Mg\(^{2+}\) Dependence of 70 S Ribosomal Protein Flexibility Revealed by Hydrogen/Deuterium Exchange and Mass Spectrometry

The ribosome from *Escherichia coli* requires a specific concentration of Mg\(^{2+}\) to maintain the 70 S complex formation and allow protein synthesis, and then the structure must be stable and flexible. How does the ribosome acquire these conflicting factors at the same time? Here, we investigated the hydrogen/deuterium exchange of 52 proteins in the 70 S ribosome, which controlled stability and flexibility under various Mg\(^{2+}\) concentrations, using mass spectrometry. Many proteins exhibited a sigmoidal curve for Mg\(^{2+}\) concentration dependence, incorporating more deuterium at lower Mg\(^{2+}\) concentration. By comparing deuterium incorporation with assembly, we have discovered a typical mechanism of complexes for acquiring both stability and flexibility at the same time. In addition, we got information of the localization of flexibility in ribosomal function by the analysis of related proteins with stalk protein, tRNA, mRNA, and nascent peptide, and demonstrate the relationship between structure, assembly, flexibility, and function of the ribosome.

The *Escherichia coli* ribosome is a super-macromolecular complex (2.3 MDa) comprised of three RNA molecules and 55 proteins. The ribosomal proteins are assembled upon the ribosome by noncovalent intermolecular interactions with three ribosomal RNAs (rRNA). It is known that the activity of the ribosome, which plays a central role in protein synthesis, is controlled by a direct interaction of the rRNA with certain positive ions (Mg\(^{2+}\), K\(^{+}\), NH\(_{4}\)\(^{+}\), etc.) and polyamines (1–5). Specifically, the effect of Mg\(^{2+}\) affects subunit association or the activity of the ribosome (6, 7), suggesting that the ribosome dynamics is exquisitely controlled through the stability and flexibility by Mg\(^{2+}\) concentration. How does the ribosome acquire these conflicting factors at the same time? It is difficult to capture such structural dynamics in solution, although some large motions of the 70 S ribosome during translation have already been determined using static structural data obtained by a combination of x-ray crystallography and cryoelectron microscopy (8–11).

In a previous study, we devised a method for detecting ribosomal protein dynamics in the 70 S ribosome (12) by using mass spectrometry to monitor hydrogen/deuterium (H/D)\(^2\) exchange on small proteins (13). The H/D exchange of backbone amide protons provides a quantitative measure of dynamics, and solvent accessibility and has been investigated using several techniques including IR, NMR, and mass spectrometry (14–17). The concomitant use of mass spectrometry has the unique advantage that the H/D exchange of diverse components in the mixture can be analyzed on the same mass spectrum. Furthermore, the recent development of matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has allowed high resolution analysis for singly charged ions of proteins and peptides; this feature also represents an advantage in the study of H/D exchange in multicomponent mixtures (12, 13, 18). Mass spectrometry has already elucidated some characteristics of the ribosome; however, prior to our previous study (12), it had not been applied to H/D exchange analysis of the constituent proteins. Monitoring H/D exchange via MALDI-MS is one of the best ways to study the dynamics of ribosome or other macrocomplexes.

In this study, we examined deuterium incorporation of the 52 ribosomal proteins in an intact 70 S ribosome at several Mg\(^{2+}\) concentrations using MALDI-MS. From those results, we created a deuterium incorporation map of the 52 proteins constituting the 30 S and 50 S subunits (here these proteins are named as “S1, S2, ...” and “L1, L2, ...”, respectively). The map displays information about the magnitude of structural flexibility as color on the x-ray structure of the ribosome. This study illustrates the structural flexibility of individual proteins in the 70 S ribosome as a function of Mg\(^{2+}\) concentration and demonstrates the relationship between structure, assembly, flexibility, and function of the 70 S ribosome.

EXPERIMENTAL PROCEDURES

Materials—Ribosomes were purified from *E. coli* A19 by hydrophobic chromatography and ultracentrifugation with a sucrose cushion buffer (19) and were stored at −80 °C after resolving in 10 mM Tris buffer, pH 8.6, including 7 mM β-mercaptoethanol and 10 mM magnesium acetate. D\(_2\)O (99.9% atomic D) and acetic acid-d (99% atomic D) were purchased from to whom correspondence should be addressed. Fax: 81-791-58-2817; E-mail: tyamamoto@spring8.or.jp.
from EURISO-TOP and IsoTec, respectively. All other chemicals were of analytical grade.

**H/D Exchange**—H/D exchange was initiated by mixing 12 μl of D₂O solution with 1.2-μl aliquots of ~10 μM ribosome solution in 1.5-ml centrifuge tubes at 22 °C; the H/D atomic ratio was 1:10. The pH of the mixture was 7.0–7.2, and the Mg²⁺ concentration was 0.38–22 mM. After 80 min, 5 μl of the reaction mixture was removed, and the H/D exchange reaction was quenched by adding 2 μl of 10% acetic acid (H/D = 1:10, pH 2.9) and freezing with liquid nitrogen. Then, the exchange of side-chain protons is terminated completely by this acid quenching. A portion of the sample solution was mixed with saturated 3,5-dimethoxy-4-hydroxycinnamic acid in 70% acetonitrile containing 0.2% trifluoroacetic acid and loaded on the sample plate for MALDI. Three minutes after loading the sample plate under a pressure of 7 Pa, the plate was set up for MALDI-TOF-MS at 10⁻⁴ Pa. All mass spectra were measured using MALDI-TOF-MS (Voyager DE-PRO, Applied Biosystems). Peaks were identified in a previous study (12), and overlapped peaks were deconvoluted to each peak as Gaussian type by graphing software. The spectrum was distributed over a wide range of masses, and hence was calibrated by separating into six regions: 3000–7000, 7000–11000, 11000–15000, 15000–20000, 20000–25000, and 25000–32000 m/z. The spectra of deuterated ribosomes showed similar peak patterns, and it was possible to detect 52 peak shifts associated with H/D exchanges. Thus, we could use MS to successfully analyze deuterium incorporation of 52 ribosomal proteins. All masses were read from the centroid values of each peak. Deuterium incorporation, D, was calculated by Equation 1,

\[
D = \left( \frac{M_{\text{80min}} - M_{\text{side}}}{M_{100\%} - M_{\text{side}}} \right)
\]

where \(M_{\text{80min}}\) is the mass of each protein after 80 min from the starting H/D exchange. \(M_{100\%}\) and \(M_{\text{side}}\) are fully deuterated masses of all residues and side chains only, respectively, at H/D = 1:10. H/D exchange was carried out three times at each Mg²⁺ concentration, and deuterium incorporation shows the average. In this examination, errors of incorporation into each protein were <0.05, and the back-exchange of H for D was calculated as <0.03% using 100% deuterated melittin.

**Dynamic Light Scattering**—Ribosome size changes were measured at a given Mg²⁺ concentration by dynamic light scattering (DynalPro, Wyatt). 40 nM ribosome solutions were set in the instrument, and all data were accumulated from more than 20 scans.

**RESULTS**

To monitor peak shifts of each protein by deuterium incorporation, the base spectrum of the undeuterated ribosome was measured under the same conditions as in the H/D exchange experiments (Fig. 1). Ionized ribosome particles by MALDI were separated into protein and rRNA components, using positive ion mode in the range of 3,000–32,000 m/z; therefore, their peaks correspond to ribosomal proteins and are not negatively charged rRNAs. We tested the time dependence at all Mg²⁺ concentrations that H/D exchange of all ribosomal proteins apparently completed after 80 min. Because the saturated incorporation value of each protein depends on the fraction of flexible region in the whole protein, we are able to interpret deuterium incorporations as flexibility quantitatively. To examine the effect of Mg²⁺ on ribosome flexibility in solution, 70 S ribosomes were reacted with deuterium oxide for 80 min at several Mg²⁺ concentrations, and the peak shifts of 52 ribosomal proteins were detected by MALDI-TOF-MS. The inset of Fig. 1 shows typical peak shifts of L30 by H/D exchange at three Mg²⁺ concentrations, 0.33, 6.03, and 21.6 mM. Fig. 2 shows deuteration incorporations of 5 typical proteins as a function of Mg²⁺ concentration. (Mg²⁺ dependence of all proteins is shown in supplemental Fig. S1.) Although more deuterium was incorporated into most ribosomal proteins at lower Mg²⁺ concentrations, the incorporation profiles showed three different patterns: “two-state transition,” “flat,” and “slope and flat.” Because Mg²⁺ concentration strongly affects the association between the 30 S and 50 S subunits, we also measured dynamic light scattering (DLS) of the ribosome solution as a function of Mg²⁺ concentration in supplemental Fig. S2. The ribosome particles were nearly 2.3 MDa, indicating almost all of the ribosomal material is present as an 70 S structure at >5 mM Mg²⁺. However, DLS at <4 mM showed that the ribosome dissociates into two subunits when Mg²⁺ concentration decreases. This result agrees well with previous reports, using a mass spectrom-
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FIGURE 3. Deuterium incorporation of ribosomal proteins. Light gray and white bars show the lowest and highest deuterium incorporations, respectively, over a range of 0.33–21.6 mM Mg$^{2+}$. The dark gray bar shows deuterium incorporations obtained by subtracting the numbers of exposed and non-hydrogen-bonding protons (calculated with PDBID: 2AVY and 2AW4) from the lowest value (22). L7, L10, L12, and L28 were not calculated because of indeterminant structures, and only L1 was calculated with 1VOR (21).

Flexibility of Individual Ribosomal Protein Depends on Mg$^{2+}$ Concentration—As shown in Fig. 5, we categorized each ribosomal protein into nine types, according to the deuterium incorporation profiles in response to shifts in Mg$^{2+}$ concentration. H/D exchange of majority of ribosomal protein (40 proteins) exhibited a sigmoidal curve as a function of Mg$^{2+}$ concentration (Type I-IV), in which the transition of H/D exchange occurred between 5 and 10 mM Mg$^{2+}$. H/D exchange of 11 proteins exhibited a "slope and flat" curve (Type V-VIII), indicating that these proteins incorporate more deuterium at lower Mg$^{2+}$ concentrations. Finally, only one protein, L4, was "flat" (Type IX), i.e. its deuterium incorporation did not depend on Mg$^{2+}$ concentration.

How do these types of H/D exchange correlate with the tertiary structure of the ribosome? Here we defined the "breakpoint," which is Mg$^{2+}$ concentration causing the collapse of inflexible conformation. Fig. 6, A and B show the breakpoints for Mg$^{2+}$ concentration in each ribosomal protein (21, 22), classified according to the nine types illustrated in Fig. 5. Because S5, S6, S8, S9, S10, and S13 have breakpoints at high Mg$^{2+}$ concentration, we conclude that the head, platform, and neck regions are flexible. On the other hand, because S3, S4, and S20 maintain an inflexible state at 6 mM Mg$^{2+}$, we speculate that the boundary line between the shoulder and head region is stable at low Mg$^{2+}$ concentrations. Specifically, S3 and S20 should hold neighbor rRNAs tightly, because these proteins had not only lower breakpoints but also lower levels of incorporation (Fig. 3). The breakpoint of S3 is very important for the

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assembly of the 30 S subunit (Fig. 6C) (23–25). If S3 is flexible at high Mg\(^{2+}\) concentration, the 30 S subunit would disassemble, because S5, S8, S9, S10, and S13 became flexible at high concentrations (Fig. 6A). The breakpoint of the 50 S subunit shows that L3, L6, L13, L17, L22, L23, and L24 locating below in backside were flexible at high Mg\(^{2+}\) concentrations (Fig. 6B). In addition, L5 and L18 in the central protuberance (CP) region are also flexible at high concentrations, and we speculate that the dynamics of the CP region are linked with those of the head region in 30 S subunit. We also studied the relationship between Mg\(^{2+}\) concentration and assembly of the 50 S subunit. Although L2, L3, L17, L22, L23, and L24 bind with 23 S rRNA in primary stages of assembly, these proteins start their transition at high Mg\(^{2+}\) concentrations (Fig. 6D) (26–28). On the other hand, assembled proteins in late stage, including L19, L27, L32, and L34, were protected from deuterium at those higher concentrations, as in the 30 S subunit. This relation between H/D exchange and assembly is viewed as the inherent mechanism in ribosome against the change of Mg\(^{2+}\) concentration, and suggested that ribosome structure is stabilized keeping the functional flexibility.

In Fig. 5, Mg\(^{2+}\) wasn’t possible to change the deuterium incorporation of L4 significantly between 0.33 and 21.6 mM. L4 is composed of 201 residue inserts, of which residues 30–100 are inserted into rRNA; L4 also binds with 23 S rRNA in a primary stage of assembly. O’Connor et al. (29) showed that mutations in L4 alter not only the structure of the 50 S subunit and associations between the subunits, but also the structure and function of the 30 S subunit. In contrast, L23 began to lose its static structure at the highest Mg\(^{2+}\) concentration, even though it is assembled with the 23 S rRNA at a primary stage. Finally, L33 and S4 (type V) maintain their static structures until the lowest Mg\(^{2+}\) concentration. This suggests that the increasing deuterium incorporation was caused by dissociation between subunits, which began to dissociate from the static structure at 4 mM.

Differences of Deuterium Incorporation between Flexible and Inflexible States—To quantify the flexibility change of each protein as a function of Mg\(^{2+}\), the differences in deuterium incorporation between the maximum and minimum are shown in Fig. 7. The flexibility of L1 and L4 changes less as a function of Mg\(^{2+}\), because those differences are lower than other for other proteins. It is possible that L1 is not sensitive to Mg\(^{2+}\) because of its functional importance as the stalk protein. Except for the molecular surface effect, L4 also exhibits little incorporation of deuterium, because it binds with 23 S rRNA in the primary stage of assembly. On the other hand, the flexibility of L6, L13, L14, L17, and L35 were significantly affected by Mg\(^{2+}\) and categorized as type II, III, and VII, which cause transitions over a wide range of Mg\(^{2+}\) concentrations. The large change of L14 may be related to the interaction with the 16 S rRNA in the 30 S subunit, which is mediated through Mg\(^{2+}\) (30).

Among proteins of the 30 S subunit, S2, S14, and S20 became a little more flexible at lower Mg\(^{2+}\) concentrations, whereas S8, S9, S13, and S15 were affected significantly (Fig. 7). These proteins exhibit a different tendency from those of the 50 S subunit, and have poor relations with H/D exchange type and assembly. S20 also incorporates a little deuterium in the spur-side region. Therefore, S20 contributes to the sta-
bility of the 30 S subunit by entangling with the 16 S rRNA. The rRNA structure around S2 and S14 is flexible, as indicated by the observation that these proteins incorporate a significant amount of deuterium (Fig. 3); these proteins are dynamic under low Mg\(^{2+}\)/H\(_{2}O\) concentrations. On the other hand, S9 and S13, in the head region, were significantly affected by Mg\(^{2+}\)/H\(_{2}O\); thus, the head region becomes flexible in low concentrations of Mg\(^{2+}\). This is reasonable, as S9 interacts with Mg\(^{2+}\) directly in the crystal structure. In addition, S15, near the terminal region also interacts with a few Mg\(^{2+}\) ions directly, incorporates deuterium more than other proteins by the dilution of Mg\(^{2+}\)/H\(_{2}O\). Because the rRNA of the terminal region becomes more flexible at low Mg\(^{2+}\)/H\(_{2}O\), it may be related that S6 in the terminal region is more highly deuterated at all Mg\(^{2+}\)/H\(_{2}O\) concentrations. The Mg\(^{2+}\)/H\(_{2}O\) dependence of S15 shows that the origins of flexibility originate in the change of dynamics between subunits, because S15 is a back ing protein of rRNA structure in the contact site, Bridge B4, with the 50 S subunit (22).

Relationship between Ribosomal Function and Flexibility—The stalk protein L7/L12 was highly deuterated at all Mg\(^{2+}\)/H\(_{2}O\) concentrations and exhibited sigmoidal curves between the two states, although these two proteins do not interact with rRNA directly. This suggests that L7/L12 is very flexible and synchronizes with L10 and rRNA. We also examined proteins located at the tRNA entrance. L16 has a breakpoint of the inflexible state at high Mg\(^{2+}\)/H\(_{2}O\) concentration, whereas S12 incorporates a significant amount of deuterium at every Mg\(^{2+}\)/H\(_{2}O\) concentration. L31 and L33, located around the tRNA exit site, were also highly deuterated at every Mg\(^{2+}\)/H\(_{2}O\) concentration. Specifically, L33 increased its deuterium incorporation under 4 mM Mg\(^{2+}\)/H\(_{2}O\); it is possible that this is linked with the dissociation of the two subunits. S9, S10, and S13, in the head region of the 30 S subunit, and L5 and L18 in the CP region of the 50 S subunit, exhibited flexibility at high Mg\(^{2+}\)/H\(_{2}O\) concentrations (Fig. 6). In addition, S9 and S13, which interacted with tRNA in the P-site, were more flexible than others as a function of Mg\(^{2+}\)/H\(_{2}O\) (Fig. 7). These results demonstrate that the association and dissociation of tRNA are carried out by flexible structures and must involve mechanisms such as induced fit. On the other hand, L27 directly interacts with tRNA in P-site and stabilizes the peptidyl transferase center, because it incorporated deuterium less than other proteins. S7 and S11 located near the mRNA exit site, incorporated
S ribosome. The Mg$^{2+}$ dependence of deuterium incorporation into ribosomal proteins is correlated with assembly; proteins assembled at the late stage play the role as stabilizers for keeping the flexibility of functional proteins, by maintaining the inflexible structure at lower Mg$^{2+}$ concentrations. This stabilizer mechanism may be introduced into not only ribosome but also other multicomponent complexes. In addition, flexibility of functional proteins also showed interesting characteristics; specifically, the association and dissociation of tRNA are carried out by flexible structures, via mechanisms such as an induced fit. On the other hand, mRNA entrance has a very inflexible structure, and proteins in the tunnel of a nascent peptide showed unique behavior as a function of Mg$^{2+}$ concentration. This study has for the first time elucidated structural flexibility as a function of Mg$^{2+}$ concentration, and the relationship between structure, assembly, flexibility, and activity of ribosome has shown that the 70 S complex is built up by detailed design despite a large size.

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