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Correlating the Chemical Modification of *Escherichia coli* Ribosomal Proteins with Crystal Structure Data

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Various chemical modifications have been applied to study protein structures. In this paper, amidination of *E. coli* ribosomal proteins was investigated to profile the structure of this large protein/RNA complex. The extent of ribosomal protein amidination was correlated with the solvent accessibility of amine groups in *E. coli* ribosome crystal structures. The modification of many residues was confirmed by CID of tryptic peptides. The amidination of proteins in the intact ribosome is very consistent with crystal structure data. The extent to which monomethylated amine groups can be amidinated was also examined. This information was used to interpret the amidination of several ribosomal proteins. Interestingly, ribosomal proteins L7 and L12, which share the same sequence and differ only by acetylation of the N-terminus, were found to be methylated to different extents. L12 is largely monomethylated but only a small portion of L7 is so modified.

Keywords: ribosomal proteins • chemical labeling • mass spectrometry • liquid chromatography • post-translational modifications

Introduction

Cellular proteins often work as part of a large complex to perform their specific function. Therefore, studying the structures of protein complexes is an essential step toward understanding their functions. For example, ribosomes contain more than 50 proteins and rRNA components. As such, they are the largest macromolecular complex in living cells. Traditionally, the structures of large proteins or protein complexes are studied using either X-ray crystallography or nuclear magnetic resonance (NMR).[1−5] Although these methods have been successfully used to investigate protein structures, they have certain well-known limitations. Some proteins, especially large protein complexes, are difficult to crystallize since crystallization condition is hard to predict. NMR has the problem of overlap between peaks if the protons have similar chemical shifts. Therefore, larger proteins (>20 KDa) are not suitable for NMR analysis since the data interpretation is complicated.[6] These two methods are time-consuming and need relatively large amounts of sample.

An alternative method to study protein structures involves combining chemical labeling with mass spectrometry. Although it does not provide the spatial resolution of NMR or X-ray crystallography, it is possible to map solvent accessible areas in native structures and derive information about interface regions of large protein complexes. Hydrogen/deuterium exchange has been employed to determine protein conformations by labeling solvent accessible backbones.[7−13] The protein mass increases by 1 Da per exchange when hydrogen atoms at backbone amides or nonaliphatic side chains are replaced by deuterium. Enzymatic digestion is often done following hydrogen−deuterium exchange, and the resulting peptides are analyzed by LC−MS/MS.[8] Although this method is straightforward and rapid for analysis of ligand binding, protein folding dynamics and protein−protein interactions in complexes,[8,10,12−15] H/D back-exchange or deuterium scrambling can complicate data interpretation.[16−18]

Residue specific chemical modifications have been developed to probe protein structural information. The use of covalent labels avoids scrambling and so data interpretation is simplified. Chemical modification can also introduce large mass shifts that obviate the need for high mass accuracy. Carbethoxylation of histidine,[19] carboxyamidomethylation of methionine,[20] nitration and iodination of tyrosine,[21−24] and cysteine modification by carboxylate amidination[25] as well as arsenous acid derivatives[26,27] have been investigated previously. A less specific modification method involving photochemical oxidation was introduced recently.[28,29] The photochemical oxidation cannot be applied to transition metal-binding proteins due to the oxidation of the metal.[30] Acetylation or succinylation of lysine residues and N-termini by acetic anhydride or succinic anhydride are particularly common protein modifications.[21,22,31−34] Lysine has also been modified by N-hydroxysuccinimidyl acetate.[35] Przybylski and co-workers modified lysine as well as cysteine by fluorescein-5′-isothiocyanate.[36] To probe protein conformation at the interface between two proteins using mass spectrometry, the choice of modification is very important. It has to be performed under mild reaction conditions that preserve the native conformation of protein and complex. Since the protein conformation is supported by noncovalent hydrogen bonds and electrostatic interactions, it can be disturbed by a derivatization that changes the charge state of a residue.[37] In addition, it would be preferable if the target residues were abundant enough in

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natural protein sequences that chemical modification could effectively probe protein conformation.

S-Methyl thioacetimide was developed as a modification reagent to probe the conformation of proteins and complexes.[38] It reacts with primary amine groups under mild conditions (pH around 8, room temperature, short reaction time). It has been found that this amidination reaction can elucidate the solvent accessible surface of folded proteins without disturbing native structures.[39,40] Amidination has been applied to probe the interfaces within ribosomal complexes.[41] The ribosome is widely investigated because of its essential function in manufacturing new proteins. Ribosome crystals for X-ray crystallography are very difficult to grow due to the huge size of the complex (>2 MDa) and the flexibility of some ribosomal proteins. After years of study, ribosome crystal structures for *Thermus thermophulus*, *Escherichia coli*, and *Deinococcus radiodurans* have been successfully solved.[2,42−45] Amidination combined with mass spectrometry is an alternative strategy to profile ribosome structure. Previously we used this approach to investigate the *Caulobacter crescentus* ribosome.[41] Labeling results were compared with crystal structures from other organisms because the *C. crescentus* ribosome has not been crystallized. This study demonstrated that the modification of most ribosomal proteins correlates well with crystal structure data. However, some of the proteins yield conflicting data. It is important to be aware of sequence variations among different organisms.[41] A better system with known crystal structures need to be selected for better correlation in order to validate the amidination labeling approach. Crystal structures of both small and large subunits of the *E. coli* ribosome have recently been reported.[2] Therefore, *E. coli* ribosome is now investigated by amidination labeling and results are correlated with the solvent accessibility of primary amines as derived from crystal structures.

Experimental Section

Chemicals. Trypsin, ammonium bicarbonate, magnesium acetate, Trizma base, ammonium chloride, formic acid, 2-mercaptoethanol, *N,N*-Diisopropylethylamine, piperidine and α-cyno-4-hydroxycinnamic acid were purchased from Sigma-Aldrich (St. Louis, MO). Glacial acetic acid, urea, trifluoroacetic acid and Fmoc amino acids for peptide synthesis were obtained from EM Science (Gibbstown, NJ). DEBPT was supplied by Midwest Biotech (Indianapolis, IN).

Amidination of Intact Ribosomes. *S*-Methyl thioacetimide was prepared as described by Beardsley and Reilly.[46] Starter cultures of *Escherichia coli* K12 were grown overnight at 37 °C. Intact ribosomes were isolated from *E. coli* following the method of Spedding.[47,48] Solutions containing intact ribosomes were mixed with equal volume of 43.4 g/L *S*-methyl thioacetimide buffered with 250 mM Trizma base at room temperature. Glacial acetic acid and 1 M $MgCl_2$ were added after 1 h incubation to stop the reaction and precipitate rRNA. The final solution contained 3:6:1(v/v/v) ribosome/glacial acetic acid/1 M $MgCl_2$. The rRNA was precipitated using centrifugation at 14100 g and clear supernatant containing modified ribosomal protein was stored in a freezer for future analysis.

Amidination of Disassembled Ribosomal Proteins. Glacial acetic acid (200 μL) and 1 M $MgCl_2$ (33.3 μL) were added to 100 μL of intact ribosome to disassemble the organelle and precipitate out rRNA. Precipitated rRNA was separated by centrifugation at 14100 g. 1.8 mL ice cold acetone was added to the supernatant and the solution was cooled on ice for 30 min. Ribosomal proteins were precipitated out and centrifuged at 1000 g for 15 s. The supernatant was removed carefully. The protein should be centrifuged at very low speed to help redissolving. The white precipitate was kept on ice for another hour to evaporate all acetone. The precipitated proteins were redissolved using 100 μL 6 M urea in buffer at pH 8.0 containing 10 mM Tris-HCl, 10 mM magnesium acetate, 60 mM NH_4Cl, and 3 mM β-mercaptoethanol. The final concentration should be similar to the intact ribosome solution. The solution of disassembled ribosomal proteins was added to an equal volume of 43.4 g/L *S*-methyl thioacetimide buffered with 250 mM Trizma base. The reaction was quenched after 1 h using glacial acetic acid and 1 M $MgCl_2$.

Chromatography and Mass Spectrometry of Ribosomal Proteins. Protein samples were loaded onto a home-built 2D-LC system for separation.[49] The first stage involved a strong cation exchange (SCE) column (Tosohaas, SP NPR). Proteins were eluted from this column using a salt gradient and fractions were directed to 20 reverse phase "trap" columns (Javelin, Thermo, 20 × 1.0 mm, C4) positioned in-line via an automated valve-switching apparatus. Salts were washed out using aqueous mobile phase before the trapped samples were successively directed into a second C4 reverse phase column (Thermo Hypersil Keystone Pioneer, 1 × 100 mm) whose eluent was electrosprayed into a micro Q-TOF (Waters, Manchester, U.K.) mass spectrometer. The mobile phases are water and acetonitrile with 0.1% formic acid. The eluent was split to 5 μL/min into mass spectrometer. The mass spectra are deconvoluted using MaxEnt algorithm.

Digestion of Amidinated Ribosomal Proteins. The modified ribosomal proteins were similarly loaded onto the traps and eluted from the 2D-LC system with high organic mobile phase and the fractions were collected using automated fraction collector. The fractions were evaporated to dryness using a speed-vac (Jouan, Winchester, VA) and redissolved in 100 mM ammonium bicarbonate buffer. Modified ribosomal proteins were digested by trypsin at 37 °C overnight. The digestions were terminated by adding TFA to 1% (v/v). Tryptic digested proteins were analyzed by nanoLC−ESI−MS/MS (LCQ and LTQ-FT, Thermo, San Diego, CA) using C18 reverse phase columns. The peptides were first trapped by a C18 trapping column and then separated by an analytical C18 column. The mobile phases are water and acetonitrile with 0.1% formic acid. CID MS/MS spectra of the top five intense peaks in every spectrum were acquired during analysis. Database searches (Mascot Search, Matrix Science, MA) were done using Swiss-Prot to identify peptides based on raw data. Amidination, methylation, acetylation, and oxidation were selected as variable modifications.

Synthesis of Monomethylated Peptides. To investigate the impact that a methyl group has on the amidination of an amine, a series of peptides containing methylated lysine or N-terminus were synthesized using standard solid phase peptide synthesis procedures.[50] The peptide sequences are indicated in Figure 2. Three of these peptides are monomethylated on either the N-terminus or a lysine side chain. Two other peptides have dual monomethylations. An unmodified peptide was also synthesized for comparison. Monomethylated amino acids were purchased from EMD.

Amidination of Synthesized Peptides. Synthesized peptides were redissolved in aqueous solution and diluted to a concentration of 100 μM. This was mixed with an equal volume of 43.4 g/L *S*-methyl thioacetimide buffered with 250 mM Trizma base. After a 1 h reaction, TFA was added to 10% (v/v) to stop

the amidination reaction. All the peptides were analyzed using MALDI-TOF. The MALDI spots were prepared by mixing 1 μL of peptide solution with 9 μL of matrix containing 10 g/L α-cyano-4-hydroxycinnamic acid in 50% water, 50% acetonitrile, and 0.1% TFA.

Results

Intact Masses of Ribosomal Proteins. Intact *E. coli* ribosomes were isolated as discussed above. Ribosomal proteins were extracted by precipitating RNA using glacial acetic acid and 1 M $MgCl_2$. The precipitate was separated by centrifugation and the supernatant was loaded onto the home-built 2D-LC system. Following their chromatographic separation, ribosomal proteins were electrosprayed and mass analyzed. Mass spectra were deconvoluted using the MaxEnt algorithm and masses measured to within 2−3 Da. The protein masses are listed in Table 1 and compared with theoretical masses from genome sequences. Post-translational modifications are tabulated and included in the calculation of the theoretical masses. Most post-translational modifications are already known for *E. coli* ribosomal proteins.[48] The identification of protein is confirmed by the corresponding mass deconvoluted using MaxEnt. Since ribosomal proteins are separated by 20 traps, the peptide identification from the same trap also confirms the presence of ribosomal proteins. S6 has a special modification and its identification was confirmed by the peptide from the same fraction of 2D-LC separation. All of the ribosomal proteins were identified except S1. The loss of S1 is expected due to the *E. coli* ribosomal protein preparation procedure.[51] All of the measured protein masses are consistent with known modifications and therefore with the theoretical mass values. Amidination results contained in Table 1 are discussed below.

Amidination of Intact and Disassembled Ribosomes. Amidinated proteins of the intact and disassembled ribosome were analyzed just as the unmodified proteins were. Figure 1 shows deconvoluted spectra of some native and amidinated ribosomal proteins. In each case, the top spectrum displays data for native proteins, while the middle and bottom ones were recorded from amidinated intact and disassembled ribosomes, respectively. It is evident that proteins of the intact ribosome are amidinated to varying extents, whereas proteins from the disassembled ribosome exhibit only one dominant modification peak. Following amidination of the intact ribosome, some proteins such as L2 exhibit wide peak distributions, and some (e.g., L9) have relatively narrow distributions. Wide distributions of modification suggest that some primary amines are partially shielded from amidination, which means that these groups are either involved in dynamic interactions or are only partially solvent accessible in the complex. Interestingly, for some proteins like L16 and L17 only a small fraction of the available sites are labeled in the intact ribosome. Others, such as L30 and L9 are almost completely amidinated in the native complex. All the spectra of modified disassembled ribosomes contain only one intense peak that corresponds to complete labeling of all available reaction sites (i.e., unmodified lysine residues and N-termini). Occasionally a low intensity peak with one fewer modification is observed as in the case of L9. Most proteins are less extensively amidinated in the intact ribosome compared to the disassembled complex. The varying extent of amidination provides some information about the position of these proteins and the structure of the intact complex. Proteins that are extensively modified are on the surface of the complex, whereas those that are sparsely labeled are buried in the complex by rRNA or other proteins. Minor peaks sometimes appear at a mass that is 46 Da higher than the one corresponding to complete amidination of the disassembled ribosome (e.g., L2 and S4). We have previously shown that this 46 Da mass shift results from the addition of an *S*-methyl group to cysteine.[39]

Table 1 summarizes the labeling of all ribosomal proteins except S1. The available sites are considered to be all of the lysine residues and free N-termini that are without methylation, acetylation or any other post-translational modification. The numbers of labeled sites for the intact or disassembled ribosome were calculated as the average modification numbers weighted by mass spectral peak intensities. As noted above, the difference in the number of labels between the intact and disassembled ribosome can be large and varies from one protein to another. The percentage of protein sites labeled in the intact ribosome varies from 22 to 100% and 60−80% is typical. Following disassembly of the ribosome, every observed protein is either completely or almost completely modified. Comparison of these results with *E. coli* crystal structures will be discussed later. Protein S22 was not detected in the amidinated intact ribosome. Even the unmodified S22 and amidinated disassembled protein appear as low intensity peaks. Recently, S22 was identified as a ribosome-associated protein and not an actual ribosomal protein since it is not an essential component of ribosomes.[52,53] This protein is less abundant than other proteins in the 30S subunit, so it is difficult to detect after ribosome extraction from *E. coli*. Consistent with this, in a previous MALDI-TOF study of *E. coli* ribosomal proteins we found that the S22 intensity was very low.[48,53]

Amidination of Peptides with Monomethylations. To test the effect that methylation of lysine and N-terminal amines has on their amidination, a series of peptides were synthesized and amidinated. All the peptides contain two lysine residues and one N-terminus some of which are monomethylated. MALDI-TOF mass spectra of these peptides appear in Figure 2. The unmodified peptides are shown on the top and their amidinated counterparts are at the bottom. Figure 2A involves the amidination of an unmethylated peptide that has three primary amine groups. After amidination, there is a 123 Da mass shift indicating derivatization at all three sites. The amidination reactivity of methylated N-terminus and lysine was studied and spectra appear in Figure 2B, C, and D. Figure 2B displays a peptide with a monomethylated N-terminus. Only two of three amine groups are modified. An MS/MS experiment demonstrated that the monomethylated N-terminus was not amidinated (data not shown). Figure 1C and D involve peptides that have been monomethylated on different lysine residues. Both peptides exhibit 123 Da mass shifts indicating that all three amine groups, including the methylated one, are amidinated. Therefore, monomethylation of lysine does not affect its reactivity. These results show that monomethylated residues are either amidinated completely or they do not react at all. Partial amidination is not observed. Figure 2E and F display another example. Eighty-two Dalton mass shifts are observed in the amidinated spectra for both peptides, indicating that only two of three sites react with the amidination reagent. MS/MS spectra confirmed that in both cases the methylated N-terminus is not amidinated but the two lysine residues are modified (see Supplementary Figures 1 and 2). We conclude that monomethylated lysine can be amidinated and the position of lysine does not affect the modification result. On the

Table 1. Summary of Labeling of Intact and Dissembled Ribosome

proteins	theoretical mass[b]	experimental mass	# of labeled intact	# of labeled dissassembled	available sites	post-translational modifications
L1	24598.6	24598.8	23.23	24.00	24	−Met
L2	29729.4	29729.3	18.27	26.00	26	−Met
L3	22257.6	22257.8	10.61	20.00	20	+methyl
L4	22086.6	22087.2	13.60	19.00	19	
L5	20170.5	20170.2	14.98	17.00	17	−Met
L6	18772.7	18772.5	15.10	17.00	17	−Met
L7[a]	12206.1	12205.6	12.71	12.81	13	−Met
L9	15769.1	15768.8	11.96	12.00	12	
L10	17580.5	17580.5	11.45	12.80	13	−Met
L11	14870.3	14870.4	12.13	13.00	13	−Met, +9 methyl
L12[a]	12164.1	12164.0	13.66	13.64	14	−Met
L13	16018.6	16018.5	8.13	15.00	15	
L14	13541.1	13540.9	9.65	12.00	12	
L15	14980.5	14980.1	5.35	14.00	14	
L16	15326.3	15327.2	6.46	16.00	16	+2 methyl and 1 hydroxyl
L17	14364.7	14364.2	2.66	9.00	9	
L18	12769.7	12769.4	8.55	10.00	10	
L19	13002.1	13001.7	9.16	12.00	12	
L20	13365.8	13366.5	7.16	15.00	15	−Met
L21	11564.4	11564.4	6.00	11.00	11	
L22	12226.4	12226.2	8.67	14.00	14	
L23	11199.2	11198.8	8.04	15.00	15	
L24	11185.1	11185.3	11.93	17.00	17	−Met
L25	10693.5	10693.5	11.00	12.00	12	
L26/S20	9553.2	9552.9	9.53	15.00	15	−Met
L27	8993.3	8993.2	9.97	12.00	12	−Met
L28	8875.3	8875.5	3.82	7.87	8	−Met
L29	7273.5	7273.3	6.37	7.00	7	
L30	6410.6	6410.2	5.42	6.00	6	−Met
L31	7871.1	7870.0	7.90	9.00	9	
L32	6315.2	6314.8	3.95	7.00	7	−Met
L33	6254.4	6254.1	10.12	12.00	12	−Met, +methyl
L34	5380.4	5380.0	1.31	5.83	6	
L35	7157.8	7157.1	4.04	15.00	15	−Met
L36	4364.4	4363.0	3.28	7.88	8	
S2	26612.6	26612.6	18.37	20.00	20	−Met
S3	25852	25852	21.49	24.00	24	−Met
S4	23338	23337.9	14.11	21.00	21	−Met
S5	17514.3	17514.7	8.08	12.00	12	−Met, +acetyl
S6[b]	15316.8	15316.0	7.00	7.00	7	glutamate additions
S7	19888.0	19887.6	11.68	15.00	15	−Met
S8	13995.3	13995.9	10.85	12.85	13	−Met
S9	14725.1	14724.9	7.60	11.00	11	−Met
S10	11735.6	11736.0	4.48	6.00	6	
S11	13727.8	13728.4	6.80	9.00	9	−Met, +methyl
S12	13651.9	13650.2	8.78	14.00	14	−Met, +β-methylthiol
S13	12968.3	12968.7	10.20	12.00	12	−Met
S14	11449.3	11450.1	8.27	12.00	12	−Met
S15	10137.6	10137.1	5.34	7.00	7	−Met
S16	9190.6	9190.4	3.22	6.00	6	
S17	9573.3	9572.8	7.97	10.80	11	−Met
S18	8897.3	8896.8	4.06	6.00	6	−Met, +acetyl
S19	10299.1	10298.6	10.24	14.00	14	−Met
S21	8368.8	8368.5	8.10	10.00	10	−Met
S22	5095.8	5095.7	−	7.00	7	

[a] Only unmethylated L7/L12 proteins were considered in this table. [b] Theretical mass is calculated from the genome sequence with post-translational modifications.

other hand, a methylated N-terminus is not amidinated under these reaction conditions. It is known that the guanidination reaction similarly modifies the ε-amine of lysines but not the peptide N-terminus.[54] One possible reason is that the α-amine is less nucleophilic than the ε-amine because of electron withdrawal by the nearby carbonyl group. In addition, the side chain attached to the α-carbon is much bulkier than the two hydrogens on the δ-carbon of lysine. The extra steric hinderance discourages guanidination of the α-amine. The amidination reagent (*S*-methylacetimidate) is more reactive than the guanidination reagent (O-methylisourea) so it modifies both primary α- and ε-amines. However, monomethylation introduces enough ad-

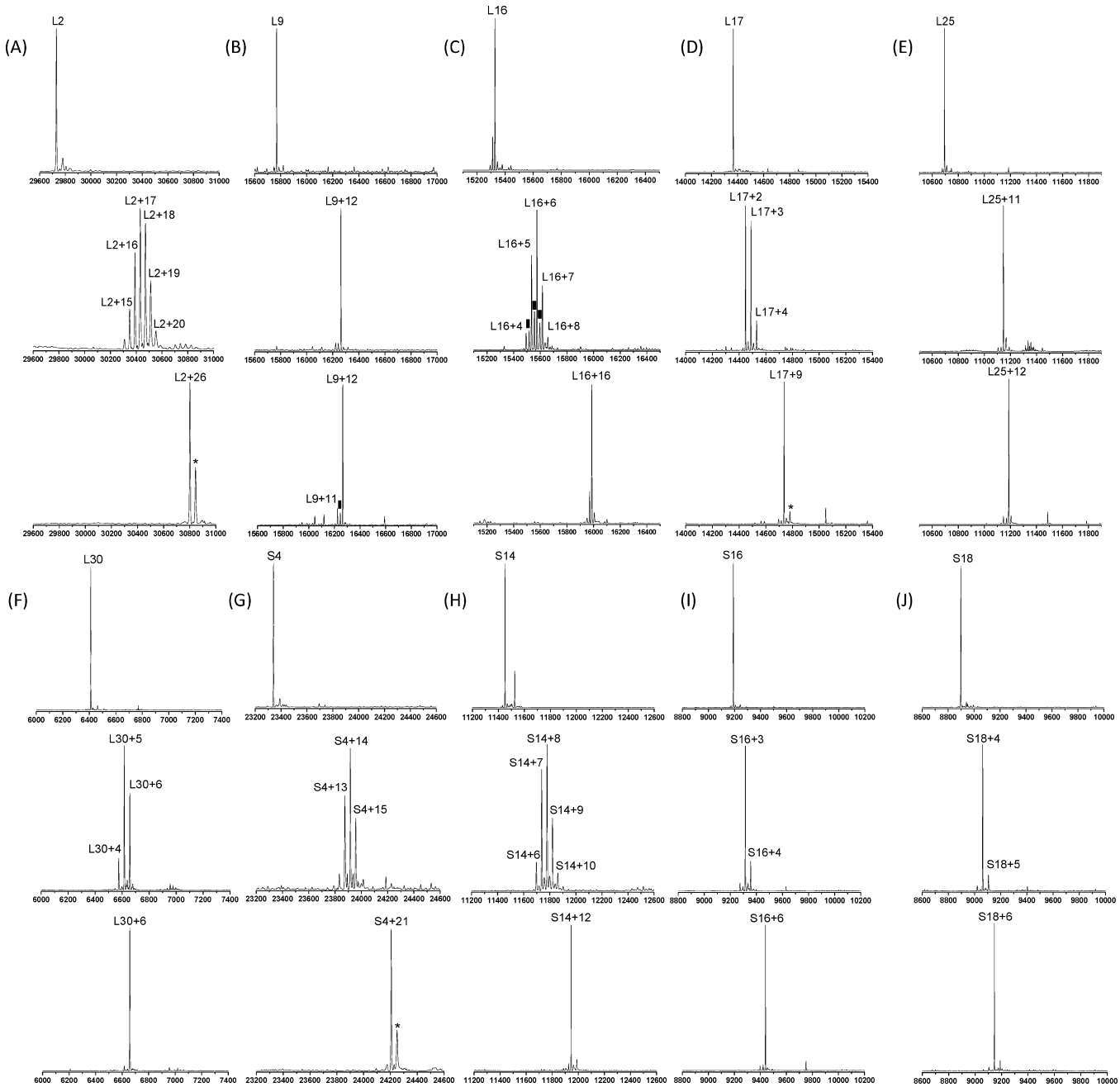


Figure 1. Deconvoluted spectra of native and modified ribosomal proteins (A) L2, (B) L9, (C) L16, (D) L17, (E) L25, (F) L30, (G) S4, (H) S14, (I) S16, (J) S18. The top spectra show the native proteins, the middle and bottom spectra are the modified proteins of intact and disassembled ribosome respectively. Stars indicate the addition of *S*-methyl group to cysteine and squares label the sodium adduct of ribosomal proteins.

ditional steric hindrance that under our conditions derivatization only occurs at the more favored N-terminus.

Ribosomal Proteins with Post-Translational Modifications. It is known that some ribosomal proteins have post-translational modifications including methylation, acetylation or other amino acid side chain modifications.[48,55−57] L3, L11, L33, and S5 are examples of modified proteins. The effects of these modifications on the amidination are shown in Figure 3A. L3 is methylated on Gln150,[57] which should not have any effect on its amidination. As seen in the spectrum, all the potential sites of L3 modification are amidinated in the disassembled ribosome. Ribosomal protein L11 contains sixteen amine groups but the N-terminus and two lysine residues are all trimethylated.[48,58] Amidination of L11 is therefore expected at thirteen sites and this is what is observed. The N-termini of L33 and S5 are methylated and acetylated respectively,[58,59] and both proteins have one fewer modification than the number of amine groups in the proteins. This is consistent with our peptide result that methylated N-termini are not be amidinated. The MS/MS spectra of S5 N-terminal peptide A2-R20 before and after amidination are displayed in Figure 3B. There are two lysine residues and one acetylated N-terminus in this peptide. The masses of the b2 and b4 fragment ions indicate that both the methylated and the acetylated N-terminus are not amidinated. The 20.5 Da mass shifts on $y10^{2+}$ and $y15^{2+}$

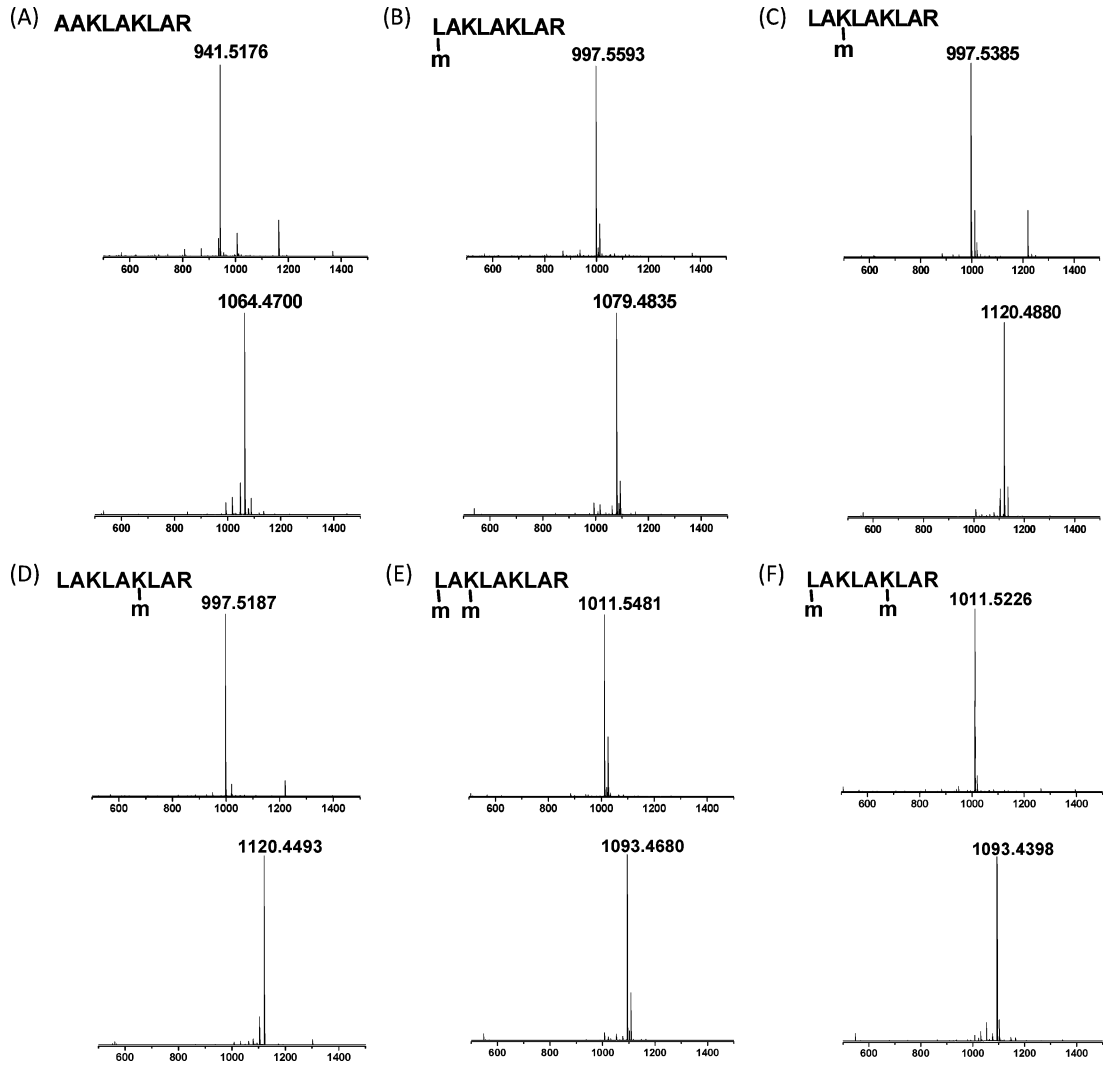


Figure 2. MALDI-TOF spectra of monomethylated peptides with and without amidination. The amidinated results are shown below the masses of native peptides. Residues labeled "m" are monomethylated.

ions prove that the two lysine residues are amidinated. This result confirms that N-terminal acetylation blocks amidination.

L7 and L12 with thirteen lysine residues and one N-terminus are two other ribosomal proteins with post-translational modifications. They share the same genome sequence and their only difference is the presence (L7) or absence (L12) of an acetyl group at the N-terminus. It has been proposed that the presence of acetylation increases the interaction with L10 in the ribosomal stalk complex.[60] After the strong cation exchange and reversed phase chromatographic separation, protein L12 was eluted about one minute before protein L7 using a 50 min gradient. The deconvoluted mass spectra of L7 and L12 before and after amidination appear in Figure 4. The top spectra are native proteins and the bottom two are amidinated proteins from the disassembled ribosome. L7 and L12 are clearly methylated to different extents. The dominant peak of L7 is the protein without methylation, but this is reversed for L12. It is assumed that one methyl or acetyl group does not significantly change the ionization efficiency of both proteins. Therefore, the extent of methylation of L7 and L12 can be accurately quantified. On the basis of this assumption, about 30% of L7 and about 70% of L12 is monomethylated. *Escherichia coli* grew at 37 °C in this experiment. It has previously been reported that L7/L12 is partially monomethylated at lysine 82[55] and that a very small portion of methylation occurs at 37 °C growth temperature.[61] The present experiment offers more accurate quantitation of the extent of methylation and better separation for L7 and L12. However, the reason why L7 and L12 are differentially methylated is still not clear. After amidination of the disassembled complex, there are thirteen amidinated sites for L7 and all the fourteen amine groups of L12 are modified as expected based on the presence or absence of N-terminal acetylation. The monomethylated proteins are amidinated to the same extent, which means that Lysine 82 is modified. There are small peaks corresponding to one fewer modification. This result is consistent with the conclusion that monomethylated lysine retains the amidination reactivity in the synthesized peptides even though the amino acid residue reactivity in peptides could be different from the reactivity in proteins.[62−64]

The MALDI mass spectrum of ribosomal protein S6 with two glutamic acid residues at the C-terminus has been reported previously.[48] However, this protein is identified to have a special post-translational modification in the present experiment. The spectra of native and amidinated S6 at two different growth times (12 and 16 h, respectively) are shown in Figure 5. S6 always coelutes with L11 in our 2D-LC separation. After 12 h of growth, it is found that most S6 molecules contain three

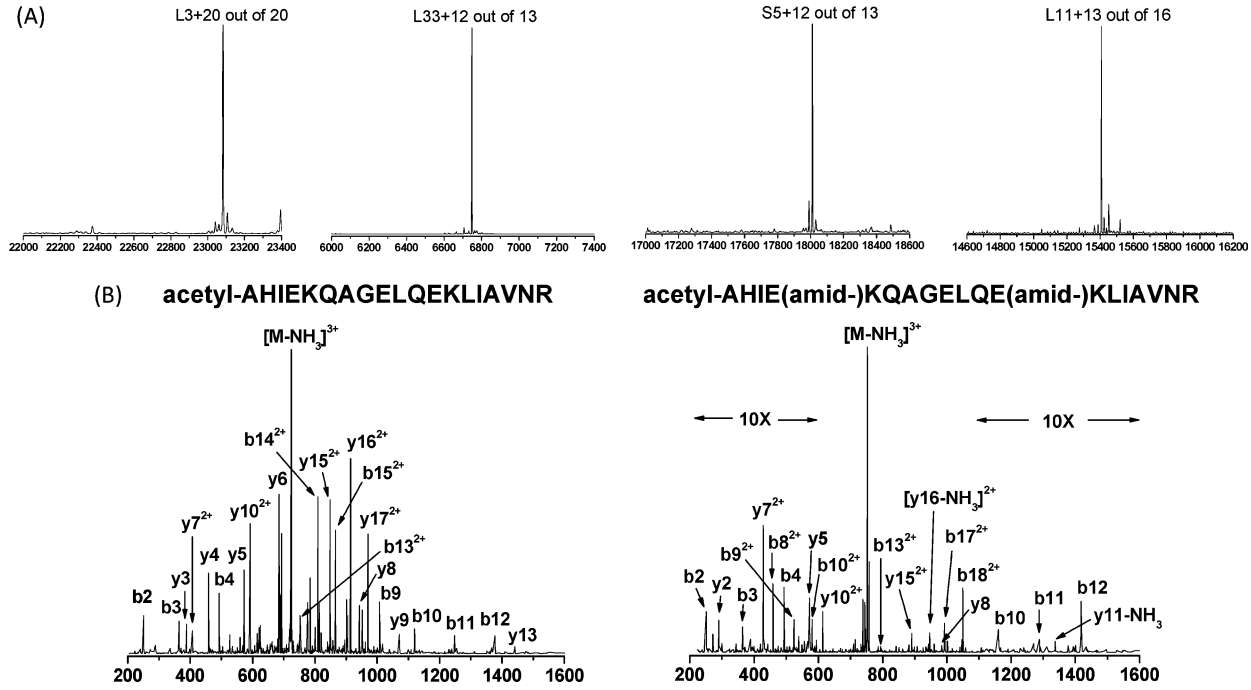


Figure 3. (A) Deconvoluted spectra of amidinated ribosomal proteins L3, L33, S5, and L11 in disassembled ribosome. L3 and L33 are methylated, S5 is acetylated, and L11 is trimethylated. (B) MS/MS spectra of S5 N-terminal peptide (acety-AHIEKQAGELQEKLIAVNR) before and after amidination. The parent ion is triply charged. Only b and y ions are assigned. The intensity of the low mass and high mass regions in the second MS/MS spectrum are enlarged by a factor of 10.

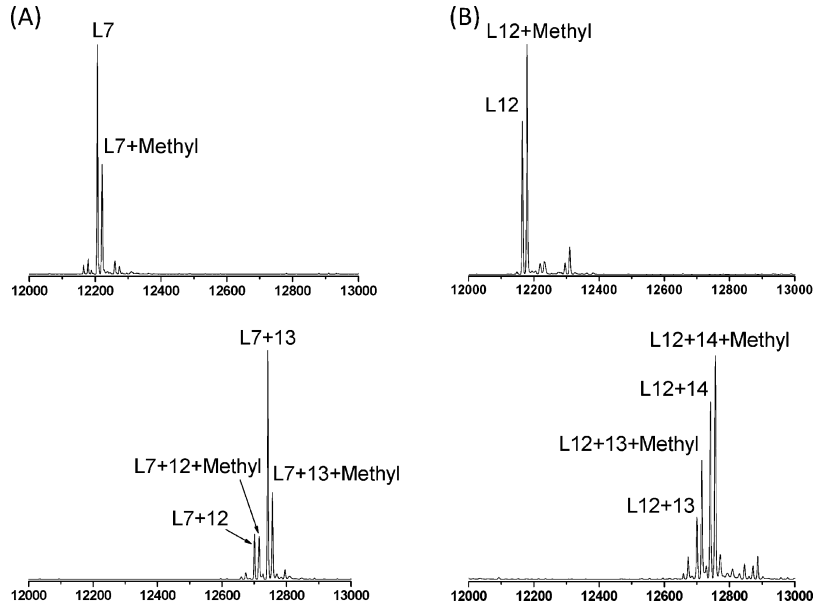
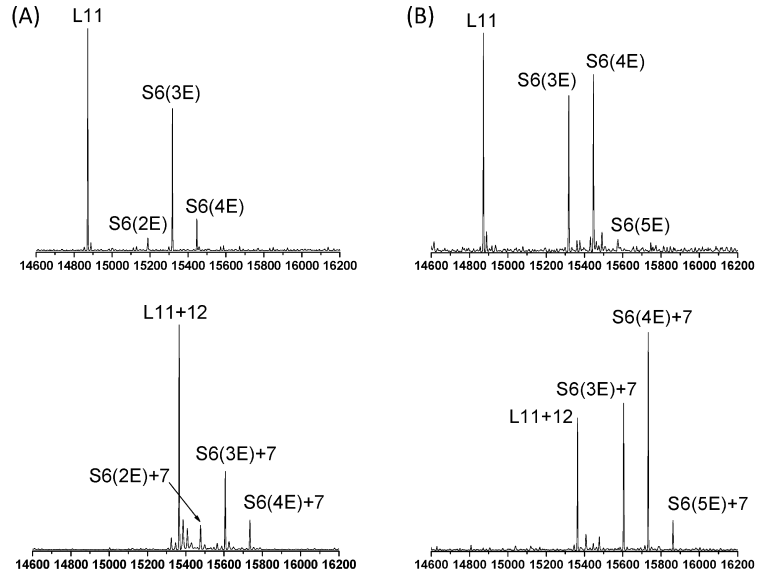


Figure 4. Deconvoluted spectra of ribosomal proteins L7 and L12. The top spectra are native proteins, and the bottom counterparts are proteins amidinated in the disassembled ribosome.

glutamic acids and a small number have two or four glutamic acids. When the growth time is extended to 16 h, the C-terminus of S6 mainly contains three and four glutamic acids and very few have five glutamic acid residues. This quantitative information about S6 at different growth times indicates that glutamic acid is added to the C-terminus of S6 as a post-translational modification. This is analogous to the previously reported addition of polyglutamic acid to folic acid.[65] The presence of different forms of S6 with up to six glutamic acid residues at the C-terminus has been reported using gel electrophoresis and amino acid analysis,[66,67] but the protein S6 with six glutamic acids was not observed in our experiments at either 12 or 16 h growth time. It may appear at longer growth time. S6 has seven potential amidination sites including six lysine residues and one N-terminus. All reaction sites of S6 are modified even in the intact ribosome. This result is consistent with *E. coli* crystal structures showing that all primary amines of S6 are solvent accessible.[2]

Comparison of Amidination Labeling of the Native Ribosome with the Crystal Structure. High quality *E. coli* crystal structures provide an excellent opportunity for comparison with the amidination results. Some ribosomal proteins



(C)

MRHYEIVFMVHPDQSEQVPGMIERYTAAITGAEGKIHRLEDWGRRQLAYPINKLHKAHYVLMNVEAPQEV
IDELETTFRFNDAVIRSMVMRTKHAVTEASPMVKAKDERRERRDDFANETADDAEAGDSEEEEEE

Figure 5. Deconvoluted spectra of S6 recorded after different growth times. (A) Twelve hours growth and (B) 16 h growth of *Escherichia coli*. The top spectra are native proteins and the bottom counterparts are the proteins amidinated in intact ribosome. (C) Primary sequence of S6.

are not shown in the crystal structures due to their flexibility. Proteins L1, L7/L12, L10, L26/S20, L28, S1, and S22 do not appear in published *E. coli* crystal structures. L31 has the wrong assignment in the structure.[68] By comparing the amidination of ribosomal proteins and their positions in the crystal structure, it is found that proteins that extend away from the 50S subunit core are highly amidinated and those that are buried in the complex structure are only modified to a small degree. For example, "stalk-like" proteins like L1, L7/L12, L9, and L10 are highly solvent accessible. But only L9 is displayed in the *E. coli* ribosome crystal structures due to their high conformational flexibility.[2,42,43,45,69,60] L1, L7/L12 and L10 appear in crystal structures from other organisms.[42,45] It is known that two copies of L7 and L12 bind with a single copy of L10 to form a pentamer, that is attached to the core of the complex.[70] Figure 6B shows the crystal structure of the 50S ribosome from the Protein Data Bank. Estimated positions of L1 and L7/L12 based on other similar organisms are marked in the figure. Mass spectra of L1, L10, and L7/L12 from the intact amidinated ribosome are displayed in Figure 6A. The modified L7 and L12 from the intact ribosome cannot be separated completely by the LC separation and the 1 Da mass difference between amidination and acetylation cannot be distinguished in the deconvoluted spectrum. Therefore, the spectrum shows a mixture of amidinated L7/L12 and the peaks are assigned as L12. For both L1 and L7/L12, the most intense peaks correspond to complete amidination. L10 is modified almost as extensively. Derivatized L9 has only one dominant peak corresponding to complete modification and its spectrum is shown in Figure 1B. The extensive labeling of L1, L9, L10, and L7/L12 is consistent with previous work indicating that these proteins interact little with rRNA.[41,69,71] In contrast, ribosomal proteins L34 and L35 are two examples that display limited amidination. Figure 6C shows the spectra of L34 and L35 from the intact amidinated ribosome. L34 only has one or two amidinated sites and the dominant peak of modified L35 corresponds to four out of fifteen available sites being labeled. These results indicate that most available amines of L34 and L35 are not solvent accessible. The crystal structures of L34 and L35 are shown in Figure 6D. They show that L34 is indeed buried inside the complex, so most potential sites are shielded from amidination. Even though L35 contains substantial solvent-accessible surface area, only a few lysine residues are exposed. Most potential sites of L35 are in the area that extends to the core of the complex. These examples illustrate how the amidination results adequately profile the positions of ribosomal proteins in the complex.

Two different conformations for the intact *Escherichia coli* 70S ribosome have been reported in X-ray crystallography experiments.[2] These two structures may relate to structural changes caused by the mRNA and tRNA movements through the ribosome during translocation of the elongation cycle.[72,73] In order to compare the labeling of intact ribosomal proteins with solvent-accessible sites in crystal structures, both conformations were considered. Numbers of solvent accessible amine groups were derived from the two *E. coli* 70S ribosome structures and the results were averaged for each protein. The number of amidinated sites for each protein was calculated by weighting modification numbers by peak intensities. For most ribosomal proteins, the number of amidinated sites correlates very well with the number of solvent exposed primary amines in the crystal structures. However, there are some proteins like L11, S3 and S19 that display obvious differences. L11 has 12 amidinated sites in our experiments, but the crystal structure shows sixteen solvent exposed amine groups. However, S3 and S19 are modified five times more than the number of solvent accessible sites in the crystal structure. Other factors like post-translational modifications and sequence incompleteness in crystals are not considered when counting the solvent accessible amine groups in the crystal structures. L11 has three trimethylated lysine residues that cannot be amidinated. These trimethylations are not apparent in the crystal structure so

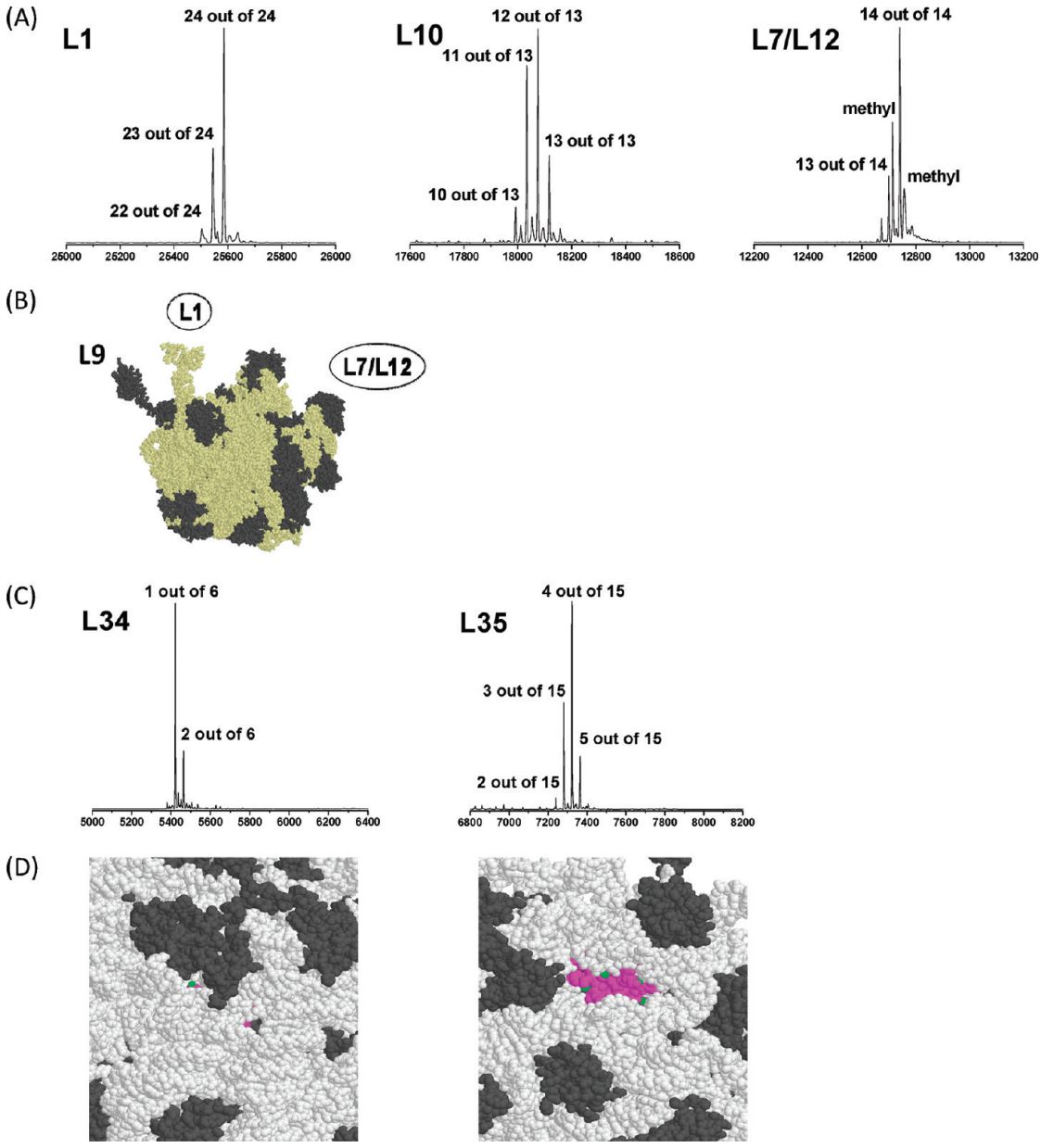


Figure 6. (A) Deconvoluted spectra of L1, L10, L7/L12 amidinated in the intact ribosome. (B) Crystal structure of 50S ribosome (PDB: 2AW4). Circled L1 and L7/L12 mark the positions of these proteins based on other similar organisms. Yellow color displays rRNA and dark gray shows ribosomal proteins. (C) Deconvoluted spectra of L34 and L35 amidinated in the intact ribosome. (D) Structures of L34 and L35 from crystal structure (PDB: 2AW4). White color shows ribosomal proteins, dark gray is rRNA, magenta is ribosomal protein L34 and L35, and the side chain amines of lysine residues are in green.

these three sites need to be subtracted from the number of solvent accessible residues in crystal structures. The C-terminal sequences of S3 and S19, which include five lysine residues respectively, do not appear in the crystal structures. Sometimes, the N- or C-terminal portions of proteins are not observed in crystal structures due to their high flexibility. For this reason, we consider these missing lysines to be solvent accessible. Incorporating these factors for all the ribosomal proteins, the numbers of solvent accessible sites are compared with the numbers of labeled sites in Figure 7. These bar graphs indicate that the numbers of amidinated sites are highly consistent with the crystal structure data. The greatest discrepancy is with L17. In this protein five lysines including K35, K56, K78, K99, and K121 appear to be solvent exposed in crystal structures, but only two to four amines are amidinated (Figure 1D). In order to determine which L17 sites were labeled, tryptic peptides were mass analyzed and fragmented. Spectra are displayed in Figure 8A and B. All these five lysines are identified as amidinated in some peptides. However, some peptides containing unmodified K56, K78, and K121 were also observed indicating that these three lysines are only partially modified. This partial protection reduces the number of amidinated L17 sites to less than four. According to the crystal structure, the side chain amines of K78 and K121 are adjacent to glutamic acid residues that form salt bridges (Figure 8C). This strong charge−charge interaction apparently prevents the primary amines from being fully amidinated.

To determine which amine groups in the intact ribosome are modified, the amidinated ribosomal proteins were digested by trypsin and analyzed using ESI-MS/MS. There are 688

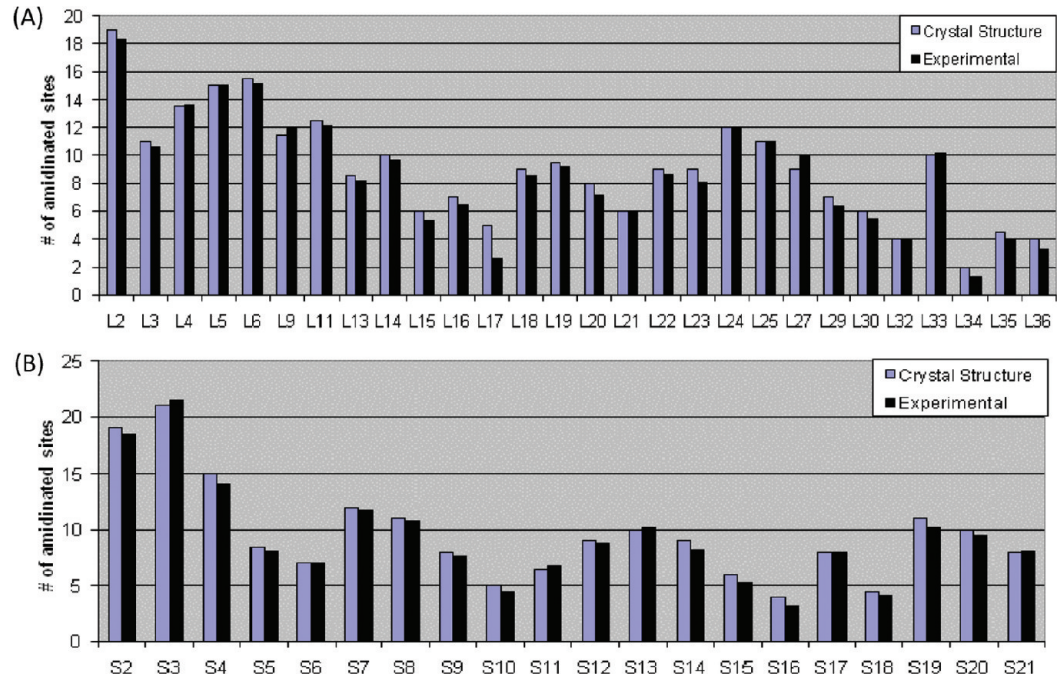


Figure 7. Corrected comparison of the labeling of (A) 50S subunit (PDB: 2AW4, 2AWB) and (B) 30S subunit (PDB: 2AVY, 2AW7) with solvent-accessible sites in crystal structures of 70S ribosome from *E. coli*.[2] Blue bars are the number of amine groups counted from crystal structure, and the black bars represent weighted average numbers of amidinated groups.

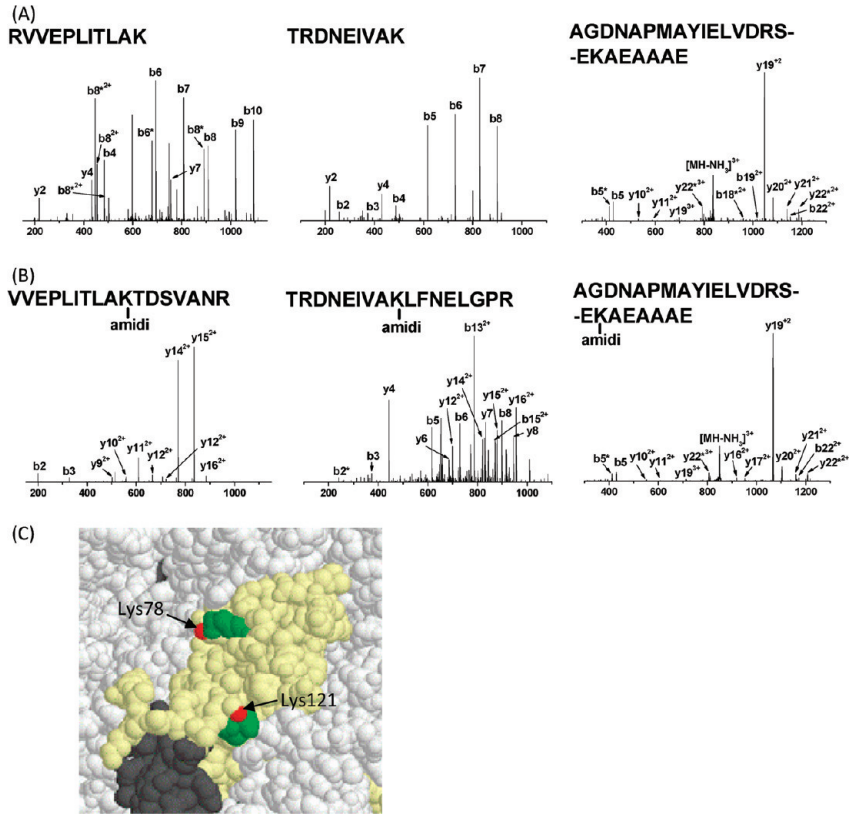


Figure 8. (A) MS/MS spectra of unmodified peptides containing K56, K78, and K121; (B) MS/MS spectra of peptides containing amidinated K56, K78, and K121; (C) crystal structure showing the interaction between lysine side chain amines and acidic residues (PDB: 2AW4). Primary amines of lysine side chains are in red, and glutamic acid residues are in green.

primary amines in all of the ribosomal proteins. Mascot Search results of CID fragmentation data indicate that 135 of these were fully amidinated, 140 were partially amidinated and 106 were unamidinated. Unfortunately, 307 of the sites were in peptides that did not appear in mass spectra, so their labeling could not be determined. Table 2 compares site modification results for two different ribosome crystal conformations. The first column lists ribosomal proteins studied in these experi-

Table 2. Comparison of Amidinated Sites Identified Using LC−MS/MS with Exposed Sites Counted from Two 70S Crystal Structures[a]

	amidinated sites				
	solvent accessible in both 2AW4 and 2AWB	only solvent accessible in 2AW4	only solvent accessible in 2AWB	not shown in either crystal structure	not solvent accessible in either structure
L2	K25,K35,K67,K70,K96,K107,K110,K146,K241,K252,K264,	K149,	K17,K124,		
L3	K55,K70,K105,K106,K190,K208,	N,K62,	K204,		K7,
L4	N,K57,K106,K123,	K185,	K194,		
L5	K2,K8,K13,K14,K46,K77,K119,K144,K178,	K32,	K71,K160,		
L6	K43,K84,K85,	−	−		
L9	N,K8,K22,K35,K41,K42,K57,K71,K83,K89,K112,	−	−		
L11	K112,	−	−		
L13	K23,K61,K121,K123,	−	K85,K106,		
L14	K111,K114,	K40,K44,K59,	K34,K118,K133,		
L15	N,K84,K129,K141,	K70,	−		
L16	K34,K62,K118,K133,	K127,	−		
L17	K35,K56,K78,K99,K121,	−	−		
L18	K17,K85,K88,	−	−		
L19	N,K5,K86,	−	−		
L20	K84,K192,K111,K113,	−	−		K41,
L21	N,K97,	−	−		
L22	K27,K48,K70,K73,K83,	K41,K42,	K28,		K16,K41,
L23	K44,K49,K88,	K64,	K9,		
L24	K16,K18,K20,K46,K60,K78,K90,	−	K42,	K103,	
L25	N,K83,K85,	−	−		
L27	K77,	−	−		
L29	K9,K44,K54,K60,	−	−		
L30	N,K2,K5,K18,K20,K55,	−	N,K2,K5,K18,K20,K55,		
L32	K31,K36,	K52,K56,	−		
L33	K26,	−	K7,K9,		K25,
L34	K37,	−	−		K11,
L35	−	−	−		
L36	−	−	−−		

	amidinated sites				
	solvent accessible in both 2AVY and 2AW7	only solvent accessible in 2AVY	only solvent accessible in 2WA7	not shown in either crystal structure	not solvent accessible in either structure
S2	K36,K44,K58,K63,K65,K104,K114,K127,	−	−		
S3	K15,K26,K37,K78,K79,K85,K88,K107,K113,	−	−		
S4	K21,K82,K155,K166,K176,	K182,	−		K206,
S5	K13,K51,K61,K65,K85,K158,	K155,	K125,	K5,K166,	
S6	K35,K53,K56,K93,	−	−	K104,K106,	
S7	K10,K16,K55,K75,K130,K148,	K34,	−	K170,	K36,
S8	K21,K30,K55,K63,K68,K88,K107,	−	K93,		
S9	N,K21,K26,K67,K99,	−	K59,		
S10	K30,K82,	K59,	−		
S11	K13,	−	−		
S12	K107,	−	−		
S13	K12,K26,K30,K43,	−	−		
S14	K18,K22,K46,K96,	−	K27,		K98,
S15	N,K9,K46,K47,K70,	−	−		K65,
S16	K13,K46,K76,K80,	−	−		
S17	K18,K29,K80,	−	−		
S18	K29,K37,	−	−		
S19	K5,K6,K16,	−	−		
S20	K15,K43,K48,K84,	K33,	K75,		
S21	K4.	−	N,		

[a] 2AW4 and 2WAB are large subunits. 2AVY and 2AW7 are small subunits. One 70S structure is formed by 2AW4 and 2AVY, and the other 70S structure is composed of 2AWB and 2AW7.

ments. L1, L7/L12, L10, L28, L31, and S22 are not included in this table since they do not appear in the crystal structures. The other columns in the table list specific lysines or N-termini whose labeling was consistent with one or both crystal structures. Of the 241 sites found to be partly or fully amidinated, 193 appear to be solvent accessible in both 70S structures. However, twenty of the amidinated sites (e.g., K40. K44, K59 of L14) appear to be solvent exposed only in one of the

ribosome structures (PDB: 2AW4 and 2AVY) and twenty eight amidinated sites (e.g., Lys85, Lys106 of L13) appear to be solvent accessible only in the other (PDB: 2AWB and 2AW7). Therefore, the observed labeling of these residues provides strong evidence that both of these ribosome structures exist in solution. Table 2 includes some sites that are missing from the crystal structures (e.g., Lys5 and Lys166 of S5) but were identified as amidinated. These sites are all at the terminal parts of ribosomal proteins that do not appear in the crystal structures due to the high flexibility. Another ten sites that are not solvent exposed according to the crystal structures were nevertheless found to be amidinated. These sites are on interfaces with rRNA or other proteins. For example, Lys25 of protein L33 was amidinated, even though it does not appear to be solvent accessible in the crystal structure. It resides at the interface between L33 and the RNA chain. This inconsistency reminds us that ribosomal proteins can be more flexible in solution than in solid crystals. Nevertheless,the data in Table 2 demonstrate that in general the labeling results are quite consistent with solvent accessibilities derived from the crystal structure.

Conclusion

Proteins in intact and disassembled ribosomes are labeled to very different extents. In the intact ribosome, stalk-like proteins are amidinated extensively, whereas those buried in the complex are only modified to a limited extent. All of the proteins in the disassembled complex are amidinated completely. Labeling of primary amines in ribosomal proteins is very consistent with crystal structure data. After examining the amidinated sites found by Mascot Search of MS/MS spectra, 265 out of 275 modified primary amines in the intact ribosome appear to be solvent exposed in crystal structures. The other 10 amidinated sites found provide an indication that ribosomes are somewhat flexible in buffer solution. Observation of certain modified sites confirms the existence of two ribosome conformations. In addition, it was determined that an N-terminus that is methylated or acetylated does not undergo amidination under our reaction conditions, whereas monomethylated lysine is still amidinated completely. Based on all of these observations, it is concluded that amidination is an effective strategy to profile the structure of a large protein complex without disturbing its conformation.

Supporting Information Available: Supporting Figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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