Dissociation of Intact *Escherichia coli* Ribosomes in a Mass Spectrometer

EVIDENCE FOR CONFORMATIONAL CHANGE IN A RIBOSOME ELONGATION FACTOR G COMPLEX*

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We used mass spectrometry to identify proteins that are released in the gas phase from *Escherichia coli* ribosomes in response to a range of different solution conditions and cofactor binding. From solution at neutral pH the spectra are dominated by just 4 of the 54 ribosomal proteins (L7/L12, L11, and L10). Lowering the pH of the solution leads to the gas phase dissociation of four additional proteins as well as the 5 S RNA. Replacement of Mg$^{2+}$ by Li$^+$ ions in solutions of ribosomes induced the dissociation of 17 ribosomal proteins. Correlation of these results with available structural information for ribosomes revealed that a relatively high interaction surface area of the protein with RNA was the major force in preventing dissociation. By using the proteins that dissociate to probe their interactions with RNA, we examined different complexes of the ribosome formed with the elongation factor G and inhibited by fusidic acid or thiostrepton. Mass spectra recorded for the fusidic acid-inhibited complex reveal subtle changes in peak intensity of the proteins that dissociate. By contrast gas phase dissociation from the thiostrepton-inhibited complex is markedly different and demonstrates release of EF-G from the ribosome and traps the ribosome in a pre-translocational state (pre-state) (8, 9). By contrast thiostrepton does not bind directly to EF-G or affect factor binding and GTP hydrolysis, rather it is reported to bind directly to RNA in a region close the L11-binding site (10) and traps the ribosome in the post-translocational state (post-state) (8, 9). The ribosome (4). Previously, we observed that the proteins that are released in the mass spectrometer (3) are among the most acidic of the ribosomal proteins (5) implying that dissociation of proteins from ribosomes in the gas phase is in part dependent on reducing the positive charge of the protein, thereby weakening interactions with negatively charged RNA.

In addition to detailed structural information, considerable insight into the mechanistic details of protein synthesis has also arisen from atomic resolution images of the ribosome. The original picture of a multistep process requiring a number of protein factors that hydrolyze GTP (6) is emerging with atomic detail (7). The elongation phase of protein synthesis begins with the insertion of an aminocyl-tRNA into the aminoacyl site by elongation factor EF-Tu. Subsequent binding of the elongation factor G (EF-G) to the ribosome triggers the movement of tRNA from the aminoacyl to the peptidyltransferase site in a process known as translocation. EF-G binding to the ribosome is labile but can be trapped using antibiotics. Fusidic acid allows translocation and GTP cleavage but prevents the release of EF-G from the ribosome and traps the ribosome in the post-translocational state (post-state) (8, 9). By contrast thiostrepton does not bind directly to EF-G or affect factor binding and GTP hydrolysis, rather it is reported to bind directly to RNA in a region close the L11-binding site (10) and traps the ribosome in a pre-translocational state (pre-state). Thiostrepton has been shown to block translocation even in the absence of EF-G (11) and recently under certain conditions to prevent complex formation (12). This is in contrast to a report where inhibition of phosphate ion and EF-G release from the ribosome was inferred (13). Biochemical studies have also revealed changes of conformation of the ribosome upon interaction with elongation factors, in particular protein L12 and its acetylated counterpart L7 (14, 15). The results were substantiated by cryo-electron microscopy studies that demonstrate large scale movements in response to binding EF-G (16, 17). The primary aim of this study is to investigate the factors that govern the loss of proteins from ribosomes in the mass spectrometer. A secondary aim is to apply the information that can be gained from such experiments to probe conformational changes in complexes of the ribosome. The recent x-ray analyses of 50 S ribosomes from *Halocarcula marismortui* (18) and...
the 30 S subunit from Thermus thermophilus (19) allows examination of the precise protein-RNA interactions and solvent accessibility for a number of ribosomal proteins and enables us to relate this information to ribosomes from E. coli. To investigate gas phase dissociation, we have manipulated samples of E. coli ribosomes, specifically the pH and metal ion content of the solution, and recorded spectra under identical MS conditions. By using the molecular weight calculated from known amino acid sequences to identify the individual ribosomal protein, we examined the relationship between pl of the protein and its interaction with other proteins and with different RNA molecules. These experiments demonstrate, through distinctive protein dissociation patterns, the sensitivity of the approach to changes in RNA structure. By having established a relationship between the surface area of interaction and the propensity for gas phase dissociation, we examined the release of proteins from ribosomes in complexes with the cofactor EF-G in the presence of two inhibitors of elongation: fusidic acid and thiostrepton freezing the ribosome in complexes analogous to post- and pre-translocation states, respectively. The results show that the two EF-G ribosome complexes are significantly different and are indicative of conformational changes in the mobile stalk region and, in the case of the thiostrepton-inhibited complex, of the 5 S RNA molecule itself.

EXPERIMENTAL PROCEDURES

Materials—Ribosomes were harvested from E. coli strain MRE600 in the log phase of the growth cycle by using standard protocols and were stored in Tico buffer (20 mM HEPES-KOH, pH 7.6, 6 mM magnesium acetate, 30 mM ammonium acetate, 4 mM β-mercaptoethanol) (20). Stock solutions were prepared immediately prior to analysis by buffer exchange at 4°C to give a final concentration of 1 μM ribosome in 10 mM ammonium acetate (pH 7.0) using Bio-Spin 6 chromatography columns (Bio-Rad) equilibrated previously with 10 mM ammonium acetate at pH 7.0 unless stated otherwise. Although some separation of 70 S particles into 50 S and 50 S subunits is observed in the mass spectrometer as the Mg2+ ion concentration is reduced, we have shown previously (21) that the majority of particles (>90%) remains associated as the 70 S. Moreover, the fact that MS conditions could be found whereby no individual proteins are observed strongly implies that protein-RNA interactions are not perturbed by this treatment (3). GTP, thiostrepton, and fusidic acid were purchased from Sigma.

EF-G—XL1 cells were transformed with the vector pQE760uA, which carries the EF-G gene under the pLac promoter. The cells were grown at 37°C at 300 rpm with aeration, in Luria Broth (LB) containing 100 mg/ml ampicillin. The 6 liters of culture was induced with isopropylthiogalactopyranoside at a final concentration of 1 mM, with 100 mg/ml ampicillin. The 6 liters of culture was induced for 3 h after induction. Cells were harvested by centrifugation at 5000 g for 20 min at 4°C and then washed twice in 1 liter of buffer A, pH 7.5, containing 50 mM KH2PO4, 10 mM NH4OAc, 1 mM dithiothreitol, and 100 mM NaCl. The final cell pellet was resuspended in 5 ml of buffer A of cell pellet. Lysozyme, DNase, and phenylmethylsulfonyl fluoride were added to a final concentration of 0.5 and 0.1 g/liter and 0.1 mM, respectively. The mixture was sonicated, and the lysate was centrifuged at 30,000 × g for 30 min at 4°C. The supernant was centrifuged at 10,000 × g for 45 min at 4°C. EF-G was purified from the cell extract using a BioCAD system (Applied Biosystems). The supernatant from the last centrifugation process was loaded onto a nickel column equilibrated in buffer A with 10 mM imidazole. The fractions containing EF-G were pooled and dialyzed five times against 30 volumes of buffer A. The final yield of EF-G was 28 μg/ml of cell pellet. EF-G produced by this process has N- and C-terminal extensions of ML and RSHHHHHHH, respectively, and two sequence changes I575V and E537A resulting in an increase in mass of 1237 over the calculated mass for the sequence of EF-G from the Swiss Protein Data Bank (code P02996).

Sample Preparation—Samples were kept at 4°C throughout buffer exchange and immediately before electrospray analysis. The precise solution pH was measured as 4.7, 7.0, and 10.8 after addition of the pH 4.5, 7.0, and 11.0 buffered solutions. For the LiCl treatment ribosomes were exchanged into 300 μM LiCl solution and incubated for 30 min. Immediately prior to analysis by MS, samples were buffer-exchanged at 4°C into 10 mM ammonium acetate solution at pH 6.5 and analyzed as described below.

EDTA in ethanol was added to 20-μl aliquots of a 1 μM solution of ribosomes equilibrated with 10 mM ammonium acetate, to give final EDTA concentrations of 50 and 300 μM and 50 mM. Each solution was incubated for 1 h at 4°C. The EDTA was removed immediately before analysis using a Bio-Spin 6 column pre-equilibrated with 10 mM ammonium acetate, and samples were analyzed as described below.

Preparation of Ribosome EF-G Complex—The ribosome-EF-G complex was prepared by using 50 μl of a stock solution of 70 S ribosomes at a concentration of 1.5 μM in 10 mM ammonium acetate, pH 7.0. 40 μl of EF-G from a stock solution at a concentration of 60 μM and 19 μl GTP of EF-G from a stock solution at a concentration of 3 μM were added to give a final volume of 119 μl. Thiostrepton or fusidic acid were added to a final concentration of 1 mM in 10 mM ammonium acetate, pH 7.0. For the thiostrepton stock solution it was necessary to add 2% v/v of Me2SO to ensure complete dissolution of the antibiotic. 1 μl of the antibiotic solution, either fusidic acid or thiostrepton, was added to the solution containing ribosomes, EF-G, and GTP after incubation on ice for 1 min. The resulting solution was kept at 4°C. The molar ratio of 70 S/EF-G/GTP/antibiotic was 1:32:760:13, closely similar to that employed in cryo-EM studies of these complexes (22). Excess antibiotic was removed before analysis by filtering the reaction mixture through a Bio-Spin 6 column equilibrated with 10 mM ammonium acetate, pH 7.0. To examine the effect of trace amounts of Me2SO on gas phase dissociation from the ribosome, mass spectra were recorded for ribosomes in 10 mM ammonium acetate containing 0.2% v/v of Me2SO (the maximum amount of Me2SO that could be present in the solution of the complex after 10-fold dilution and after Bio-Spin chromatography). The spectra recorded were identical to those recorded in the absence of Me2SO confirming that at these low concentrations Me2SO does not affect the pattern of gas phase dissociation from ribosomes.

Further control experiments were carried out in which a single component from the reaction was omitted, and the solutions were prepared and incubated as described above. In the absence of the antibiotics fusidic acid and thiostrepton, but with identical solvent composition to the inhibited EF-G ribosome complexes, the spectra showed similar patterns of peaks observed routinely for naked ribosomes. In the absence of GTP, EF-G was observed in mass spectra but showed no hydrolysis of GTP. For EF-G in the absence of ribosomes no appreciable hydrolysis of GTP to GDP could be detected.

Mass Spectrometry—All samples were analyzed by nanoflow electrospray using capillaries prepared as described previously (23). The high viscosity of the ribosomal samples necessitated the use of needles with large tip orifices and a backing pressure of 10–20 pounds/square inch. 2-μl aliquots of sample were loaded into capillaries, and spectra were recorded on a modified Micromass QToF2 mass spectrometer (Micromass, Manchester, UK). This modified mass spectrometer has been described in detail elsewhere (24) and consists of a nanoflow electrospray source, a low frequency extended mass range quadrupole, a double-focusing magnetic sector of isolating ions up to m/z 32,000, followed by a hexapole collision cell, and a time-of-flight (ToF) mass analyzer. The capillary and cone voltages were maintained at 1800 and 80 V, respectively, throughout this investigation. Pressure conditions were maintained at 9 × 10−3 mbar in the hexapole ion guide. For the MS/MS experiments of the 5 S RNA, the peak centered at m/z 3070 was isolated with a window of ±30 m/z units. A voltage of 40 V was applied to the collision cell at an argon pressure of 3.0 × 10−2 mbar.

For the comparison between the EF-G complexes and naked ribosome sample, the same batch of ribosomes was used throughout, and the mass spectra were recorded on the same day to ensure that conditions were entirely reproducible. In addition the fact that each of ribosomes ensures that the tRNA content is identical in each of the complexes formed. Control experiments with re-associated ribosomes, in which RNA is absent, have shown that the dissociation pattern is not the same as that observed for naked ribosomes used in this study.

Data Analysis—The calculated masses of ribosomal proteins from E. coli were calculated from the known amino acid sequence by using the Prot data base. For the spectra recorded from solutions at different pH values results were calculated from three replicate experiments. The spectra recorded from LiCl solutions were repeated twice, whereas those for complexes were repeated at least in triplicate. The intensities of three charge states from each series were summed, and the average Signal-to-noise ratio of the peaks associated to each precursor ion in each experiment was calculated. These values were expressed as a percentage of total intensity assigned to protein peaks in the spectra. The signals were not possible to deconvolute the peaks assigned to L7/L12 because of extensive metal ion binding under neutral pH solution conditions. However,
because low pH conditions confirm their equal population on the ribosome, the intensity calculated for L12 is displayed.

Calculation of Surface Areas of Interaction—Because only three small subunit proteins (S1, S8, and S10) are released in mass spectra from the different solution conditions, it was difficult to draw conclusions about the extent of protein dissociation from 16S RNA. This study therefore focuses on interactions in the large subunit. For the large subunit 16 proteins were released from the 23S and 5 S RNA molecules. To calculate the extent of protein-protein and protein-rRNA interactions, the solvent-accessible surface areas of the protein, buried in the respective contact, was calculated ($\text{sASA}$). These values were calculated using a modified version of the program NACCESS (25), which implements the algorithm of Lee and Richards (26). Default atomic radii (27) and a standard probe radius of 1.4 Å were used. The buried surface area $\text{sASA}$ was computed for protein-rRNA and protein-protein interactions in the 50S subunit. The results showed the same trends for $\text{sASA}$ when both protein backbone and side chain atoms are computed as when only backbone atoms are considered. Because only a limited data set is available for the three-dimensional structure of protein backbone and side chains (12 proteins from the 50S as opposed to 21 for which only backbone coordinates have been deposited, Protein Data Bank accession code 1KQS), the larger data set was used to enable trends to be established. Both protein-RNA and protein-protein interactions in the 50S subunit were calculated. However, given that L12 is associated solely through protein-protein interactions and is readily observed in mass spectra, such interactions are clearly not the driving force for maintaining the gas phase ribosome. Moreover with the exception of L7, L3, and L14, all other proteins interact exclusively with rRNA and no other proteins. Therefore, to examine the correlation between the extent of protein dissociation in the mass spectrometer and protein-rRNA interactions, protein-protein interactions were not included. Considering only protein-rRNA interactions the values calculated for L7, L3, and L14 are reduced by 65, 2.6, and 45%, respectively, from the values calculated for protein-protein and protein-rRNA interactions. Protein interaction surface area calculated in this way will depend at least in part on the size of the protein. In order to reduce this dependence, the $\Delta\text{sASA}$ was divided by the molecular mass.
of the protein. This value is then plotted against the extent of protein dissociation calculated from the mass spectra from the different solution conditions.

RESULTS AND DISCUSSION

Solutions of E. coli ribosomes were prepared over a range of pH values to examine the influence of pH on the dissociation of proteins (see “Experimental Procedures”). An example of a typical spectrum recorded for a solution of E. coli ribosomes at pH 7.0 is shown (Fig. 1A). Assignment of the peaks together with the known masses of the ribosomal proteins shows that spectra are dominated by L10, L11, and L7/L12. Under these MS and solution conditions, no intact stalk complex was observed, in contrast to our previous experiments, as in this case the mass spectrometer was optimized for dissociation of proteins (2, 3). Observation of these individual proteins from solution at neutral pH is attributed to their preferential gas phase dissociation from the intact ribosome. These proteins are located in the 50 S stalk region together with the protein L11, located immediately at the base of the stalk complex. The intensity of the peaks assigned to the proteins L10 and L11 is much lower than those of L7/L12. Intensity in a mass spectrum is governed by the interplay of many different factors, and in this case can be attributed at least in part to the fact that four copies of L7 or L12 are present on ribosomes. However, the peaks assigned to L7/L12 are a factor of ~10 greater than those of both L11 and L10, and this relatively high intensity is therefore consistent with their more favorable release from ribosomes. Close examination of the peaks assigned to L7/L12 in the spectrum recorded for E. coli ribosomes from pH 7.0 shows multiple peaks each separated by 22 Da (Fig. 1C). This mass difference could be attributed either to sodium ion binding, in which a monovalent sodium ion replaces a single proton, or divalent magnesium ion binding in which two protons are replaced. To distinguish these two possibilities, EDTA was added in increasing amounts to chelate the divalent cation and spectra recorded. The resulting spectra showed a substantial reduction in the intensity of the multiplet with increasing concentrations of EDTA. Removal of the multiple peaks under these conditions identifies the ion binding as magnesium.

We also examined solutions of E. coli ribosomes at unit pH values from 7 to 11 to investigate further the effect of charging proteins and rRNA on the propensity for release. Although little is known of the solution structure of ribosomes at these high pH values, the proteins that were observed (L7/L12, L10, and L11) are the same subset as those released under neutral pH conditions. These results suggest therefore that reducing the positive charge of the proteins at high pH does not alter their dissociation pattern from that observed at pH 7.0. By contrast at low pH (4.5), the spectra reveal a number of additional proteins not seen at neutral pH that correspond in mass to the proteins L1, L6, L9, and S8 (Fig. 1B). Unlike L7/L12, L10, and L11 stalk proteins that dissociate at neutral pH, the proteins released from ribosomes at pH 4.5 are known from x-ray crystallography to have no mutual interactions, see structure in Fig. 1. Moreover the peaks assigned to L7/L12 are of comparable intensity to those of other ribosomal proteins implying that at pH 4.5 additional proteins dissociate from the ribosome as readily as L7/L12. The multiplets assigned to L7/L12 recorded from low pH solutions, Fig. 1D, give rise to fewer peaks than were seen at neutral pH. Their m/z values are consistent with their identity as L12 and its acetylated counterpart L7. The similarity in their intensity under these solution conditions confirms their equal population on the ribosome. Removal of the multiple peaks due to Mg$^{2+}$ binding also allows an examination of the post-translational modifications of the proteins and reveals the extent of methylation of both proteins as <10%.

Also apparent in the spectra recorded from pH 4.5 solution conditions are signals at m/z 3,600–4,400 (data not shown). It is difficult to assign a mass to this series due to the broad nature of the peaks (100 m/z units at half-height for the peak at m/z ~3500). In order to obtain a precise mass measurement of these peaks, a single charge state was isolated in the quadrupole of the QToF mass spectrometer (see “Experimental Procedures”) and subjected to increased acceleration in the collision cell. Under these conditions the width of the peaks

### TABLE I

| Protein | Measured mass | Calculated mass | pI |
|---------|---------------|-----------------|----|
| L12     | 12,162 ± 0.3  | 12,164          | 4.0 |
| L12-Me  | 12,177 ± 4.2  | 12,178          |    |
| L12-Mg$^{2+}$ | 12,186 ± 3.5 | 12,188          |    |
| L7      | 12,206 ± 1.0  | 12,206          | 4.8 |
| L7-Me   | 12,219 ± 1.9  | 12,220          |    |
| L10     | 17,578 ± 1.2  | 17,580          | 7.5 |
| L11     | 14,869 ± 2.5  | 14,870          | 9.7 |
| L1      | 24,597 ± 4.8  | 24,598          | 9.2 |
| L6      | 18,771 ± 0.8  | 18,773          | 10.6|
| L9      | 15,768 ± 0.1  | 15,769          | 6.4 |
| S1      | 61,167 ± 8.7  | 61,158          | <7.6|
| S8      | 13,992 ± 2.6  | 13,995          | 12.2|
| 5 S RNA | 39,039 ± 24   | 38,731          |    |
| L33*    | 6240 ± 1.0    | 6240            | >12.0|
| L32*    | 6318 ± 0.4    | 6315            | 11.3|
| L29     | 7276 ± 0.6    | 7274            | 10.0|
| L28*    | 8877 ± 1.2    | 8875            |    |
| L27*    | 8994 ± 2.6    | 8994            | >12.0|
| L25*    | 10,691 ± 5.4  | 10,693          | 9.4 |
| L24     | 11,184 ± 2.7  | 11,155          | 10.7|
| L21     | 11,571 ± 2.9  | 11,564          | 8.2 |
| S10*    | 11,734 ± 7.7  | 11,735          | >12.0|
| L12-Li* | 12,172 ± 0.5  | 12,170          | 4.0 |
| L12-Mg  | 12,186 ± 0.1  | 12,188          |    |
| L7-Li*  | 12,213 ± 1.0  | 12,212          | 4.8 |
| L7-Mg-Li| 12,230 ± 1.0  | 12,234          |    |
| L7-Mg2* | 12,250 ± 1.0  | 12,250          |    |
| L18*    | 12,770 ± 1.2  | 12,770          | 12.0|
| L11*    | 14,880 ± 1.5  | 14,873          | 9.7 |
| L9*     | 15,778 ± 3.3  | 15,769          | 6.4 |
| L10*    | 17,590 ± 3.1  | 17,580          | 7.5 |
| L8*     | 18,780 ± 4.0  | 18,772          | 10.6|
| L5*     | 20,181 ± 1.3  | 20,170          | 9.4 |
| L4*     | 24,606 ± 5.0  | 24,598          | 9.2 |
| Pentamer| 66,296 ± 21   | 66,312          |    |
| EF-G    | 78,690 ± 16   | 78,687          |    |
| EF-G GDP| 79,138 ± 26   | 79,120          |    |

* Denotes proteins that are known to be released stoichiometrically (28) in solution by 2 mM LiCl treatment.
* Denotes proteins that are known to be partially released in solution by 2 mM LiCl treatment.

Large and small subunit proteins from E. coli ribosomes were released during gas phase dissociation from different solution conditions as follows: A, from neutral solution at pH 7.0; B, additional proteins released from acidic solution pH 4.5; C, proteins released after treatment with 200 mM LiCl solution; D, additional proteins and complexes released from the ribosome EF-G complexes in the presence of fusidic acid and thiostrepton. RNA protein contacts are taken from the crystal structures of the 30 S and 50 S subunits (18, 19). pI values are taken from Ref. 5.

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decreases (40 \textit{m/z} units at half-height for the +11 charge state) enabling the mass to be measured as 39,038 ± 24 Da (Fig. 1B and Table I). This value is in reasonable agreement to that calculated from the sequence of the 5 S RNA of 38,731 Da. The broad appearance of the peaks and increased molecular mass over that calculated from the sequence are consistent with multiple metal ions adducting to negatively charged RNA. Acceleration within the mass spectrometer and multiple collisions are sufficient to remove small molecule binding strongly suggesting the identity of the species as 5 S RNA.

Solution based methods for removing subsets of proteins from ribosomes have been devised to investigate proteins required to establish ribosomal function. These experiments have recognized that it is possible to dissociate proteins from the ribosome in solution by addition of LiCl to leave a stable structural nucleus (28). To compare the dissociation of proteins in the mass spectrometer with those destabilized by LiCl treatment in solution, a similar protocol was employed, although excess Li$^+$ was removed before mass spectra were recorded (see “Experimental Procedures”). After this treatment a large number of additional proteins were released (Fig. 2). 13 large subunit proteins are known to remain tightly bound to the 50 S subunit after addition of 2 M LiCl in solution (L2, L3, L4, L13, L14, L17, L19, L21, L22, L23, L24, L29, and L30) (28). From similar treatment and analysis by MS, only three of the tightly binding proteins were observed in mass spectra (L21, L24, and L29). Four and twelve proteins, identified previously as partially and fully released, respectively, by treatment with LiCl, were all observed in mass spectra with the exception of L15, L16, and L26 (Table I). In summary, 10 of the 13 proteins known to remain bound to RNA after LiCl treatment do not dissociate in the gas phase, whereas 16 of the 19 proteins released by this protocol are identified in mass spectra. Thus the majority of proteins removed from ribosomes in LiCl solutions are substantially destabilized by this treatment and consequently readily observed by mass spectrometry.

Examination of the intensity of the peaks in the spectra recorded after LiCl treatment shows that the proteins L7/L12 are no longer dominant; L11, L10, and L29 are released as readily under these conditions. It is also apparent from this spectrum that the multiplets assigned to L7/L12 appear less well resolved than those observed in spectra recorded when Mg$^{2+}$ is the predominant cation (Fig. 2B). This is explained by the fact that the molecular masses of both L7 and L12 recorded after LiCl treatment are consistently higher (7–10 Da see Table I) than those recorded from the solution containing Mg$^{2+}$ or from the low pH solution conditions. This increased mass is consistent with binding of a single Li$^+$ to these proteins. Under these solution conditions L7/L12 are only observed in the Li$^+$ -bound form. Consequently, the mass difference between the peaks corresponding to L7/L12-Li and L7/L12-Mg is reduced compared with the corresponding mass difference observed for the L7/L12 and L7/L12-Mg when Mg$^{2+}$ is the predominant cation. From the spectra recorded under these solution conditions, we can conclude that Mg$^{2+}$ ions are replaced partially by Li$^+$ and that as a result of this cation exchange only a subset of proteins remains firmly attached to the RNA scaffold in the gas phase. It is well established that Mg$^{2+}$ is crucial for the structural integrity of RNA interacting either by diffuse binding in which fully hydrated Mg$^{2+}$ binds to RNA via nonspecific long range electrostatic interactions or by site-specific interactions with anionic ligands facilitated by the RNA fold (29).
binding of Mg\(^{2+}\) to the acidic stalk proteins observed in the present study is presumably nonspecific, because multiple ad-
ducts are observed, and demonstrates the presence of the dif-
fuse Mg\(^{2+}\) cloud surrounding rRNA. It is also established that monovalent cations such as Li\(^{+}\) can compete for Mg\(^{2+}\)-binding sites (30). This was also observed in this study because both Li\(^{+}\) and Mg\(^{2+}\)-bound L7 and L12 were observed indicating the presence of both cations in the diffuse cloud.

To establish the factors that govern protein dissociation from the ribosome in the gas phase, we first examined the relationship between the pI of the protein and its intensity in mass spectra. The fact that proteins L7, L12, and L10 are readily observed in spectra recorded from neutral pH solution conditions and have pI values of 4.8, 4.0, and 7.5, respectively (Table I), suggests a correlation in which reduced electrostatic interactions between RNA and proteins with a greater extent of negatively charged residues lead to their preferential release.

However, from neutral solution conditions dissociation of L11 with a pI of 9.7, a value greater than 8 of the ribosomal proteins listed in Table I that are not observed, is not explained by this correlation. Moreover, the proteins that are observed in spectra recorded at low pH have pI values that range from 4.0 to 12.2 (Table I). An explanation based solely on the loss of electrostatic interactions does not therefore explain the observed dissociation behavior from solutions at different pH values.

The high resolution atomic structures of ribosomes of 50 S subunit from *H. marismortui* (18) as well as those reported from the 70 S of *T. thermophilus* (31) enable us to examine protein-RNA interactions and, because of the widely accepted conservation of structure across bacterial ribosomes (4), to draw conclusions about those from *E. coli*. The interactions between individual large subunit proteins and RNA were examined using a modified version of the program NACCESS (see “Experimental Procedures”). A plot of the ΔSASA against the extent of gas phase dissociation measured from the spectra under different solution conditions is shown in Fig. 3. From these data we have used an arbitrary classification of proteins, based on the rate of change of the extent of ΔSASA as high (>0.030 Å\(^2\)/Da), intermediate <0.030 Å\(^2\)/Da, >0.015 Å\(^2\)/Da), and low (<0.015 Å\(^2\)/Da). By using this classification, it can be seen that proteins with a high ΔSASA do not dissociate from ribosomes under the different solution conditions employed in this study. Similarly proteins with a relatively low ΔSASA undergo gas phase dissociation from either neutral or low pH solutions of ribosomes or after cation exchange with Li\(^{+}\). There are only two exceptions to these generalizations, L15 and L19. L15 falls within the category of having low ΔSASA but is not observed in mass spectra recorded from any solution conditions described in this study. Given the fact that ΔSASA of L15 is smaller per unit mass than that calculated for six proteins that are released from 23 S RNA, this result is surprising. However, examination of the pI of this protein (5) reveals that it has an anomalously high value (>12.0). This is the highest pI value of all the proteins that fall in the low surface area of the interaction category (Table I) implying that high positive charge in this case may prevent its release from 23 S RNA.

Between these extremes of proteins with high and low surface area of interactions are those with intermediate interaction surface areas, and L19 borders this group. Examination of this subset of proteins in conjunction with the x-ray structure reveals that those that are released (L1, L11, and L24) interact with only one of the six folding domains in the 23 S RNA (31). By contrast L2, L19, and L23 interact with 4, 4, and 2 domains, respectively. Thus for those proteins with intermediate surface area of interaction the number of contacts with different domains and the extent of interaction appear to play a role in maintaining protein-RNA interactions in the gas phase. Proteins with higher surface areas of interaction than L24 are not released under any of the solution conditions examined here. It was not possible to calculate interaction surface areas for L10, L21, L27, L28, L32, and L33 because x-ray structure data are not available for these proteins. Given the fact that proteins with a high interaction surface area are not released by this treatment and that L10, L21, L27, L28, L32, and L33 are readily observed strongly implies that these proteins have either intermediate or low interaction surface areas with the 23 S RNA.

Because it is well established that the Li\(^{+}\)-bound RNA struc-
Mass Spectrometry of E. coli Ribosomes

Mass spectrum of the ribosome EFG complex in the presence of the antibiotic fusidic acid. The doublets at high m/z correspond to EF-G and EF-G-GDP. Also present at high m/z are peaks assigned to the stalk pentamer labeled P and the protein S1, the largest ribosomal protein. The stalk proteins dominate the low m/z region of the spectrum from 1500 to 2500. The break in the axis at m/z 3000 represents a 2-fold magnification of the intensity of the higher m/z range.

To investigate further the sensitivity of gas phase dissociation to protein-RNA interactions and hence to changes in the conformation of ribosomes, we have investigated the effects of complex formation on proteins that are released. By using cryo-EM, it has been established that complexes formed between ribosomes and EF-G can undergo major conformational changes depending on the particular translocation stage that is inhibited by a given antibiotic (32, 33). These complexes therefore provide excellent model systems to test our hypothesis that gas phase dissociation is a sensitive probe of protein-RNA interactions. Ribosome EF-G complexes were prepared as described under “Experimental Procedures” and inhibited with either fusidic acid or thiostrepton. The spectra recorded for the fusidic acid-inhibited complex, Fig. 4, display additional peaks at high m/z. The doublets that appear at m/z 3,800–4,400 correspond in mass to EF-G and the high m/z component of the doublet to EF-G GDP in which the majority of the nucleotide has undergone hydrolysis of GTP to GDP and remained associated to the elongation factor. Under the conditions used here EF-G is present in excess, and it is therefore not clear whether the peaks observed in the mass spectra and assigned to EF-G are due to the protein factor that is bound to ribosomes or free in solution. Control experiments, in which GTP is added to EF-G in the absence of ribosomes, however, show no appreciable hydrolysis of GTP under these conditions. This implies that under the conditions used here EF-G is in complex with ribosomes, at least transiently, during GTP hydrolysis.

In the mass spectrum of the fusidic acid-inhibited complex, peaks can also be assigned to the largest ribosomal protein S1 and to the stalk pentamer ([L7/L12], L10), despite the fact that the conditions within the mass spectrometer were optimized to promote dissociation. This observation suggests that, in the ribosome EF-G complex, interactions between these stalk proteins are enhanced. Changes in the interactions of the stalk complex and in the release of S1 are in fact the only significant differences between the spectra recorded for fusidic acid-inhibited complex and those recorded for the 70 S particle alone. This indicates that this ribosome EF-G complex is in a conformation that is largely indistinguishable from naked ribosomes save the changes that persist in both the stalk region and in the binding of protein S1. The high m/z region of the spectrum obtained for the thiostrepton-inhibited complex was closely similar to that obtained with fusidic acid demonstrating the same GTP hydrolysis product associated to EF-G as well as the presence of S1 and the pentameric complex of the stalk proteins.

At the low m/z region of the spectra recorded for the two complexes there are marked differences (Fig. 5). For the spectrum recorded for the fusidic acid-inhibited complex the dissociation products are primarily those from the mobile stalk region and are of comparable intensity to those observed for solutions of ribosomes at neutral pH (cf. Fig. 1). Minor increases in the intensity of L11 relative to those of L7/L12 and L10 are consistent with the enhanced interactions of L7/L12/L10 in the pentameric stalk complex reducing the extent of dissociation to individual proteins. By contrast the low m/z region of the spectrum recorded in the presence of thiostrepton (Fig. 5B) shows that the proteins L7/L12 that dominate spectra obtained in fusidic acid and naked ribosomes are virtually absent under these conditions. Moreover, a number of additional peaks are observed and identified as the proteins L1, L5, L6, and L18.

Of the additional proteins that are released, the loss of L1 may be accounted for under these conditions because it has an intermediate interaction surface area with rRNA (Fig. 3), extends significantly beyond the RNA envelope, and interacts with only one RNA domain (18). The combination of these effects would suggest that it is likely to be sensitive to perturbations in protein-RNA interactions. Similarly, the binding site of L6 is established in close proximity to the factor binding site (34) (Fig. 5). Its release can be interpreted in terms of a conformational change to the RNA induced by binding of EF-G. It is interesting to note, however, that both L1 and L6 do not appear to be destabilized by binding of EF-G in the presence of fusidic acid providing evidence for a difference in the protein-RNA interactions in the two complexes.

The proteins L5 and L18, which are also released from the thiostrepton-inhibited ribosome EF-G complex, have the largest surface area of interaction with the 5 S RNA (Fig. 3). Their dissociation is therefore consistent with conformational changes, specifically to the 5 S RNA, reducing the surface area of interaction between the RNA and both L5 and L18. Interestingly, L25 which also interacts with 5 S RNA, although with a significantly reduced surface area of interaction compared with those of L5 and L18, is not released from this complex. This implies destabilization of protein 5 S RNA interactions in the region of the binding sites of L5 and L18 (Fig. 5) rather than at the base of the central protuberance where the binding site of L25 is located. In summary the four additional proteins that are released from the thiostrepton-inhibited complex are located either on the periphery of the particle (L1), at the factor binding site (L6), or interact exclusively with the 5 S RNA (L5 and L18).

As the observation of additional proteins can be interpreted in terms of changes to protein-RNA interactions, so too can the...
presence of non-covalent associations of proteins. For example, the observation of the pentameric stalk complex ([L7/L12]₅), L10) in the presence of EF-G is consistent with enhanced interactions between these proteins. Examination of the x-ray structure of the ([L7/L12], L10) stalk complex reveals that L7/L12 consists of distinct N- and C-terminal domains separated by a hinge region (35). The N-terminal domains are responsible for binding to L10, whereas the highly mobile C-terminal domains are implicated in the translation process, interacting with EF-G and visiting multiple sites on the ribosome (36). Moreover, cryo-EM analysis has established that the stalk proteins become elongated in the presence of EF-G (16, 37). The fact that the pentameric stalk complex is observed in mass spectra in the presence of EF-G in both complexes is therefore in accord with the observation of elongated forms of the stalk region observed by cryo-EM (17, 37).

It is also of interest to note the virtual absence of individual proteins in the mass spectra recorded for the thiostrepton-inhibited EF-G ribosome complex. The L7/L12 proteins that typically dominate all mass spectra often forming the base peak under neutral pH conditions examined here and in previous investigations (2, 3) are barely detectable in the mass spectra of this complex. This strongly implies that the highly mobile C-terminal domains of L7/L12 are anchored to the ribosome in some way. This observation is consistent with a previous finding of a covalent cross-link between L7/L12 and the protein L5 (38). This cross-link has been established both in the presence and absence of EF-G. For L7/L12 to bind at this location at least one dimer of the L7/L12 tetramer would have to be in an extended conformation, lying across the body of the 50 S subunit (39). By contrast the presence of individual proteins in the fusidic acid-inhibited complex indicates that a significant population of the stalk proteins is less constrained and therefore free to dissociate into individual monomers.

In summary the observation of both L5 and L18, two proteins that interact exclusively with 5 S RNA, together with the absence of L7/L12 proteins in mass spectra of the thiostrepton-inhibited complex is consistent with binding of the C-terminal domains of L7/L12 to the 5 S rRNA scaffold itself in the vicinity of the L5/L18-binding site. These results therefore suggest that in the ribosome EF-G thiostrepton-inhibited complex, L7/L12 is capable of destabilizing L5 and L18 5 S RNA interactions, which in turn lead to a markedly different gas phase dissociation pattern. We propose therefore that in the thiostrepton-inhibited complex, contacts between the elongated form of the L7/L12 stalk promote dissociation of L5 and L18 through long range interactions with the 5 S RNA.

CONCLUSION

The dissociation of proteins in the gas phase of the mass spectrometer in response to solution conditions has been investigated. The results show that increasing pH values from pH 7 to 11 does not change the dissociation pattern. By contrast for ribosomes analyzed from low pH solutions, many more proteins are observed in the mass spectra. Similarly replacement of Mg²⁺ ions by Li⁺ brought about significant changes in gas phase dissociation. The dependence of protein dissociation on the magnitude of protein-RNA interactions allows us to investigate solution conditions where the precise contacts are not well defined. The results recorded for ribosome EF-G complexes inhibited by thiostrepton and fusidic acid reveal markedly different dissociation patterns allowing us to demonstrate interactions between mobile regions of the ribosome with the RNA architecture which result in the dissociation of additional proteins. More generally these results highlight a novel application of MS to probe protein-RNA interactions, an area likely to be of increasing importance not only with the growing interest in ribozymes (40), but also for macromolecular complexes for which the precise protein-nucleic acid interactions have yet to be defined.

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Dissociation of Intact Escherichia coli Ribosomes in a Mass Spectrometer: EVIDENCE FOR CONFORMATIONAL CHANGE IN A RIBOSOME ELONGATION FACTOR G COMPLEX

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