structure, and domain II exists as a three layer βαβ sandwich structure. Except for E. coli RRF, the relative angles of these two domains are −90 ° and thus their overall structures are arranged in an L-shaped conformation very similar to that of tRNA. Based on this similarity, a concept of molecular mimicry was proposed. It was suggested that domains I and II of RRF correspond to the anticodon and acceptor arms of tRNA, respectively (7). Thus it was proposed as a hypothetical mechanism that RRF would be bound first to the A-site of the ribosome and then translocated by EF-G to the P-site in a manner similar to that of tRNA, leading to the disassembly of the post-termination complex (7). Meanwhile the tertiary structure of E. coli RRF determined by an x-ray analysis showed that even though the secondary structure elements of each domain was almost the same as those of the other RRFs, the spatial arrangement of the two domains is in an obtuse angle so that it does not look like tRNA (8). One could explain that the difference in the structure might be caused by a large difference between the species of origin because E. coli is a mesophilic bacterium but the other three are thermophilic bacteria.

However, the above mentioned concept of molecular mimicry presents a problem. RRFs whose structures have been determined to be a strict L-shape, were obtained from thermophilic bacteria and did not show polysome breakdown activity in vitro system containing EF-G and puromycin-treated polysome fraction from E. coli (11–13). RRFs that demonstrated activity in that system were only mesophilic bacteria, i.e. E. coli and Pseudomonas aeruginosa (14). It has been also demonstrated in

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† The on-line version of this article (available at http://www.jbc.org) contains Fig. S1 titled “The sensorgrams on Biacore of RRF-DI and wild-type RRF binding to ribosome.”

§ The atomic coordinates and structure factors (code 1IS1) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

¶ The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB064319.

** Member of the Structural Biology Sakabe Project.

†† To whom correspondence should be addressed. Tel.: 81-6-6879-8220; Fax: 81-6-6879-8224; E-mail: yujik@protein.osaka-u.ac.jp.

The abbreviations used are: RRF, ribosome recycling factor; EF-G, elongation factor G; kbp, kilobase pair; DTT, dithiothreitol; MES, 4-morpholineethanesulfonic acid; r.m.s.d., root mean square deviations; CD, circular dichroism.
TABLE I

| Data collection statistics | Value | Completeness (%) | Structure refinement statistics | Value |
|----------------------------|-------|------------------|--------------------------------|-------|
| Space group                | R32   |                  | Resolution limit/Å             | 20–2.2 |
| molecules/asymmetric unit  |       |                  | No. of reflections             | 2946   |
| a = b = 84.799, c = 142.727 | 7.3 (25.1) |                  | R-factor/Rfree                 | 0.203/0.273 |
| Number of reflections      | 82069 |                  | R.m.s.d. from standard geometry | 0.005  |
| Number of unique reflections | 10326 |                  | Bond length/Å                  | 0.978  |
| Completeness (%)           | 99.6  |                  | No. of nonhydrogen atoms       | 1441   |
| Protein                    |       | 227              | Solvent                        |       |

Crystallographic data statistics for V. parahaemolyticus RRF

Values in parentheses are for the highest resolution shell.

![Table and text](image-data)

Structure and Binding Mode of RRF

vivo by Janosi et al. using temperature-sensitive mutant of E. coli that RRFs from mesophilic bacteria exhibit an RRF activity but RRFs from thermophilic bacteria do not (15).

To clarify the problem, we would like to know if the unique structure of E. coli RRF is common in mesophilic bacteria and essential for RRF activity, or if the unique structure is just an exception because of some artifact like detergent binding. Actually, the detergent molecule, which was required to crystallize the protein, was reported to attach to the hinge region connecting the two domains of E. coli RRF (16).

Here, we obtained a crystal of RRF from Vibrio parahaemolyticus that was free of detergent. This is a mesophilic bacterium that is a relative of E. coli, and its RRF was expected to show activity in the E. coli system. The molecular cloning and characterization will be described, and the crystal structure of this RRF at 2.2 Å resolution will be described as well. To obtain deeper insight into the mechanism of disassembly of the termination complex, we prepared the domain I portion of RRF without domain II and investigated the interaction between domain I and ribosome. Here we propose a new binding mode between ribosome and RRF.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Conditions—V. parahaemolyticus strain KXX237 (O3:K6) (17) was used for molecular cloning. E. coli strain DH5α was used as a host strain for cloned plasmid DNA. E. coli strain BL21 (DE3) was used for protein expression. Luria-Bertani (LB) broth (Nakalai Tesque) was used in liquid media and solid agar media (1.5%) for routine cultivation of bacteria. The media were supplemented with 100 µg/ml ampicillin.

Molecular Cloning—To select V. parahaemolyticus RRF DNA (fr) clones, a partial E. coli fr fragment was used as a DNA probe. The DIG system (Roche Molecular Biochemicals) was used for labeling of the DNA probe. Primers (5′–CATGGGACATGCTGCTAGAAAGCGC–3′ and 5′–CTCTTTTCTGCTGCAGGCGCC–3′) for PCR were designed to amplify the DNA fragment encoding residues Met–13–Glu–179 of E. coli RRF. The pET22b (+) plasmid (Novagen) carrying E. coli fr was used as a PCR template.

NotI fragments of genome DNA of V. parahaemolyticus KXX237 were hybridized with the probe. Pulse field gel electrophoresis and subsequent Southern hybridization methods have been applied as described previously (18). The luminescent fragment of ∼168 kbp was collected and digested with PstI. Digested products were subjected to electrophoresis on agarose gel (0.8%) and hybridized again. The PstI fragment of 4.1 kbp was collected and inserted into PstI site of pBluescript II KS(−) vector. A restriction map of the 4.1-kbp PstI fragment was created with conventional methods (19). The fragment of 1.2 kbp between SpeI and HindIII sites included the fr gene. The fragment was reconstructed into pBluescriptII KS(−) vector and sequenced. DNA sequencing was performed by an automated DNA sequencer (Shimadzu) with a fluorescent dye primer method. The DNA sequence was analyzed with the GeneWeb II program (20).

Construction of RRF-DI (E. coli Domain I)—To elucidate the role of domain I in RRF, we constructed an expression vector coding a recombinant protein with the Met–1–Gly 30 and Thr–106–Phe 185 segments of E. coli RRF that correspond to domain I. The two segments were connected by a flexible linker composed of glycine residues. In our preliminary result of E. coli BL21G mutant, the distance between Gly-30 and Thr-106 was 9.8 Å (21). Thus three glycine residues were sufficient to connect the two segments. The expression vector, (1–30–)

Circular Dichroism (CD) Measurement—Circular dichroism spectra of E. coli RRF and RRF-DI were recorded on an AVIV model 202 spectrophotometer (AVIV) with a 1-mm path-length cuvette at 10°C using 5 µM protein solutions in a buffer (20 mM sodium acetate, pH 5.0, and 1 mM NaCl). The far-UV CD spectra were measured at 195–250 nm and the data were obtained at a scan rate of 100 nm/min with a bandwidth of 1.0 nm.

The Functional Assessment of RRFs—Purified recombinant V. parahaemolyticus RRF, E. coli RRF, and RRF-DI were assayed with puromycin-treated polysome as a substrate (11). The polysome fraction was prepared from E. coli Q13 strain and used for the post-termination...
complex disassembly reaction by RRF as described previously (22). The reaction mixture (150 μl) contained 10 mM Tris-HCl at pH 7.4, 8.2 mM MgCl₂, 80 mM NH₄Cl, 1 mM DTT, 0.16 mM GTP, 0.05 mM puromycin, 1.3 A₂₆₀ units of polysomes, 1.6 μM EF-G, and various amounts of RRF. The mixture was incubated at 30 °C for 20 min, followed by separation of the mixture into polysome and monosomes with 5–45% sucrose density gradient ultracentrifugation (250,000 × g, 45 min at 4 °C). Quantitative measurements of dissociation products were determined from the absorbance at 260 nm with a Piston Gradient Fractionater (Biocomp Inc.). The inhibitory effect of RRF-DI on the recycling activity of E. coli RRF was estimated by the addition of an excess amount of RRF-DI in the presence of 1 μM E. coli RRF.

Crystallization and Data Collection—The V. parahaemolyticus RRF crystals were grown at 4 °C using the hanging drop vapor diffusion method. Drops consist of 2 μl of protein solution (10 mg/ml) with 2 μl of reservoir solution containing 50 mM MES-NaOH at pH 6.2, 200 mM sodium acetate, and 23–25% w/v PEG 8000. During crystallization, fusidic acid and GTP were added to the drop to a final concentration of 0.75 and 5 mM, respectively. This condition produced several crystals within 1 day, and crystals reached full size (typically 0.2 × 0.2 × 0.2 mm) within 3 days. The crystal was transferred into a cryoprotectant consisting of 50 mM MES-NaOH at pH 6.2, 200 mM sodium acetate, 25% w/v PEG 8000, 15% v/v 2-methyl-2,4-pentanediol, and 5% v/v 2-propanol and flash frozen in liquid nitrogen. The x-ray diffraction data set at 2.2 Å resolution were collected using synchrotron radiation with a crystal-to-detector distance of 430 mm at station BL18B of the Photon Factory in the High Energy Accelerator Research Organization (Tsukuba, Japan). Data frames were collected on a Weissenberg camera with an imaging plate for macromolecular crystallography (23). The data were processed using the DENZO and SCALEPACK programs of the HKL package (24). The crystal of the V. parahaemolyticus RRF belongs to the trigonal space group R32 with unit-cell parameters a = b = 84.80 and c = 142.73 Å. Assuming one molecule per asymmetric unit, the calculated Matthews coefficient Vₘ value is 2.40 Å³/Da (25). The solvent content of the crystal was therefore calculated to be 48.8%. Data collection statistics are given in Table I.

Structure Determination and Refinement—Molecular replacement calculations were performed on the V. parahaemolyticus RRF using the program AMoRe in the CCP4 suite (26). As the search model, we used the crystal structure of E. coli RRF mutant, R132G, which has been reported elsewhere (21). In generating the search model, the residues that are not identical between sequences were replaced by Ala residues. As a result, a single solution was found with a correlation coefficient of 0.316 and R-factor of 55.2% (8.0–4.0 Å) after the translation-function

FIG. 1. Comparison of amino acid sequences from prokaryotic RRFs. The amino acid sequences of RRFs were aligned with the Gene Web II program (18). The numbering at the top is according to the sequence from V. parahaemolyticus RRF. Amino acid sequences are shown in single letter codes. Residues that are conserved in all prokaryotic RRFs are shown in red. The secondary structure of RRF is depicted below the alignment. V. pa, Vibrio parahaemolyticus; V. ch, Vibrio cholerae; E. co, Escherichia coli; P. ae, Pseudomonas aeruginosa; C. je, Campylobacter jejuni; H. py, Helicobacter pylori; M. tu, Mycobacterium tuberculosis; M. le, Mycobacterium leprae; S. au, Staphylococcus aureus; A. ae, Aquifex aeolicus; T. th, Thermus thermophilus; T. ma, Thermotoga maritima.

FIG. 2. Ribosome recycling activity of RRFs. The decomposition of the post-termination complex by RRF from different sources was examined using E. coli polysome and EF-G. Closed circles, E. coli; open circles, V. parahaemolyticus; closed squares, T. thermophilus; open squares, T. maritima.
calculation. To improve the accuracy of the solution, the resultant structure was subjected to 40 cycles of rigid body refinement using data from 8.0 to 3.0 Å resolution. The R-factor was refined to 46.2.

Structure refinement was carried out further using the programs X-PLOR (27), CNS (28), and TOM (29). After a number of cycles of model fitting and refinement, it was possible to trace almost the entire molecule. In this step, the alanine-replaced residues were restored to the original amino acid residues. Later refinement steps included the refinement of grouped or individual temperature factors. Omit maps were used to check the model. Water molecules were identified using waterpick procedure in the CNS program.

The final model included all 185 amino acids and 227 water molecules. Under crystallization conditions, the solution contains fusidic acid and GTP. Although these compounds are essential for crystallization of *V. parahaemolyticus* RRF, only protein and water molecules were observed in electron density maps. The final R-factor and Rfree were 20.3% and 27.3%, respectively, with a mean positional error of 0.24 Å (30). This model had excellent stereochemistry when it was evaluated with the program PROCHECK (31). On the Ramachandran plot, 94.7% of the non-glycine residues are in the most favored region, and none of the residues lie in the generously allowed and disallowed regions. Root mean square deviations (r.m.s.d.) of bond lengths and angles are 0.005 Å and 1.078 °, respectively. Table I summarizes the refinement statistics. The final coordinates and structure factors have been deposited in the Protein Data Bank (PDB number 1IS1).

Ribosome Binding Assay—Surface plasmon resonance experiments were carried out on a Bicore 2000 biosensor system (Biacore AB) to qualitatively examine the binding of 70 S ribosome or its subunits to RRF at 25 °C. Immobilization of RRF on a CM5 sensor chip (Biacore) was carried out according to the manufacturer’s standard protocol using an amine coupling method, by which the protein is covalently immobilized with N-hydroxysuccinimide-based chemistry via lysine ϵ-amino groups. Acetate buffer (10 mM sodium acetate, pH 4.5) was used as the running buffer at a flow rate of 10 µl/min. To immobilize RRF-DI onto flow cell 1 of the CM5 sensor chips, RRF-DI (0.1 mg/ml) was injected as a 24-min pulse. The level of immobilized RRF-DI was ~5000 resonance units. To prepare a blank cell, flow cell 2 was also activated but only running buffer was injected. Unreacted N-hydroxysuccinimide groups on both cells were blocked with ethanoldine hydrochloride.

For monitoring the interaction, all procedures were automated to create repetitive cycling of sample injection and regeneration. Ribosomes, over a range of concentrations between 60 and 900 nm, were injected simultaneously into cells 1 and 2. Then the response from cell 1 was corrected by subtracting the response from cell 2, which arose from nonspecific interactions between ribosomes and the dextran matrix on the sensor chip. The HEPES buffer (10 mM HEPES-NaOH, pH 7.4, 50 mM NH4Cl, 8.2 mM Mg(OAc)2, 1 mM DTT, and 0.005% Tween 20) was used for sample dilution and running buffer at a flow rate of 10 µl/min.

To investigate inhibition of wild-type RRF binding to the 50 S subunit by RRF-DI, the 50 S subunit was preincubated with various concentrations of RRF-DI (0.05–3 µM). Then the mixture was injected on a CM5 sensor chip. In this experiment, wild-type *E. coli* RRF was immobilized on the sensor chip using the above procedure.

Binding of RRF-DI to 70 S ribosome or its subunits was further measured by a filtering technique. RRF-DI was incubated with 0.25 µM ribosome in 40 µl of Tris buffer (10 mM Tris-HCl, pH 7.4, 8.2 mM MgSO4, 50 mM NH4Cl, 1 mM DTT, and 0.3 mM EDTA) at 30 °C for 10 min. The mixture was applied onto Microcon YM-100 (Millipore) and centrifuged for 5 min at 3,000 × g to trap the ribosome-bound RRF-DI. 40 µl of the same buffer was loaded into the column and centrifuged for 10 min at 3,000 × g to wash out the unbound RRF-DI. The washing was performed two times. Then the filter was incubated with 40 µl of the buffer for 1 min at room temperature. The ribosome-bound RRF-DI was collected from the inverted column by centrifugation. The recovered RRF-DI was detected by Western blotting with anti *E. coli* RRF rabbit antibody (1:10,000 dilution). Bound RRF-DI was quantified by the blotting of known amounts of RRF-DI standards. To quantify nonspecific binding between RRF-DI and filter apparatus, control experiments without ribosomes were done. Binding of wild-type RRF to 70 S ribosome or its subunits was measured in the same way.

RESULTS

Nucleotide Sequence of the frr Gene and Comparison of Its Amino Acid Sequence with Other RRFs—The 1.2 kbp SpeI–HindIII fragment containing *V. parahaemolyticus* frr was sequenced. The fragment had only one major open reading frame.
Pribnow box, −35 element and Shine-Dalgarno sequence were also included upstream of the open reading frame in this fragment. The open reading frame consisted of 555 bp and coded a protein that consists of 185 amino acids. The calculated molecular weight was 20570.45. Homology between the *V. parahaemolyticus* and *E. coli* RRFs was 70.1% at the amino acid level.

The DNA sequence of *V. parahaemolyticus* frr has been deposited in the DDBJ/GenBank™/EMBL (accession number AB064319).

The amino acid sequence of *V. parahaemolyticus* RRF was compared with other previously reported RRF sequences (Fig. 1). Most RRFs contain 185 amino acids. The amino acid residues conserved in other species are also conserved in *V. parahaemolyticus* RRF. All reported RRFs are highly homologous (e.g. *E. coli* 70.1%, *P. aeruginosa* 63.2%, *Staphylococcus aureus* 45.4%, *T. thermophilus* 42.7%). Such high level homology strongly suggests that RRFs have similar tertiary structures.

**V. parahaemolyticus RRF Is Functional in the E. coli System**—The ribosome recycling activity of recombinant *V. parahaemolyticus* RRF was tested in a polysome breakdown assay. As shown in Fig. 2, *V. parahaemolyticus* RRF shows polysome breakdown activity in the present assay system containing *E. coli* polysomes. The fraction of polysome converted into 70 S ribosome increases as the amount of *V. parahaemolyticus* RRF increases. The fraction of converted polysome reaches a constant value of ~40% with the addition of a sufficient amount of *V. parahaemolyticus* RRF (>3 μM). This converted fraction by *V. parahaemolyticus* RRF is somewhat smaller than, but close to, that processed by *E. coli* RRF where 50% of polysome was processed with the same amount of *V. parahaemolyticus* RRF. The initial slopes of RRFs from *E. coli* and *V. parahaemolyticus* in this assay indicate that the order of specific activity is similar between these two RRFs. In contrast, RRF from thermophilic bacteria show no activity using this assay system (12).

**Overall Architecture**—The *V. parahaemolyticus* RRF structure has a strictly L-shaped conformation with two domains and dimensions of ~60 × 51 × 25 Å (Fig. 3). The overall structure of *V. parahaemolyticus* RRF is similar to those of *T. maritima* (7), *E. coli* (8), *T. thermophilus* (9) and *A. aeolicus* (10) RRFs. Domain I (residues 1–30 and 104–185) is a three-helix bundle. Domain II (residues 31–103) is composed of two α-helices and six β-strands. The topology of *V. parahaemolyticus* RRF is nearly identical to other RRFs. The dimensions of domain I are ~60 × 25 × 25 Å. This domain is composed of helices α-1, α-4, and α-5 forming a three-helix bundle, which consists of residues 1–24, 107–144, and 150–182. The three
Fig. 7. Binding of RRF to 70 S ribosome or each subunit. A, sensorgrams on Biacore of RRF-DI or RRF binding to ribosome. Various concentrations of 70 S ribosome or its subunits (blue, 60 nM; green, 200 nM; red, 900 nM) were injected over surface with immobilized RRF-DI and wild-type RRF. Bindings of RRF-DI to the 30 S subunit (a), to the 50 S subunit (b), and to 70 S ribosome (c) were represented. Bindings of wild-type RRF to the 30 S subunit (d), the 50 S subunit (e), and 70 S ribosome (f) were also shown. B, prohibition of wild-type RRF to the 50 S subunit by
helices are tightly packed against each other through hydrophobic interactions. Domain II has a globular structure with a four-stranded anti-parallel β-sheet, a two-stranded anti-parallel β-sheet, and two short α-helices. These secondary structure elements form a hydrophobic core. The dimensions of domain II are about 26 × 22 × 20 Å.

The number of hydrogen bonds was calculated by the Insight II program (Accelrys Inc.) based on atomic distances. Besides the hydrogen bonds in α-helices or β-sheets, twelve other hydrogen bonds were identified in the structure. Eight interhelix hydrogen bonds contribute to the packing of the α-helix bundle of domain I. Four other hydrogen bonds stabilize domain-domain interactions. The O-γ of Thr-29, located at the end of domain I, forms a hydrogen bond to the O-ε2 of Glu-181, located in the helix 5 of domain I. A hydrogen bond is also present between the main chain N of Ala-32 in the hinge region and the main chain O of Ala-62 in the loop region of domain II. The guanidinium moiety of Arg-110 forms hydrogen bonds to the main chain O of Glu-184 and O of Leu-182. The significance of these interactions is shown by the fact that the R110H mutant of E. coli RRF is nonfunctional (15). The interdomain salt bridge between the N-ε atom of Arg-28, located in the hinge region, and C-γ of Asp-86, located in the loop of domain II, also contributes to the L-shaped structure. The contact area between the two domains is 1066 Å², which corresponds to 9.6% of the total surface area (11,086 Å²) of RRF. This small contact area and the small number of interdomain interactions suggest that the interaction between domains I and II is relatively weak and hence the relative configuration of domains I and II is expected to be flexible.

Comparison to Other RRFs—As shown in Fig. 4A, the overall folds and dimensions of domains I and II of V. parahaemolyticus RRF are similar to those RRF structures that were previously determined by x-ray crystallography and NMR spectroscopy. Furthermore, during the survey of crystallizing conditions of RRFs, we succeeded in crystallizing one RRF mutant from E. coli, R132G, that is free of detergent. The preliminary study on this mutant has been reported elsewhere (21). The x-ray analysis on V. parahaemolyticus RRF and this RRF mutant revealed that both RRFs from mesophilic bacteria have a strict L-shaped structure very close to those of the above-mentioned RRFs from thermophilic bacteria. On the other hand, spatial arrangements of domains I and II are different among all of RRFs.

The relative orientation of these domains in RRFs for which tertiary structures have been reported are plotted in Fig. 4B using three spherical polar angles: Φ, Ψ, and Χ defined by Yoshida et al. (10). In the crystal structures, the zenith angles (θₛ) are almost 90°, which corresponds to precise L-shaped structure. The 110° angle for E. coli RRF is an exception, and it has an open L-shaped structure. Even in the ensemble of solution structures of A. aeolicus RRF, θₛ are ~90°. Thus a detergent molecule (decyl-β-D-maltopyranoside), which was reported to attach on E. coli RRF, may be responsible for the altered angle in the crystal structure of E. coli RRF. We concluded that RRFs from both mesophilic and thermophilic bacteria have a strict L-shaped structure. We have reported for A. aeolicus RRF that the Φ angle fluctuates from −30° to 30° in the ensemble of solution structures. Interestingly, azimuth angles (Φₛ) among the four crystal structures are spread over a wide range of −50°, but these values fall within the range of solution structures. Thus we postulated an intramolecular movement within the RRF molecule where only the Φ angle fluctuates, while the Ψ angle is maintained at 90°. This movement was confirmed by molecular dynamics calculations. Therefore these crystal structures are regarded as snapshots with variable Φ angle in solution.

Ribosome Binding of RRF-DI—We have concluded above that the RRF molecules consist of two domains in a strict L-shaped structure where intramolecular movement is restricted in a plane. This raised the question whether each domain plays a different role. To clarify this question, we first tried to split the molecule at the hinge region by inserting a scissile sequence for suitable proteinases, but we failed. This may be because the inserted portion was not exposed enough for enzymes to access it. Then we designed the recombinant proteins corresponding to each domain. For the one corresponding to domain II, we constructed an expression vector coding Arg-31-Leu-105, but the desired polypeptide precipitated as an inclusion body in transformed cells. However, we succeeded in expressing a polypeptide consisting of the fragments; Met-1-Gly-30 and Thr-106-Phe-185, with Gly-Gly-Gly, as a linker between the fragments. This polypeptide, named RRF-DI, was characterized by CD measurements.

As shown in Fig. 5, the CD spectrum of RRF-DI showed the characteristic profile of an α-helix with a double minimum at 208 and 222 nm. The ellipticity at 222 nm of RRF-DI represents a higher α-helical content than wild-type RRF. The deconvolution analysis on this CD curve with the DICROPROT program (32) indicated an α-helix content of 91%. This indicates that a major part of RRF-DI consists of α-helix and that the inserted tripeptide fits nicely into the loop connecting the two α-helical fragments to fulfill a three-stranded α-helical bundle, which is likely to be quite similar to that of domain I in wild-type RRF.

We wondered whether RRF-DI exhibits the activity of disassembly of the post-termination complex. According to Fig. 6, RRF-DI does not show any ribosome recycling activity. Furthermore RRF-DI inhibited wild-type RRF activity. By mixing of RRF-DI with wild-type RRF (~50:1) in the reaction mixture, the yield of monosome was reduced to ~30% compared with that converted by wild-type RRF in the absence of RRF-DI. These results indicate that both RRF-DI and wild-type RRF share the same binding site.

Furthermore we have shown that the positively charged cluster region around Arg-132 on domain I plays a crucial role in binding to the 50 S subunit of ribosome (33). Consequently another question arose whether domain I, where Arg-132 is located, interacts with the ribosome or with the 50 S subunit. Bicistronic experiments have been shown to be useful for investigations of interactions involving ribosomes (34). To examine

**Table II**

| Dissociation constants of RRF-DI and wild-type RRF from the ribosome and its subunits |
|---------------------------------|------------------|------------------|------------------|
|                                 | 70 S             | 50 S             | 30 S             |
| RRF-DI                          | 0.16 ± 0.07      | 0.52 ± 0.22      | ND               |
| Wild-type RRF                   | 0.42 ± 0.09      | 0.16 ± 0.03      | ND               |

Dissociation constants were estimated from the Scatchard analysis. Standard deviations for three experiments are presented as errors. ND, not detected.

2 T. Yoshida, S. Oka, S. Uchiyama, H. Nakano, T. Ohkubo, and Y. Kobayashi, submitted for publication.
Fig. 8. **RRF-ribosome binding model.** A, comparison of surface representation of *V. parahaemolyticus* RRF (blue) and yeast tRNA\(^{\text{phe}}\) (green) (45). A new concept of mimicry between RRF and tRNA is shown. Domain I of RRF corresponds to the acceptor arm of tRNA. B, the models of RRF and ribosome complex were constructed according to Selmer’s proposal (8). C, new model for the A-site bound RRF according to A. This arrangement is consistent with biological findings that domain I of RRF interacts not with the 30 S subunit but with the 50 S subunit.
the interactions between RRF and ribosomes, we carried out Biacore experiments. Although, nonspecific interactions, which were estimated in blank cell, were not negligible in the experiments (see supplemental Fig. S1 at http://www.jbc.org), qualitative results were reproducible. As shown in Fig. 7A, resulting sensorgrams showed qualitatively that RRF-DI is bound to 70 S ribosome and the 50 S subunit but not to the 30 S subunit. The prohibition on the binding of the 50 S subunit to the immobilized wild-type RRF by RRF-DI was also observed (Fig. 7B). The response for the binding of wild-type RRF to 70 S ribosome was relatively low. Presumably, the immobilization of wild-type RRF efficiently reduced the accessibility for such a large particle as 70 S ribosome. Dose-dependent responses were observed in these experiments; however, the responses were not proportional to the amounts of injected ribosomes. The apparent affinity might be affected by the nonspecific interactions and the immobilization procedure. Their effects were estimated in the blank cell and were not negligible in these studies (supplemental Fig. S1). Because the apparent affinity might be affected by such nonspecific interactions, the affinities e.g. between wild-type RRF and RRF-DI cannot be quantitatively compared with the Biacore technique.

Thus, to examine the binding affinities of wild-type RRF and RRF-DI to ribosomes more quantitatively, we performed binding assays using a filtering technique (Fig. 7, C and D, and Table II). Various amounts of wild-type RRF or RRF-DI were mixed with 70 S ribosome, the 50 S subunit, or the 30 S subunit, and then the amounts of bound RRFs were determined. In these experiments, nonspecific binding of wild-type RRF or RRF-DI to filter apparatus was found to be negligible (Fig. 7C). Binding of both proteins to 70 S ribosome and the 50 S subunit was observed, but was not observed to the 30 S subunit. Although these measured dissociation constants may be overestimated because the filter technique is a non-equilibrium technique, these values should be relatively meaningful (see discussion by Hirokawa et al. (35)). The apparent dissociation constant of RRF-DI from the 50 S subunit was estimated to be 0.52 μM. This value is slightly higher than that of wild-type RRF, 0.16 μM. For 70 S ribosome, the apparent dissociation constant of RRF-DI was 0.16 μM, which was slightly lower than 0.2 μM of wild-type RRF. The similarity of dissociation constants for RRF-DI and wild-type RRF indicates that the binding modes of these molecules are likely to be comparable to each other. Our results appear to contradict a previous report by Ishino et al. that RRF interacts weakly with the 30 S subunit (33). This is likely due to the fact that the low ionic strength buffer (10 mM Tris-HCl, pH 7.4, 8.2 mM MgSO₄, 10 mM NH₄Cl, 1 mM DTT) was used in their experiment and that at such a condition nonspecific interactions easily occur.

**DISCUSSION**

Structures of the ribosome and its subunits have been elucidated by cryo-electron microscopy and x-ray crystallography (36). Recent crystallographic studies revealed the structure of the ribosome at high resolution and the location of ribosome-bound tRNAs (37). In the present study, the crystal structure of *V. parahaemolyticus* RRF and the binding mode of RRF to the ribosome were determined. The resemblance of the structure of RRF to that of tRNA in terms of shape and size has been recognized as representative of molecular mimicry (7). To examine this mimicry we attempted to construct the RRF-ribosome complex model by replacing tRNA in the ribosome structure with RRF.

It is generally accepted that domains I and II of RRF correspond to the anticodon and acceptor arms of tRNA, respectively. This assumption, which has been suggested by Selmer et al., is based on the structural similarity between *T. maritima* RRF and tRNA (7). In Selmer’s model, domain I of A-site-bound RRF would point toward the decoding area of the 30 S subunit and domain II would point toward the peptidyl transferase region of the 50 S subunit. A cross section of the RRF-ribosome complex derived from Selmer’s model, as shown in Fig. 8B, clearly shows that domain I of RRF contacts primarily the 30 S subunit, whereas domain II contacts the 50 S subunit.

In this study, we have shown that wild-type RRF binds to the 50 S subunit and that it does not bind to the 30 S subunit. According to Selmer’s model, domain II of RRF should be responsible for the binding to the 50 S subunit. However, our experiments using RRF-DI clearly show that the affinity of RRF domain I to the 50 S subunit is equivalent to that of RRF. Therefore we conclude that binding between RRF and the ribosome depends on the interaction between domain I of RRF and the 50 S subunit, and Selmer’s model is excluded. In Selmer’s model, Arg-132, which is essential for binding to the ribosome in domain I, is located near the concave cavity of 30 S subunit and does not contribute to binding to the 50 S subunit (Fig. 8B).

This finding led us to consider the other aspect of the mimicry model where domain I was superimposed on the acceptor arm of tRNA. Interestingly, although correspondence between domains of RRF and arms of tRNA in our model is in just the reverse relation to that in Selmer’s model, these two molecules are nearly identical in both shape and size, as shown in Fig. 8A.

We constructed the ribosome-RRF complex model by replacing the A-site-bound tRNA in the *T. thermophiles* 70 S ribosome structure (37) with RRF according to this new concept. In this model, the RRF molecule could be placed in a tight fit in the ribosomal A-site, avoiding any spatial overlap with the ribosome. As shown in Fig. 8C, this model, in which domain I binds to the 50 S subunit, is consistent with our biochemical results in this study. Furthermore, all conserved Arg residues, such as Arg-110, Arg-129, Arg-132, and Arg-133 of RRF domain I, which aligns along the long axis of domain I, would be exposed to 23 S rRNA. In RRF, these positively charged residues are important for the interaction with the ribosomal RNA.

In our superposition, the spatial orientation of ribosome-bound RRF is such that β-strands 4 and 6 would face the ribosomal P-site and the α-3 helix of domain II would face the factor binding site where EF-G is bound.

It was noted that a hydrophobic patch is located on the tip of domain II (10). This region consists of conserved aromatic residues, such as Tyr-44, Tyr-45, and Phe-70. These residues are unusually exposed to solvent and are surrounded by hydrophilic residues containing conserved residues such as Gly-46 and Asp-71. The tip region of domain II may play a crucial role in recognition of the target molecule. Recently, the significance of the interactions of RRF with EF-G has been reported based on the fact that *Mycobacterium tuberculosis* RRF is inactive in *E. coli*, but it regains activity upon co-expression of *M. tuberculosis* EF-G (38). From the mutational studies of RRF and EF-G, Ito et al. have proposed that EF-G motor action is transmitted to RRF (39). As described under “Results,” azimuth angles (Φ) between domains can vary in the range of ~50°. Such a domain movement or conformational change may occur upon EF-G binding. Biochemical and structural studies showed that the flexibility of the relative orientation of domains I and II may be important for RRF function (9, 10, 12).

It has been proposed as a hypothetical mechanism that RRF may be bound first to the A-site of the ribosome and then translocated by EF-G to the P-site in a manner similar to that of tRNA, leading to the disassembly of the post-termination complex (7). This model has been based on the fact that RRF...
binds to the A-site (40) and that tRNA is released in the recycling step (41). We examined whether the mechanism is consistent with the new model. Joseph and Noller reported that the anticodon stem loop of tRNA is required in the A-site for translocation by EF-G during the elongation step (42). However in our model, RRF lacks the part corresponding to the anticodon stem loop of tRNA. Therefore RRF is not likely to be translocated from the A-site to the P-site by EF-G. Furthermore it was shown that the release of tRNA from post-termination complex partially takes place with EF-G alone (35). Therefore, we propose that RRF does not go through a translocation from the A-site to the P-site with the help of EF-G. In this respect, RRF is not a perfect functional tRNA mimic. Movement toward the P-site or conformational change of domain II might assist tRNA release from post-termination complex by EF-G, while domain I still keeps the A-site occupied to protect the A-site against the incoming EF-Tu-aminoacyl-tRNA complex during the disassembly reaction. Using the post-termination complex involving short synthetic mRNA with a Shine-Dalgarno sequence, Karimi et al. have shown in another sequence of ribosome recycling that RRF, EF-G, and GTP catalyze the dissociation of the 50 S subunit from the post-termination complex followed by tRNA removal from the 30-S-deacylated tRNA-mRNA complex by IF3 (43). This may be due to the strong Shine-Dalgarno sequence and may be different from the natural long mRNAs discussed in this study (35).

We have pointed out that movement of the ϕ angle that maintains the l-shaped structure is important for RRF action (10). Based on this view, the physicochemical study to elucidate the difference in RRF activity between mesophilic and thermoacidophilic bacteria is in progress. In this paper, we have proposed a new concept for molecular mimicry by RRF and a new model for RRF action.

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REFERENCES
1. Hirashima, A., and Kaji, A. (1970) Biochem. Biophys. Res. Commun. 41, 877–883
2. Pavlov, M. Y., Freistroffer, D. V., MacDougall, J., Buckingham, R. H., and Ehrenberg, M. (1997) EMBO J. 16, 4134–4141
3. Kaji, A., Teyssier, E., and Hirokawa, G. (1998) Biochem. Biophys. Res. Commun. 250, 1–4
4. Janosi, L., Richter, R., and Kaji, A. (1996) Biochimie (Paris) 78, 959–969
5. Ryoi, M., Berland, R., and Kaji, A. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 5973–5977
6. Janosi, L., Mottagui-Tabar, S., Isakkson, L. A., Sekine, Y., Ohtsubo, E., Zhang, S., Goon, S., Nelken, S., Shuda, M., and Kaji, A. (1998) EMBO J. 17, 1141–1151
7. Selmer, M., Al-Karadaghi, S., Hirokawa, G., Kaji, A., and Liljas, A. (1999) Science 286, 2349–2352
8. Kim, K. K., Min, K., and Suh, S. W. (2000) EMBO J. 19, 2362–2370
9. Toyoda, T., Tan, O. F., Ito, K., Fujiwara, T., Kumasaka, T., Yamamoto, M., Garber, M. B., and Nakamura, Y. (2000) RNA 6, 1432–1444
10. Yoshida, T., Uchiyama, S., Nakano, H., Kashiwagi, H., Kimura, H., Ohshima, T., Saihara, Y., Ishino, T., Shimahara, H., Yoshida, T., Yokose, K., Ohkubo, T., Kaji, A., and Kobayashi, Y. (2001) Biochemistry 40, 2387–2396
11. Hiroshima, A., and Kaji, A. (1972) Biochemistry 11, 4057–4044
12. Aratahari, K., and Kaji, A. (2000) J. Bacteriol. 182, 6154–6160
13. Fujiwara, T., Ito, K., and Nakamura, Y. (2001) RNA 7, 64–70
14. Ohnishi, M., Janosi, L., Shuda, M., Matsutomo, H., Hayashi, T., Terawaki, Y., and Kaji, A. (1999) J. Bacteriol. 181, 1281–1291
15. Janosi, L., Morii, H., Sekine, Y., Abragn, J., Janosi, R., Hirokawa, G., and Kaji, A. (2000) J. Mol. Biol. 295, 815–829
16. Yan, J., Kim, W., Ha, S. C., Eom, S. H., Sub, S. W., and Kim, K. K. (2000) Acta Crystallogr. Sect. D Biol. Crystallogr. 56, 84–85
17. Nasu, H., Iida, T., Sugahara, T., Yamaishi, Y., Park, K. S., Yokoyama, K., Makino, K., Shimagawa, H., and Honda, T. (2000) J. Clin. Microbiol. 38, 2156–2161
18. Iida, T., Suthienkul, O., Park, K. S., Tang, G. Q., Yamamoto, R. K., Ishibashi, M., Yamamoto, K., and Honda, T. (1997) J. Mol. Microbiol. 46, 639–645
19. Selmer, M., Al-Karadaghi, S., Hirokawa, G., Kaji, A., and Kobayashi, Y. (2000) Genomics Informatics 19, 164–165
20. Nakano, H., Uchiyama, S., Yoshida, T., Ohkubo, T., Kato, H., Yamagata, Y., and Kobayashi, Y. (2002) Acta Crystallogr. Sect. D Biol. Crystallogr. 58, 124–126
21. Hirashima, A., and Kaji, A. (1972) J. Mol. Biol. 65, 43–58
22. Sakabe, N. (1991) Nucl. Instrum. Methods Phys. Res. A 303, 448–463
23. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
24. Matthews, B. W. (1968) J. Mol. Biol. 13, 491–497
25. Navaza, J. (1994) Acta Crystallogr. Sect. A 50, 157–163
26. Brünger, A. T. (1992) X-PLOR Version 3.1 A System for X-ray Crystallography and NMR. Yale University, New Haven, CT
27. Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., G., P., Gros, P., Grosse-Kunstleve, R. W., Jiang, J.-S., Kuszwinksi, J., Nilges, N., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1994) Acta Crystallogr. Sect. D Biol. Crystallogr. 50, 760–768
28. Cambillau, C., Horjaies, E., and Jones, T. A. (1984) J. Mol. Graph. 2, 53–54
29. Luzzati, P. V. (1952) Acta Crystallogr. 5, 802–810
30. Laskowski, R. A., MacArthur, M. W., Moss, D., and Thornton, J. M. (1993) J. Appl. Cryst. 26, 283–291
31. Deléage, G., and Gerourjion, C. (1993) Comp. Appl. Biosc. 9, 197–199
32. Ishino, T., Atarashi, K., Uchiyama, S., Yamami, T., Saihara, Y., Yoshida, T., Harai, H., Yokose, K., Kobayashi, Y., and Nakamura, Y. (2000) Genes Cells 12, 953–963
33. Karlsson, M., Pavlov, M. Y., Malmqvist, M., Persson, B., and Ehrenberg, M. (1995) Biochimie (Paris) 78, 995–1002
34. Hirokawa, G., Kiel, M. C., Muro, A., Selmer, M., Raj, V. S., Liljas, A., and Janosi, L. (1997) EMBO J. 16, 3483–3492
35. Joseph, S., and Noller, H. F. (1998) EMBO J. 17, 3478–3483
36. Karlsson, M., Pavlov, M. Y., Buckingham, R. H., and Ehrenberg, M. (1999) Mol. Cell 3, 601–609
37. Kuzoh, J. J. (1991) J. Appl. Crystalllogr. 24, 946–950
38. Jack, A., Ladner, J. E., and Klug, A. (1976) J. Mol. Biol. 108, 619–649