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The Ribosome Modulation Factor (RMF) Binding Site on the 100S Ribosome of *Escherichia coli*[1]

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During the stationary growth phase, *Escherichia coli* 70S ribosomes are converted to 100S ribosomes, and translational activity is lost. This conversion is caused by the binding of the ribosome modulation factor (RMF) to 70S ribosomes. In order to elucidate the mechanisms by which 100S ribosomes form and translational inactivation occurs, the shape of the 100S ribosome and the RMF ribosomal binding site were investigated by electron microscopy and protein-protein cross-linking, respectively. We show that (i) the 100S ribosome is formed by the dimerization of two 70S ribosomes mediated by face-to-face contacts between their constituent 30S subunits, and (ii) RMF binds near the ribosomal proteins S13, L13, and L2. The positions of these proteins indicate that the RMF binding site is near the peptidyl transferase center or the P site (peptidyl-tRNA binding site). These observations are consistent with the translational inactivation of the ribosome by RMF binding. After the "Recycling" stage, ribosomes can readily proceed to the "Initiation" stage during exponential growth, but during stationary phase, the majority of 70S ribosomes are stored as 100S ribosomes and are translationally inactive. We suggest that this conversion of 70S to 100S ribosomes represents a newly identified stage of the ribosomal cycle in stationary phase cells, and we have termed it the "Hibernation" stage.

Key words: hibernation stage, 100S ribosome, ribosome modulation factor (RMF).

When bacterial cells such as *Escherichia coli* are cultured under normal laboratory conditions, the stationary phase is reached after about three hours of exponential growth. During the transition from exponential growth to the stationary phase, the expression of genes important for rapid growth is generally stopped, and a set of genes required for the stationary phase are expressed (*1*). As a consequence, the composition and conformation of various cellular complexes are altered, most notably the composition of the RNA polymerase and the conformation of nucleoids (*2*).

Until recently, the components of the ribosome have been considered to be relatively stable through different phases of cell growth, with the exception of the ribosomal protein L12, which is acetylated during the stationary phase (*3*). However, evidence of other dynamic changes is accumulating. Four novel proteins have been shown to bind to the *E. coli* ribosome during the stationary phase: the ribosome modulation factor (RMF) (*4*), the protein YfiA, the protein YhbH (*5*), and the stationary phase induced ribosome-associated protein (SRA, formerly termed ribosomal protein S22) (*6, 7*). Furthermore, changes are found not only in the protein components but also in the behavior of the subparticles. The most remarkable change is the conversion of 70S ribosomes to 100S ribosomes (*4*). 100S ribosomes arise through the dimerization of 70S ribosomes mediated by RMF, which binds to the 50S subunit of the 70S ribosome during the stationary phase. Besides those in *E. coli*, 100S ribosomes have been found in other enterobacteria, including *Salmonella typhimurium*, *Serratia marcescens*, and *Proteus mirabilis*, and in some soil bacteria (*8*). In these bacteria, the 70S ribosomes also dimerize, suggesting that ribosomal monomer-dimer interconversion is a basic cellular mechanism in many prokaryotes. RMF seems to play a critical role in the reorganization of subparticles. In eukaryotes and archaebacteria however, RMF homologues and the dimerization of ribosomes have not been observed.

RMF is a small basic protein (6,475 Da, pI = 11.3) that can be released from 100S ribosomes by high-salt washing. The expression of *rmf* increases remarkably during the transition from the exponential to the stationary phase, and the association of RMF with 70S ribosomes parallels the formation of 100S ribosomes (*8*). The *rmf* gene is induced by ppGpp (*9*). We here show that when stationary phase *E. coli* cells are transferred to fresh medium, the RMF is immediately released from 100S ribosomes, and these 100S ribosomes dissociate back into 70S ribosomes. This process is very quick and completed within two minutes. After the process, the cells reinitiate protein synthesis and proliferation within six minutes (*6*). An *in vitro* trans-

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lation assay demonstrated that 100S ribosomes have no translational activity, which we have attributed, at least in part, to the inhibition of aminoacyl-tRNA association with ribosomes caused by RMF binding (*10*). The interconversion between active 70S ribosomes and inactive 100S ribosomes is therefore a phase-dependent cellular mechanism that controls translation.

E. coli rmf mutants do not form 100S ribosomes. These mutants survive up to the early stationary phase, but die rapidly afterwards with the degradation of ribosomal particles. Their life is shortened by about 4 days compared to the wild-type parent cells (*11*). This suggests that the conversion of 70S ribosomes to 100S ribosomes may serve not only to store inactive ribosomes, but also to protect them from degradation by proteases and/or nucleases that are induced during the stationary phase.

Without detailed knowledge of the molecular structure and topography of 100S ribosomes, the translational inactivation process cannot be fully understood. In order to investigate the structure of 100S ribosomes, we observed negative-stained 100S ribosomes by electron microscopy. Electron micrographs of 100S ribosomes were published in 1960 and 1982 (*12, 13*), but the 100S ribosomes observed in these studies were formed artificially under conditions of low ionic strength, and not during a natural stationary phase. In the present work, we observed for the first time native 100S ribosomes formed by RMF.

Second, in order to investigate the RMF binding site, we cross-linked neighboring proteins on 100S ribosomes using 2-iminothiolane. This allowed the identification of 3 ribosomal proteins that exist near the RMF binding site, suggesting that RMF binds near the peptidyl transferase center. In this paper, we include the results of our observations of the structure of 100S ribosome.

EXPERIMENTAL PROCEDURES

E. coli Strains—The *E. coli* K-12 strains used in this work are listed in Table I. The W3110 strain was used for electron micrography. MG1655, KY1702 and WY2001 strains were used for the cross-linking study. KY1702, an *rmf* gene disruptant of MG1655, was constructed in the same way as described in a previous work (*7*). The expression vector pQE-9 (QIAGEN), which contains a 6 × His-tag at the N-terminus, was used to express His-tagged RMF. The ORF from the Kohara clone #222 was amplified by PCR with the following primers: 5′-GCGGATCCATGAAGAGACAAAAACGAGA-3′ (forward) and 5′-GCCGAAGCTTTTCAGGCCATTACTACCCTGT-3′ (reverse). WY2001 was constructed by transforming the ligated vector [pQE-9(*rmf-lacZ*)] into the *rmf* deleted strain KY1702.

Electron Microscopy—W3110 cells were grown in medium E (*14*) containing 2% polypeptone at 37°C with shaking at 100 cycles per min for 4 days, harvested by centrifugation at 4°C, and then stored at –80°C until use. Preparation of crude ribosomes from the cell pellet was carried out essentially according to the method of Noll *et al.* (*15*), with slight modifications as described by Horie *et al.* (*16*).

Crude ribosomes were fractionated by sucrose density gradient centrifugation as previously described (*4*). The 70S and 100S fractions were collected and then concentrated as a pellet by centrifugation at 50,000 rpm for 90 min. After resuspension in Association buffer (100 mM CH_3COONH_4, 15 mM $(CH_3COO)_2Mg{\cdot}4H_2O$, and 20 mM Tris-HCl at pH 7.6) with 6 mM 2-mercaptoethanol, 2% glutaraldehyde was added to stabilize ribosomal subunit association, and the solution was incubated on ice for 20 min. The addition of glutaraldehyde to ribosomes extracted from exponentially growing cells did not lead to the artificial formation of 100S ribosomes. Negative staining with 1% uranyl acetate was used for electron microscopy. Specimens were observed in a Nihon-denshi JEM-100-CX electron microscope.

Growth Conditions of MG1655, KY1702, and WY2001 Cells—MG1655, KY1702, and WY2001 cells were grown in 2× YT medium at 37°C with shaking at 100 cycles per min. The medium for KY1702 cells contained 25 μg/ml kanamycin, and that for WY2001 cells contained 25 μg/ml kanamycin and 100 μg/ml ampicillin. Expression of His-tagged RMF in WY2001 cells was weakly induced by adding IPTG [isopropyl-β-D(–)-thiogalactopyranoside, Wako] to a final concentration of 0.03 mM at the early stationary phase (OD_{600}: about 0.8). Cells were grown for 24 h and harvested by centrifugation at 4°C, and then stored at –80°C until used. Crude ribosomes from these strains were prepared in the same way as for W3110.

Sucrose Density Gradient Centrifugation—Crude ribosomes were subjected to centrifugation on 5–20% linear sucrose density gradients in Association buffer with 6 mM 2-mercaptoethanol. After centrifugation in a SW40Ti rotor (Beckman) at 40,000 rpm for 80 min at 4°C, ribosome profiles were observed at 260 nm with a UV-180 spectrometer (Shimazu) using a flow cell.

After centrifugation, the crude ribosomes prepared from WY2001 cells were divided into 12 fractions, and analyzed by Western blotting using an anti–His tag antibody.

Protein-Protein Cross-Linking—Cross-linking was performed according to the method of Kenny *et al.* (*17*). The 70S and 100S ribosome pellet was suspended in Buffer A (100 mM KCl, 15 mM $MgCl_2$, 50 mM triethanolamine·HCl, and 5 mM dithiothreitol, pH 8.0), and then dialyzed against Buffer A in order to remove free amines that might react with 2-iminothiolane. The concentration of the ribosomes was adjusted to OD_{260nm} 45 with Buffer A. Following the addition of 1.2 mM 2-iminothiolane (Sigma), the solution was incubated for 2.5 h at 0°C. Hydrogen peroxide was added to 40 mM, and the incubation was continued for 30 min at 0°C to promote cross-linking between adjacent sulfhydryl groups by disulfide bond formation. Unreacted hydrogen peroxide was removed by the addition of catalase (15 μg/ml) and by incubation for 15 min at 0°C. Unreacted 2-iminothiolane was removed by dialysis against Buffer B (100 mM KCl, 15 mM $MgCl_2$, and 50 mM triethanolamine·HCl, pH 8.0). Iodoacetamide (40 mM) was added in order to alkylate free sulfhydryl groups and the mixture was incubated for 30 min at 30°C. Proteins were extracted from the treated ribosomes by the acetic acid method (*18*). After dialysis against 2% acetic acid, the proteins were lyophilized.

Identification of Cross-Linked Proteins—The cross-linked

TABLE I. ***E. coli* strains used in this work.**

Strains	Relevant genotype
W3110	F^-; prototroph
MG1655	F^-; prototroph
KY1702	MG1655 *rmf::kan*
WY2001	KY1702 pQE-9 (*rmf*)

proteins were suspended in Binding buffer (20 mM NaH_2PO_4, 500 mM NaCl, 8 M urea, and 10 mM imidazole), and passed through a column filled with 1 ml nickel-nitrilotriacetic acid-agarose (Ni-NTA, HiTrap column from Amersham Pharmacia Biotech.) to capture protein complexes cross-linked to the His-tagged RMF. The resin was washed with 10 ml Washing buffer (20 mM NaH_2PO_4, 500 mM NaCl, 8 M urea, and 20 mM imidazole). The protein complexes were eluted from the column with 3ml Elution buffer (20 mM NaH_2PO_4, 500 mM NaCl, 8 M urea, and 500 mM imidazole) and analyzed by Tricine SDS-PAGE (*19*).

The proteins in each band were identified by amino acid sequencing (PE Biosystems, 491 Protein Sequencer). In this analysis, the lysine signal could not be detected due to modification by 2-iminothiolane.

RESULTS

The 100S Ribosome is a 70S Ribosome Dimer—The ribosomes prepared from *E. coli* W3110 cells in the stationary phase were separated by sucrose density gradient centrifugation to examine the subparticle organization. The OD_{260nm} ratio of the 100S peak to 70S peak was about two to one (data not shown). The fractions corresponding to 70S and 100S ribosomes were examined by electron microscopy. 70S ribosomes typically appeared to be 23 × 31 nm in size (Fig. 1a). On the other hand, 100S ribosomes appeared as rods of nearly twice the length, 23 × 53 nm (Fig. 1b). Nearly half of the 100S ribosomes dissociated back into 70S particles due to the severe conditions of preparation for microscopic examination. The shapes of the 100S ribosomes in a magnified vision (Fig. 1c) are dimeric 70S particles in which the two 30S subunits make face-to-face contact (Fig. 1d). A similar structure was observed for 100S ribosomes artificially prepared at low ionic strength, and also appeared as a 70S ribosome dimer in which face-to-face contact was made between the two 30S subunits (*12, 13*).

Formation of 100S Ribosomes by His-Tagged RMF—We constructed a disruptant strain of *rmf*, KY1702, from MG1655, and complemented it with a plasmid-born His-tagged RMF in WY2001. Crude ribosome fractions from these cells were prepared from stationary phase MG1655, KY1702, and WY2001 cells, and analyzed on sucrose density gradients (Fig. 2). MG1655 cells formed 100S ribosome because this strain has native RMF, which is essential for the formation (*4*). As expected, the dimer formation was observed as shown in Fig. 2a. On the other hand, KY1702 cells did not form dimers (Fig. 2b), consistent with their lack of RMF. The absence of the dimer was complemented by introducing the plasmid-encoded His-tagged RMF formed 100S ribosomes, as shown in Fig. 2c. The centrifugal ribosomes of WY2001 cells were divided into 12 fractions (Fig. 2c), and analyzed by Western blotting, using the monoclonal anti-His tag antibody (Fig. 3). The distribution of this antibody coincided with that of 100S, indicating that the His-tagged RMF binds exclusively to 100S ribosomes. Fractions No. 6–9 were mixed together, and this sample was used for cross-linking studies. Figure 4a shows the

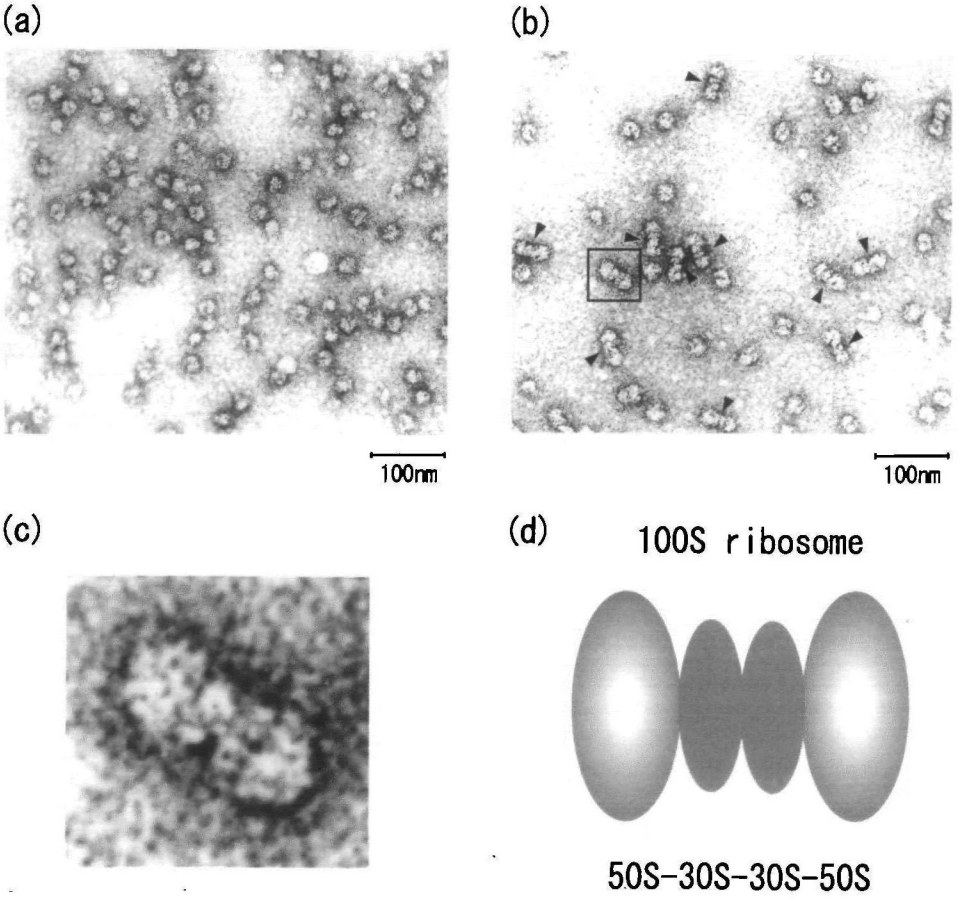

Fig. 1. **Electron micrographs of ribosomes in the stationary phase.** Ribosomes in the stationary phase were separated into 70S and 100S fractions by sucrose density gradient centrifugation. (a) Ribosomes in the 70S fraction. (b) Ribosomes in the 100S fraction. The dimers of 70S ribosomes are indicated by arrows. (c) A magnification of the region indicated by the square in (b). (d) A binding model of the 100S ribosome. The 100S ribosome is a dimer of 70S ribosomes in which two 30S subunits make face-to-face contact.

ribosome profiles of this combined sample after sucrose density centrifugation. The molar ratio of 70S to 100S in Fig. 4a is estimated to be approximately 1:2.5. To determine the amounts of His-tagged RMF in the sample, we carried out 2-D PAGE as shown in Fig. 4b. The copy number of His-tagged RMF was about 0.8, which is estimated by normalizing spot density per molecular weight to the average values of those of L27, L28, L29, and L30. In the previous study it was found that RMF binds exclusively to 100S ribosomes (*4*). If RMF binds to the 70S in the 100S ribosome one by one, the copy number of RMF in the sample can be calculated roughly at about 0.8 by using the molar ratio of 70S to 100S. This calculated value is consistent with the experimental value. Therefore, it is reasonable to suppose that all, or most, of the His-tagged RMF binds to 100S ribosomes.

Identification of Cross-Linked Proteins—We used the protein-protein cross-linking method to determine the RMF binding site on the 100S ribosome, and the His-tag on RMF to separate cross-linked proteins. The proteins in the combined fractions, corresponding to the 100S ribosomes shown in Fig. 2, were cross-linked by 2-iminothiolane. The eluents from a Ni-NTA column were analyzed with Tricine SDS-PAGE (Fig. 5, lanes 2 and 3). The 100S fractions prepared from WY2001 contained the His-tagged RMF (lane 3), but no proteins were detected in the corresponding fraction prepared from cells lacking His-tagged RMF (lane 2).

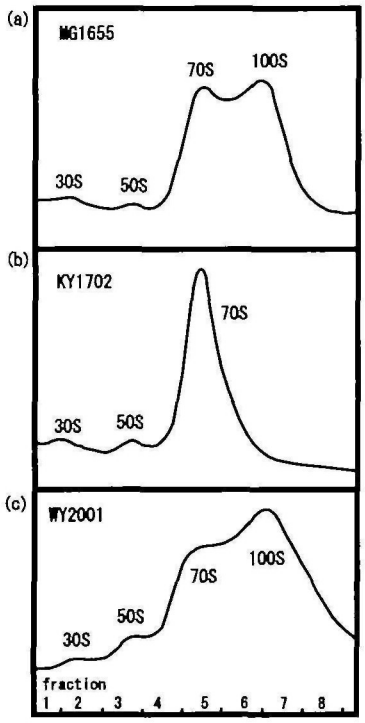

Fig. 2. ***In vivo* formation of 100S ribosomes.** Crude ribosomes were prepared from stationary phase MG1655, KY1702, and WY2001 cells and centrifuged on 5–20% linear sucrose density gradients at 40,000 rpm for 80 min after incubation at 37°C for 30 min. (a) In MG1655 cells, the native RMF can promote 100S ribosome formation. (b) KY1702 cells cannot form 100S ribosomes because they lack RMF. (c) WY2001 cells form 100S ribosomes in the presence of His-tagged RMF. The direction of the gradient is from left to right.

We identified the proteins induced in bands 1-3 of lane 3 by N-terminal amino acid sequencing (see Table II). Band 1 contained the His-tagged RMF exclusively, which was identified as a single signal in each cycle of sequencing. Band 2 included the signals of ribosomal proteins S13 and L13, and band 3 included those of ribosomal protein L2 in addition to His-tagged RMF. No other interpretations of the detected sequences were possible due to the weakness of the other signals. These identifications were readily made because the proteins in each band consisted solely of *E. coli* ribosomal proteins or His-tagged RMF. Although the sig-

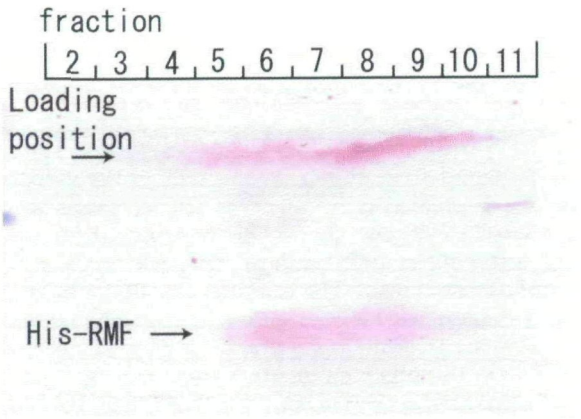

Fig. 3. **Western blotting using anti-His tag antibody.** The centrifugal ribosomes of WY2001 cells were divided into 12 fractions (see Fig. 2c). Each fraction was analyzed by Western blotting using the monoclonal anti-His tag antibody. The distribution agreed with those of 100S ribosomes.

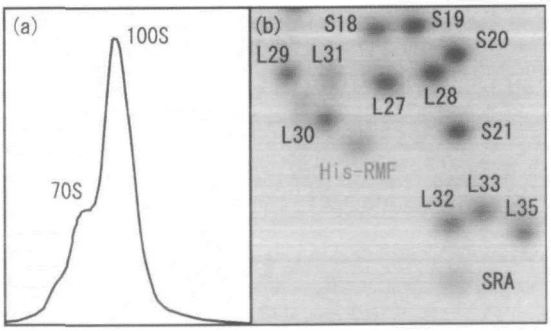

Fig. 4. **Ribosome profile and 2-D PAGE of the fractions used for cross-linking study.** (a) Fractions No. 6–9 in Fig. 2c were combined and subjected again to centrifugation on a 5–20 % sucrose density gradient. It is estimated that the molar ratio of 70S to 100S is approximately 1:2.5. (b) The proteins included in the fractions were analyzed by 2-D PAGE. This results show that the copy number of His-tagged RMF is about 0.8.

TABLE II. **Detected sequence signals.**

Cycle	1-2-3-4-5-6-7-8-9-10-	Identified proteins
Band 1	M-R-G-S-H-H-H-H-	His-RMF
Band 2	M-R-G-S-H-H-H-H-	His-RMF
	A-R-I-A-G-I-N-I-	S13
	M-X-T-F-T-A-X-P-	L13
Band 3	M-R-G-S-H-H-H-H-	His-RMF
	A-V-V-X-X-X-P-T-S-P-	L2

X: unidentified.

nals of cycles 2 and 7 of the identified L13 were not detected, the sequence is perfectly consistent with that of L13, when Lys residues, which are modified by 2-iminothiolane, are applied to the cycle 2 and 7. In the case of the identified L2, the sequence is consistent with that of L2 when K-C-K is applied to cycle 4-5-6. There is a possibility that the N-terminal amino groups of ribosomal proteins also react with 2-iminothione. In this case, the modified proteins are not detectable by sequencing. Therefore, it is thought that the detected proteins are cross-linked at Lys residues with His-tagged RMF. Each of the bands in lane 3 has a molecular weight that is approximately equal to the sum of the molecular weights of each ribosomal protein (M_r = 12,968, 16,018 and 29,729 to S13, L13, and L2, respectively) and RMF (M_r = 6,475). Complexes of S13-RMF and L13-RMF were not resolved in band 2. These results show that S13, L13, and L2 are near the RMF binding site on the 100S ribosome. The positions of these proteins suggest that the RMF binding site is near the peptidyl transferase center or the P site (peptidyl-tRNA binding site).

DISCUSSION

In order to elucidate the mechanism of 100S ribosome formation and the translational inactivation of 70S ribosomes by RMF, we investigated the shape of the 100S ribosome by electron microscopy, and identified the RMF binding site on the 100S ribosome by protein-protein cross-linking. The binding of RMF promotes the establishment of contacts between the 30S subunits of 70S ribosomes, thereby allowing the formation of 100S ribosomes (Fig. 1). The shape of the 100S ribosomes as seen in this study is similar to that of the 100S ribosome artificially formed under conditions of low ionic strength (*12, 13*). It had been observed that free 30S subunits tend to dimerize at high Mg_2^+ concentrations or low monocation concentrations. This dimerization is believed to be the result of conformational changes in the 30S subunits, since their composition does not change during dimerization, suggesting that the formation of 100S ribosomes under conditions of low ionic strength might be caused by conformational changes in the 30S subunits as well.

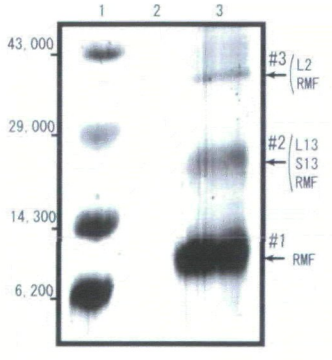

Fig. 5. **SDS-PAGE of cross-linked proteins.** Cross-linked proteins associated with 100S ribosomes prepared from MG1655 and WY2001 cells were purified by Ni-NTA affinity chromatography and resolved by Tricine SDS-PAGE (lanes 2 and 3, respectively). Lane 2 is free of bands because 100S ribosome formation in MG1655 cells is promoted by native RMF. In lane 3, three bands can be detected. Amino acid sequencing is shown in Table 2. Band 1 corresponds to His-tagged RMF. In addition to His-tagged RMF, band 2 includes the ribosomal proteins S13 and L13, and band 3 includes the ribosomal protein L2. Lane 1 contains a molecular weight markers.

By protein-protein cross-linking studies, three ribosomal proteins located near the RMF binding site were identified, as shown in Fig. 5. Among these proteins, two proteins (L2 and L13) belong to the large subunit and one (S13) to the small subunit. This observation shows that RMF is located in the interface between the two subunits. The intercalation of RMF in the interface may induce a conformational change in the 30S subunits, which causes two 70S ribosomes to dimerize. However, it is difficult to imagine how RMF directly mediates the interaction between two 30S subunits.

The ribosomal proteins S13, L13, and L2 can be directly cross-linked to RMF in native 100S ribosomes (Fig. 5). These proteins can also be detected in cross-linking experiments using 100S ribosomes formed *in vitro*. We prepared 100S ribosomes by incubating 70S ribosomes and His-tagged RMF *in vitro* and cross-linked proteins with 2-iminothiolane. In this study, S13, L13, and L2 were also identified as proteins that cross-link directly to RMF (data not shown). Ribosomal proteins S13, L13, and L2 play interesting roles in translation. It is well known that L2 is involved in peptidyl transferase activity (*20–22*) and that it is located about 2.35 nm from the peptidyl transferase center (*23*). It has been reported that the ribosomal protein L13 is also located near the peptidyl transferase center (*22–26*). The position of these proteins indicates that the RMF binding site is near the peptidyl transferase center, as shown in Fig. 6. RMF consists of 55 amino acids including three lysines (Lys-2, -5, and –26), which have ε–amino residues involved in cross-linking. The maximal distance between Lys-2 and Lys-26 is estimated to be about 8 nm, and the length of the bridging reagent is about 1.4 nm. As the distance between L2 and L13 is estimated to be less than 8 nm (*23*), RMF bound to the peptidyl transferase center can be directly cross-linked to L2 and L13. Another ribosomal protein detected, S13, is located on the head of the 30S subunit, and may face or make contact with the peptidyl transferase center of the 50S subunit (*27*). In addition, it has been reported that S13 can be cross-linked to tRNA at the P site (*28*). Although the structural properties of the ribosome can vary depending upon the conditions used in their preparation or the organisms from which they are isolated, it is clear that RMF is located near the peptidyl transferase center or the P site. Finding that RMF binds to the peptidyl transferase center or to the P site is consistent with the inactivation of translation or the inhibition of the ribosomal aminoacyl-tRNA association by RMF (*10*).

Recently it has been shown that in the ribosome cycle, a "Recycling" stage follows the "Termination" stage. The ribosome recycling factor (RRF) (*29*) is involved in this recycling. During exponential growth, ribosomes are readily transferred to the "Initiation" stage after the "Recycling" stage. During the stationary phase however, many 70S ribosomes are converted to 100S ribosomes and lose translational activity, suggesting that 100S ribosomes form after the "Recycling" stage (see Fig. 7).

When the cells are transferred to fresh medium, the 100S ribosomes readily dissociate back into 70S ribosomes by releasing RMF, and the 70S ribosomes begin to synthesize proteins. Therefore, the 100S ribosome is a temporary

and reversible form of the resting ribosome. We term the formation of 100S ribosomes the "Hibernation" stage in the ribosome cycle.

It has been reported that the ribosomal proteins L2 and S13 detected in this work cross-link with IF-3 (http://www.mpimg-berlin-dahlem.mpg.de/~ag_ribo/ag_brimacombe/drc/). This suggests that the ribosomal binding site of IF-3 is in close proximity to that of RMF. During the exponential phase, IF-3 and IF-1 bind to the 70S ribosome after the "Recycling" stage and cause the 70S ribosomes to dissociate into 50S and 30S subunits prior to the initiation of translation. During the stationary phase however, RMF binds to the 70S ribosome after the "Recycling" stage, resulting in the formation of the 100S ribosome, and in the inactivation of translation (the "Hibernation" stage). RMF and IF-3 appear to have opposing functions in the organization of ribosomal subunits. These factors may compete for binding sites on the ribosome during the stationary phase.

In conclusion, this work demonstrates that RMF inactivates *E. coli* ribosomes by binding near the peptidyl transferase center or P site during stationary phase. It is noteworthy that *E. coli* encodes proteins that reversibly inhibit protein synthesis by binding to the peptidyl transferase center, and thereby control translational activity in order to survive a longer stationary phase. This is reminiscent of the mode of action of some antibiotics, such as chloramphenicol or lincomycin, which inhibit protein synthesis by binding to the peptidyl transferase center and causing cell death. RMF homologues have not yet been found in eukaryotes, but higher cells may also have an RMF-like factor or a process similar to "Hibernation" that serves to reversibly inactivate translation during periods of quiescence.

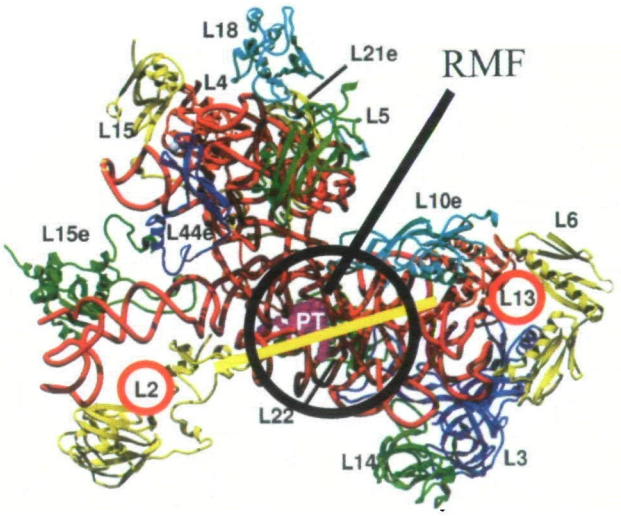

Fig. 6. **The position of RMF on a three-dimensional model based on crystal structure analysis of the peptidyl transferase center of the *Haloarcula marismortui* large ribosomal subunit.** This figure is adapted from Ref. 22. RMF is cross-linked to ribosomal proteins L2 and L13, suggesting that RMF binds near the peptidyl transferase center.

REFERENCES

1. Hengge-Aronis, R. (1993) Survival of hunger and stress: the role of RpoS in early stationary phase gene regulation in *E. coli*. *Cell* **72**, 165–168
2. Ishihama, A. (1999) Modulation of the nucleoid, the transcription apparatus, and the translation machinery in bacteria for stationary phase survival. *Genes Cells* **4**, 135–143
3. Ramagopal, S. and Subramanian, A.R. (1975) Growth-dependent regulation in production and utilization of acetylated ribosomal protein L7. *J. Mol. Biol.* **94**, 633–641
4. Wada, A., Yamazaki, Y., Fujita, N., and Ishihama, A. (1990) Structure and probable genetic location of a "ribosome modulation factor" associated with 100S ribosomes in stationary-phase *Escherichia coli* cells. *Proc. Natl. Acad. Sci. USA* **87**, 2657–2661
5. Maki, Y., Yoshida, H., and Wada, A. (2000) Two proteins, YfiA and YhbH, associated with resting ribosomes in stationary phase *Escherichia coli*. *Genes Cells* **5**, 965–974
6. Wada, A. (1998) Growth phase coupled modulation of *Escherichia coli* ribosomes. *Genes Cells* **3**, 203–208
7. Izutsu, K., Wada, C., Komine, Y., Sako, T., Ueguchi, C., Nakura, S., and Wada, A. (2001) *Escherichia coli* ribosome-associated protein SRA, whose copy number increases during stationary phase. *J. Bacteriol.* **183**, 2765–2773
8. Wada, A., Mikkola, R., Kurland, C.G., and Ishihama, A. (2000) Growth phase-coupled changes of the ribosome profile in natural isolates and laboratory strains of *Escherichia coli*. *J. Bacteriol.* **182**, 2893–2899
9. Izutsu, K., Wada, A., and Wada, C. (2001) Expression of ribosome modulation factor (RMF) in *Escherichia coli* requires ppGpp. *Genes Cells* **6**, 665–676
10. Wada, A., Igarashi, K., Yoshimura, S., Aimoto, S., and Ishihama, A. (1995) Ribosome modulation factor: stationary growth phase-specific inhibitor of ribosome functions from *Escherichia*

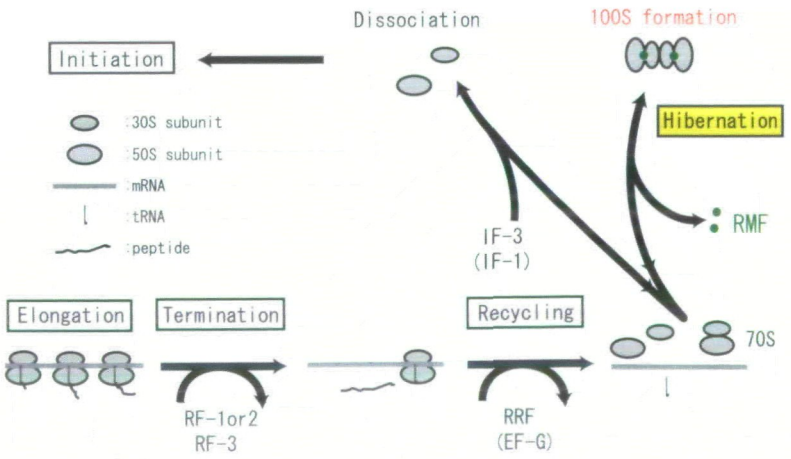

Fig. 7. **The *E. coli* ribosome cycle.** The ribosome cycle is characterized by the "Initiation," "Elongation," "Termination," and "Recycling" stages. It is thought that during stationary phase 100S ribosome formation occurs between the "Recycling" and "Initiation" stages. We term this stage of 100S ribosome formation the "Hibernation" stage. After the "Recycling" stage, the binding of RMF to ribosomes drives them into the "Hibernation" stage, resulting in the loss of translational activity. When translation can resume, the 100S ribosomes readily dissociate back into 70S ribosomes by releasing RMF.

coli. *Biochem. Biophys. Res. Commun.* **214**, 410–417
11. Yamagishi, M., Matsushima, H., Wada, A., Sakagami, M., Fujita, N., and Ishihama, A. (1993) Regulation of the *Escherichia coli rmf* gene encoding the ribosome modulation factor: growth phase- and growth rate-dependent control. *EMBO J.* **12**, 625–630
12. Huxley, H.E. and Zubay, G. (1960) Electron microscope observation on the structure of microsomal particles from *Escherichia coli*. *J. Mol. Biol.* **2**, 10–18
13. Bernabeu, C. and Lake, J.A. (1982) Packing of 70 S ribosomes in dimers formed at low ionic strength. Images of an unusual ribosome projection. *J. Mol. Biol.* **160**, 369–373
14. Vogel, H.J. and Bonner, D.M. (1956) Acetylornithinase of *Escherichia coli*: partial purification and some properties. *J. Biol. Chem.* **218**, 97–106
15. Noll, M., Hapke, B., Schreier, M.H., and Noll, H. (1973) Structural dynamics of bacterial ribosomes. I. Characterization of vacant couples and their relation to complexed ribosomes. *J. Mol. Biol.* **75**, 281–294
16. Horie, K., Wada, A., and Fukutome, H. (1981) Conformational studies of *Escherichia coli* ribosomes with the use of acridine orange as a probe. *J. Biochem.* **90**, 449–461
17. Kenny, J.W. and Traut, R.R. (1979) Identification of fifteen neighboring protein pairs in the *Escherichia coli* 50 S ribosomal subunit crosslinked with 2-iminothiolane. *J. Mol. Biol.* **127**, 243–263
18. Hardy, S.J.S., Kurland C.G., Voynow, P., and Mora, G. (1969) The ribosomal proteins of *Escherichia coli*. I. Purification of the 30S ribosomal proteins. *Biochemistry* **8**, 2897–2905
19. Schagger, H. and von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**, 368–379
20. Levieva, I., Levieva, S., and Garrett, R.A. (1995) Role for the highly conserved region of domain IV of 23S-like rRNA in subunit-subunit interactions at the peptidyl transferase centre. *Nucleic Acids Res.* **23**, 1512–1517
21. Uhlein, M., Weglohner, W., Urlaub, H., and Wittmann Liebold, B. (1998) Functional implications of ribosomal protein L2 in protein biosynthesis as shown by in vivo replacement studies. *Biochem. J.* **331**, 423–430
22. Vladimirov, S.N., Druzina, Z., Wang, R., and Cooperman, B.S. (2000) Identification of 50S components neighboring 23S rRNA nucleotides A2448 and U2604 within the peptidyl transferase center of *Escherichia coli* ribosomes. *Biochemistry* **39**, 183–193
23. Nissen, P., Hansen, J., Ban, N., Moore, P.B., and Steitz, T.A. (2000) The structural basis of ribosome activity in peptide bond synthesis. *Science* **289**, 920–930
24. Alexander, R.W., and Cooperman, B.S. (1998) Ribosomal proteins neighboring 23 S rRNA nucleotides 803-811 within the 50 S subunit. *Biochemistry* **37**, 1714–1721
25. Muralikrishna, P. and Cooperman, B.S. (1995) Ribosomal components neighboring the 2475 loop in *Escherichia coli* 50S subunits. *Biochemistry* **34**, 115–121
26. Gregory, S.T., and Dahlberg, A.E. (1999) Erythromycin resistance mutations in ribosomal proteins L22 and L4 perturb the higher order structure of 23 S ribosomal RNA. *J. Mol. Biol.* **289**, 827–834
27. Montesano-Roditis, L., McWilliams, R., Glitz, D.G., Olah, T.V., Perrault, A.R., and Cooperman, B.S. (1993) Placement of dinitrophenyl-modified ribosomal proteins in totally reconstituted *Escherichia coli* 30 S subunits. Localization of proteins S6, S13, S16, and S18 by immune electron microscopy. *J. Biol. Chem.* **268**, 18701–18709
28. Osswald, M., Döring, T., and Brimacombe, R. (1995) The ribosomal neighbourhood of the central fold of tRNA: cross-links from position 47 of tRNA located at the A, P or E site. *Nucleic Acids Res.* **23**, 4635–4641
29. Hirashima, A. and Kaji, A. (1970) Factor dependent breakdown of polysomes. *Biochem. Biophys. Res. Commun.* **41**, 877–883