Published in final edited form as:

Biopolymers. 2009 June ; 91(6): 410–422. doi:10.1002/bip.21161.

LIMITED PROTEOLYSIS ANALYSIS OF THE RIBOSOME IS AFFECTED BY SUBUNIT ASSOCIATION

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Abstract

Our understanding of the structural organization of ribosome assembly intermediates, in particular those intermediates that result from mis-folding leading to their eventual degradation within the cell, is limited due to the lack of methods available to characterize assembly intermediate structures. Because conventional structural approaches, such as NMR, X-ray crystallography and cryo-EM, are not ideally suited to characterize the structural organization of these flexible and sometimes heterogeneous assembly intermediates, we have set out to develop an approach combining limited proteolysis with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) that might be applicable to ribonucleoprotein complexes as large as the ribosome. This study focuses on the limited proteolysis behavior of appropriately assembled ribosome subunits. Isolated subunits were analyzed using limited proteolysis and MALDI-MS and the results were compared to previous data obtained from 70S ribosomes. Generally, ribosomal proteins were found to be more stable in 70S ribosomes than in their isolated subunits, consistent with a reduction in conformational flexibility upon subunit assembly. This approach demonstrates that limited proteolysis combined with MALDI-MS can reveal structural changes to ribosomes upon subunit assembly or disassembly, and provides the appropriate benchmark data from 30S, 50S and 70S proteins to enable studies of ribosome assembly intermediates.

Keywords

MALDI-MS; 30S subunits; 50S subunits; ribosome structure; ribosome topography; mass spectrometry

INTRODUCTION

The bacterial ribosome, a 2.5 MDa macromolecule, catalyzes protein synthesis in bacterial organisms. The 70S ribosome is composed of two unequal parts, a smaller 30S subunit and a larger 50S subunit. The 30S subunit contains 16S ribosomal RNA (rRNA) and more than 20 proteins, while the 50S subunit contains 5S and 23S rRNAs and more than 30 proteins. Our understanding of ribosome structure and function has benefited tremendously since high-resolution X-ray crystal structures of the ribosome were obtained in the early 2000's.^{1–5} In addition, improvements in cryo-electron microscopy (cryo-EM) continue to reveal details on ribosome dynamics and the various functional states associated with protein synthesis.^{6–11}

Another area of interest, which is more challenging to study, is that of ribosome biogenesis and assembly.^{12,13} Proposed 30S and 50S subunit assembly pathways were reported

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previously,^{14–17} and more recently a kinetic model for the assembly of bacterial 30S subunits has been reported using pulse-chase labeling and mass spectrometry.¹⁸ Holmes and Culver used chemical modification and primer extension analysis to characterize differences in 30S assembly intermediates, including the RI and RI* intermediates.^{19,20} These last two studies have increased our understanding of 30S ribosomal protein – 16S rRNA interactions during the assembly process, but have primarily probed changes to assembly intermediates at the RNA level. Despite these recent reports, our understanding of the structural organization of assembly intermediates, in particular those intermediates that result from mis-folding leading to their eventual degradation within the cell, is limited due to the lack of methods available to characterize assembly intermediate structures.

Because conventional structural approaches, such as NMR, X-ray crystallography and cryo-EM, are not ideally suited to characterize the structural organization of these flexible and sometimes heterogeneous assembly intermediates,²¹ we have set out to develop an approach combining limited proteolysis with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) that could be applied to ribonucleoprotein complexes as large as the ribosome. By limiting the proteolytic digestion, the higher order structure of proteins and protein-ligand complexes can be probed.²² Unlike other probing techniques, such as H/D exchange²³ or protein amidation²⁴ which provide information on the topography of a protein or protein-ligand complex such as ribosome subunits, limited proteolysis reveals areas of enhanced conformational flexibility rather than strict surface accessibility.²⁵ Among its various applications, limited proteolysis has been successful at characterizing the unfolding or refolding of particular proteins and the effects of ligand binding on protein conformation.^{22,26} Thus, by combining a surface probing technique, such as H/D exchange, with a conformational flexibility technique, such as limited proteolysis, one should be able to obtain structural information from complexes that are too large for characterization by NMR yet too flexible for study by X-ray crystallography or cryo-EM. Mass spectrometry can be used as the detection step for both H/D exchange and limited proteolysis, and the advantages of mass spectrometry are particularly important when attempting to characterize multi-component complexes, such as ribosome assembly intermediates.

To date, very few limited proteolysis studies of ribosomal proteins either in their presumed native structure or within ribosome subunits or 70S ribosomes have been reported, and no limited proteolysis data is available on ribosomal protein structure within assembly intermediates. Littlechild and co-workers previously reported limited proteolysis results from ribosomal proteins extracted from *Escherichia coli* 70S ribosomes.²⁷ In their work, ribosomal proteins S15, S16, S17 and L30 were found not to be susceptible to limited proteolysis, while proteins S2, L2, L27, L29 and L33 were found to be completely degraded. The other remaining ribosomal proteins that were detected in that work were classified into two groups – those producing large molecular weight fragments (molecular weights at least 60% of that of the intact protein) and those producing small molecular weight fragments. However, these results only reflect the structure of the protein obtained after extraction from the 70S ribosome, rather than the native ribosomal structure, which may be influenced by the presence of rRNA and other ribosomal proteins.

Soon thereafter, Kruft and Wittman-Liebold performed limited proteolysis studies on isolated 50S subunits from *E. coli*, *Bacillus stearothermophilus* and *Haloarcula marismortui*.²⁸ In that report, 14 large subunit *E. coli* proteins were found to undergo limited proteolysis, with the majority of the digestion sites occurring at the N- or C-terminal of the ribosomal protein. Among those characterized by these researchers, L1, L4, L5, L9, L11 and L15 yielded specific cleavage patterns for internal lysine residues, to generate small molecular weight fragments. While this work was the first to characterize limited proteolysis behavior of 50S ribosomal proteins, similar information for 30S ribosomal proteins remains

unknown, nor is it known if limited proteolysis is sensitive to conformational differences that might arise during ribosome biogenesis and subunit assembly.

Given our interest in characterizing ribosome assembly intermediates, we recently developed an approach combining limited proteolysis with MALDI-MS and applied this approach to bacterial 70S ribosomal proteins.²⁹ Because 70S ribosomes contain over 50 proteins and multiple RNAs, this first study focused on the experimental and instrumental parameters necessary to obtain limited proteolysis data that can be assigned to the level of individual ribosomal proteins without prior electrophoretic or chromatographic separation of the digested protein mixture. That first work was restricted to the 70S conformation of ribosomes from *Escherichia coli* and *Thermus thermophilus* to simplify development of the approach. Specifically, 70S ribosomes were incubated with trypsin and Proteinase K under limited proteolysis conditions. 70S conformations were isolated from dissociated 30S and 50S subunits via sucrose density gradient (SDG) ultracentrifugation, and the status of the 70S ribosomal proteins was assessed by MALDI-MS through detection of m/z values corresponding to intact ribosomal proteins or corresponding to specific proteolytic fragments. From those experimental conditions, six small subunit and 22 large subunit proteins were found to be resistant to proteolysis in *E. coli* 70S ribosomes. The other remaining ribosomal proteins that could be detected exhibited differing levels of stability to limited proteolysis. For nearly all of the ribosomal proteins that were not stable to limited proteolysis, identified sites of proteolysis corresponded to N- or C-termini of the ribosomal proteins.

Our previous method development work was the first to demonstrate the applicability of limited proteolysis and MALDI-MS on a large ribonucleoprotein complex. While that work demonstrated that the combination of techniques can be applied to complexes such as the ribosome, questions still remain relating to the utility and structural sensitivity of the approach. For example, the previous study did not provide for any comparison to determine whether the information obtained via limited proteolysis was reflective of changes in complex structure and conformational flexibility. Furthermore, before limited proteolysis and MALDI-MS can be applied to the study of ribosome assembly intermediates, baseline data for ribosomal subunits and intact ribosomes are necessary to provide the appropriate benchmark for evaluation of differences in proteolysis behavior. Therefore, one goal of the present work is to establish the limited proteolysis behavior of ribosomal subunits from a model organism, thereby allowing future studies on assembly intermediates. Ultimately, the utility of limited proteolysis combined with mass spectrometry for the study of ribosome assembly intermediates will require that the data accurately report differences in conformational flexibility at the level of individual proteins between different assembly states. Thus, another goal of the current study is to use ribosome subunit association as a simple model system to reveal whether limited proteolysis is sensitive to differences in conformational flexibility detected for individual proteins that would arise as the global organization of the subunit changes.

In this work, we have used limited proteolysis with MALDI-MS to examine the limited proteolysis behavior of ribosomal proteins from isolated 30S and 50S subunits of *E. coli*. The ribosomal proteins from 30S and 50S subunits were monitored as a function of digestion time and these results were compared to those previously obtained from 70S ribosomes.²⁹ Several ribosomal proteins exhibited significant differences in stability to limited proteolysis between isolated subunits and intact ribosomes. These results establish the baseline data for limited proteolysis of ribosomal subunits and demonstrate that limited proteolysis data from ribosomes differs between subunits and intact ribosomes. Such results illustrate how MALDI-MS can be used to identify specific ribosomal proteins whose conformational flexibility changes upon subunit assembly.

RESULTS

The conformational flexibility of isolated 30S and 50S subunits ribosomal proteins of *E. coli* was determined by limited proteolysis as described in the Experimental methods. These data were then compared to limited proteolysis data previously obtained from intact 70S ribosomes.²⁹ Comparisons were made by examining the stability of individual ribosomal proteins to limited proteolysis in both cases. Where stability in the 70S structure decreased relative to stability in the isolated 30S or 50S structures, those proteins may display increased conformational flexibility at particular regions in the intact ribosome. Likewise, increased stability of specific ribosomal proteins in the 70S structure relative to isolated 30S or 50S subunits suggests a less flexible conformation in the isolated subunit structure. Comparisons were made, where possible, between the stable proteolytic fragments identified here from ribosomal subunits and those identified during limited proteolysis of 70S ribosomes. In addition, the structural significance of the ribosomal proteins found to exhibit differential limited proteolysis behavior between isolated subunits and intact ribosomes was examined through use of existing data from recent crystal structures.

Limited Proteolysis Behavior of Small Subunit Ribosomal Proteins

Initially, limited proteolysis using trypsin of isolated 30S subunits from *E. coli* was performed. The digestion profiles of 30S ribosomal proteins from isolated subunits grouped according to their region in the small subunit are plotted in Figure 1. Fig. 1 also contains the digestion profiles of the small subunit ribosomal proteins within intact 70S ribosomes as reported previously by our lab.

Among the eight 30S proteins that are located in the Head region of the 30S subunit, four proteins, S7, S9, S13 and S14 are stable to limited proteolysis, both before and after ribosome assembly. S2 is completely digested in both states, with S19 also exhibiting minimal stability in these two states. A small difference in stability is seen for S3, although in either state it is readily digested by trypsin. The only protein in this region exhibiting a significant difference towards limited proteolysis is S10. In the 30S state, this protein is detected intact up to the maximum digestion period of 500 min. However, upon assembly into 70S ribosomes, this protein becomes significantly less stable and the intact protein cannot be detected after 120 min of digestion. No specific proteolytic fragments could be assigned to S10 (Table 1), thus the particular sites of proteolysis are not known for this protein.

There are six ribosomal proteins that can be grouped into the Body region of the 30S subunit. Five of these proteins, S4, S12, S16, S17 and S20 are detected intact up to 500 min incubation in both the 30S and 70S states. S5 is no longer detected after 60 min incubation in either state. Thus, there appear to be no significant changes to the limited proteolysis behavior of ribosomal proteins that constitute this region of the 30S subunit.

The Platform region of the 30S subunit contains six ribosomal proteins, S6, S8, S11, S15, S18 and S21, and exhibited variable behavior among ribosomal proteins between the 30S and 70S states. The two proteins with the greatest stability to limited proteolysis are S8 and S15, which were detected intact up to 500 min incubation in both states. Two proteins, S21 and S18, exhibit intermediate stability in both states, with both being detected intact between 1 and 3 h of incubation. S6 was not detected intact in either state at any incubation period, although stable proteolytic fragments primarily associated with loss of the N-terminus were detected for this protein in the 70S ribosome. S11 was found to be more stable in the 70S state, being detected intact up to 120 min of incubation, yet could not be detected intact when the 30S subunit was analyzed.

Although proteolytic fragments could be identified for S11, no difference in the specific limited proteolysis products for S11 was detected when comparing the results between the 30S and 70S states (Table 1). In both states, the primary tryptic products identified correspond to cleavage at the N- and C-termini. For both states, the detected fragment(s) were found to be stable to further limited proteolysis, as they were detected even after 500 min of incubation.

Overall, two small subunit ribosomal proteins, S10 and S11, were found to exhibit reproducible differences in stability between isolated 30S subunits and assembled 70S ribosomes. Specifically, S10 was found to be more stable to limited proteolysis in isolated 30S subunits while S11 was found to be more stable within intact 70S ribosomes. The remaining small subunit ribosomal proteins, S2, S3, S4, S5, S6, S7, S8, S9, S12, S13, S14, S15, S16, S17, S18, S20, and S21, displayed essentially identical limited proteolysis stability between isolated 30S subunits and assembled 70S ribosomes.

Limited Proteolysis Behavior of Large Subunit Ribosomal Proteins

Similarly, limited proteolysis of isolated 50S subunits from *E. coli* was performed with the results shown in Figure 2 in comparison with previous data on the large subunit ribosomal proteins analyzed in assembled 70S ribosomes. The 50S proteins can be generally divided into several regions. Peptidyl transferase proteins consist of L3, L6 and L16. Proteins located at the core of the 50S subunit include L24 and L30. Proteins involved in various stalk regions are L1, L7/L12, L10 and L11. Exit tunnel proteins are L4, L22 and L29. Proteins involved in salt bridges with 30S are L5, L14 and L19. The other remaining proteins which do not appear in any functional domain are L2, L4, L9, L13, L15, L17, L18, L20, L21, L23, L25, L27, L28, L31, L33, L34, L35 and L36.

Proteins L4, L7/L12, L9, L20 and L23 were not detected in the isolated 50S subunits analyzed at time = 0. Sucrose density gradient ultracentrifugation confirmed sedimentation corresponding to 50S subunits with no evidence of subunit dissociation (data not shown). The absence of these ribosomal proteins in the mass spectral data likely reflects instrumental limitations rather than the absence of these proteins from the initial sample,²⁹ thus the limited proteolysis behavior of 50S ribosomal proteins should reflect the native structure of this isolated subunit. The absence of these proteins in the mass spectral data means that no comparisons can be made for L7/L12, L9, L20 and L23 between 50S limited proteolysis behavior and that obtained on 70S ribosomes. No comparison can be made for L4, either, although it was also not detected during analysis of 70S ribosomes.

The majority of the ribosomal proteins from the large subunit are stable to limited proteolysis in either the 50S or 70S state. Specifically, large subunit proteins L2, L13, L15, L17, L18, L21, L25, L28, L31, L33, L34, L35 and L36, which are not assigned to specific functional locations of the large subunit, were detected intact up to 500 min incubation in both states. L27 also is not assigned to a specific functional location of the large subunit, and was no longer detected as an intact protein after 120 min of incubation in either state. Similarly, the three ribosomal proteins located in the peptidyl transferase region, L3, L6 and L16, along with the two proteins in the core region, L24 and L30, were all detected intact up to 500 min incubation in both states.

The three functional regions which yielded ribosomal proteins exhibiting greater stability to limited proteolysis in the 70S state versus the isolated 50S state were proteins in stalk regions, proteins involved in intersubunit bridges with 30S, and one of the exit tunnel proteins. Ribosomal proteins L1, L10 and L11 are involved in various stalk regions of 50S. L10 was detected intact up to 500 min incubation in both states. However, both L1 and L11 exhibited significantly greater stability towards limited proteolysis when in the 70S state. L1

was not detected as an intact protein when isolated 50S subunits were analyzed, and L11 was found to be stable only up to 120 min of incubation with trypsin when in the 50S conformation. Both proteins were detected intact up to 500 min of incubation in the 70S state. For three ribosomal proteins known to be involved in intersubunit bridges with 30S, only L14 was found to yield the same limited proteolysis behavior between the 50S and 70S states, being detected intact up to 500 min of incubation in both cases. L5 and L19 were both significantly more stable to limited proteolysis in the 70S state, being detected intact up to 500 min of incubation for that conformation. However, in the 50S state, L5 was extremely unstable to limited proteolysis and L19 was only detected intact up to 120 min of incubation. For L22 and L29, proteins that are located at the exit tunnel of the 50S subunit, L29 was stable to limited proteolysis in both states. L22, however, cannot be detected intact upon limited proteolysis of the 50S conformation, but is detected intact even after 500 min of incubation when in the 70S conformation.

As before for the small subunit ribosomal proteins, identification of specific limited proteolysis fragments for large subunit ribosomal proteins was also undertaken where possible. The only ribosomal protein whose limited proteolysis products were detected in both the 50S and 70S analyses was L19, and in both cases a very stable limited proteolysis product was detected that corresponded to cleavage of the C-terminus. For the other proteins exhibiting differential stability, the specific cleavages detected in the 50S state are listed in Table 1. No corresponding fragments could be detected for these proteins in the 70S conformation, which is not completely surprising given the enhanced stability of these proteins when bound within intact ribosomes.

While the majority of the large subunit proteins (22 of the 27 detected) are reproducibly the same in their limited proteolysis behavior between the isolated 50S subunit and the intact 70S ribosome, ribosomal proteins L1, L5, L11, L19 and L22 did display differences in stability. Interestingly, all of these large subunit ribosomal proteins were significantly more stable within intact 70S ribosomes than when present in isolated 50S subunits.

DISCUSSION

Limited proteolysis is an experimental technique that can probe the conformational features of proteins and reveal local sites of protein disorder. Recently, a method combining limited proteolysis with MALDI-MS was developed and applied to 70S ribosomes, known to contain more than 50 proteins and several RNAs. In a continuing effort to further develop limited proteolysis with MALDI-MS and to understand the information ultimately obtainable from this method, here we characterized the limited proteolysis behavior of 30S and 50S ribosomal subunits from *E. coli* and compared those results to prior results obtained from *E. coli* 70S ribosomes. One goal of this work was to identify and benchmark the limited proteolysis behavior of ribosomal proteins from 30S and 50S ribosomal subunits using *E. coli* as our model organism. The other goal of this work was to use subunit association as a model to determine whether limited proteolysis data from ribosomal proteins would be sensitive to organization rearrangements and changes.

Data from the available crystal structures for 30S and 50S ribosomal subunits does not reveal a significant protein presence in the interface regions (i.e., sites where the subunits associate together to form the 70S structure) consistent with the view that the functional activity of ribosomes resides in the rRNA components.^{30–33} Moreover, as limited proteolysis reports the conformational flexibility of surface accessible proteins, limited proteolysis differences between ribosomal subunits and intact ribosomes cannot be inferred simply based on existing crystal structures and solvent exposure. However, if differences in limited proteolysis behavior are noted, such differences may reflect a global reorganization

of subunit structures upon association which leads to changes in conformational flexibility of particular ribosomal proteins.

A total of seven ribosomal proteins were found to exhibit significantly different stability towards limited proteolysis between isolated 30S and 50S subunits and intact 70S ribosomes. Ribosomal proteins S11, L1, L5, L11, L19 and L22 were all found to be more stable in 70S ribosomes than in their respectively isolated subunits. Ribosomal protein S10 was the only protein found to be less stable upon subunit assembly than in the isolated 30S subunit. After subunit assembly nearly 80% of the ribosomal proteins (37 out of 47 identified by MALDI-MS) were still detected intact even up to 500 min of incubation, suggesting bacterial 70S ribosomes are structurally resistant to proteolytic attack.

Comparison of Limited Proteolysis Behavior Between 30S Subunits and 70S Ribosomes

Global locations of ribosomal proteins from 30S are shown in the crystal structure images in Figures 3 and 4. Two proteins, S10 and S11, shown on the crystal structure plot, were found to have digestion times that varied from 30S to 70S. The 16S rRNA is shown in dark blue, protein S7 in yellow, protein S10 in green, S11 in orange, and the remaining proteins in pink. Figure 4 denotes the structure of the assembled 70S ribosome with the 50S subunit in brown. Protein S1, which is a large acidic protein, is lost in sample preparation steps and was not detected nor shown in the crystal structure. Out of the 20 detected proteins, 9 (45%) were found to be digested before 500 minutes. These proteins are S2, S3, S10, S19, S5, S6, S11, S18, and S21.

In 30S subunits, protein S10 was detected intact even after 500 minutes of digestion with trypsin. From the 30S crystal structure, S10 is located at the top of the subunit next to the stem loop of H39, and 20% of its residues and 25% of its surface area are involved in binding RNA.⁵ After assembly into 70S ribosomes, S10 was only detected intact until 125 minutes. Thus, ribosome assembly leads to greater conformational flexibility for ribosomal protein S10. As S10 is implicated in tRNA binding,⁵ the reduced stability in the 70S state may reflect the conformational changes necessary to facilitate tRNA interactions during translation.

Protein S11 is located on the platform of the 30S subunit and bridges several disparate RNA helices of the 16S RNA. According to the crystal structure of 30S, ~26% of S11 is packed against RNA.⁵ In 30S subunits, protein S11 was not detected intact after even 10 minutes of digestion. In the 70S ribosome, S11 forms part of the Shine-Delgarno cleft, is known to have interactions with S7 and S18, and has been cross-linked to initiation factor-3 (IF-3). S11 likely maintains 30S platform-head interactions via S7, and should experience conformational changes upon translocation.³⁴ After assembly into 70S ribosomes, ribosomal protein S11 is more stable and is detected intact up until 125 minutes of digestion, although the N-terminus is still sufficiently flexible to be digested during extended periods of incubation with trypsin. Interestingly, S7, which is believed to maintain protein-protein interactions with S11, is stable to limited proteolysis in both the 30S subunit and 70S ribosome. Thus, the conformational changes occurring during subunit assembly only affect the limited proteolysis stability of S11 suggesting this protein is more mobile than S7.

Comparison of Limited Proteolysis Behavior Between 50S Subunits and 70S Ribosomes

Global locations of ribosomal proteins from 50S are shown in the crystal structure images in Figures 5 and 6. The 50S ribosomal proteins exhibiting differential behavior are highlighted, except L1, which is not present in the crystal structure. Protein L11 is shown in red, L5 in green, L19 in yellow and L22 in orange. The remaining 50S proteins are shown in pink and the rRNAs are shown in blue. Figure 6 denotes the structure of the assembled 70S ribosome

with the 30S subunit in brown. Out of the 27 detected proteins, 6 (22%) were found to be digested before 500 minutes in the 50S conformation, with only L27 being digested before 500 minutes when in the 70S state.

Ribosomal protein L1 is a component of a mobile stalk region of the large subunit that has been implicated in translocation of deacylated tRNAs from the P to the E site.⁷ In the 50S subunit, L1 could not be detected as an intact protein upon limited proteolysis, suggesting it is immediately cleaved by trypsin into smaller fragments. One stable fragment, corresponding to cleavage of the N- and C-termini was detected. In the 70S state, L1 appears to be very stable, as it is still detected intact even after 500 minutes of incubation with trypsin.

Protein L5 binds and possibly mediates the attachment of 5S RNA into the 50S subunit. In the 50S subunit, L5 was detected intact only up to 30 minutes of digestion, suggesting a flexible conformation. No significant limited proteolysis products could be detected, which likely reflects multiple sites of proteolytic cleavage for this protein. When 50S is bound to 30S, L5 becomes part of the B1B bridge, contacting S13 and S19. There have been several different models published to account for the protein-protein interactions in the B1B intersubunit bridge.^{35,36} The limited proteolysis results finds that L5 becomes significantly more stable in the 70S state, as intact protein can be detected even up to 500 minute of incubation.

Protein L11 is a component of the GTPase-associated center (GAC), is known to facilitate binding of the L10(L12)₄ stalk, and is involved in binding of elongation factors. In isolated 50S subunits, L11 was detected intact up until 125 minutes of digestion, with only one stable fragment, corresponding to cleavage of the N- and C-termini, detected. As with the other 50S proteins, when analyzed in the 70S state, L11 is detected intact after 500 minutes of digestion with trypsin.

Protein L19 is located at the interface of the 30S and 50S subunits. In isolated 50S subunits, L19 is no longer detected intact after 125 minutes of incubation, with several N- and C-termini fragments produced. Such results are not surprising as this protein should be accessible in the 50S state. L19 is hypothesized to make two contacts with the 16S RNA, forming bridges B6 and B8, when present in 70S ribosomes. When present in the 70S state, this protein remains stable as it is detected intact after 500 minutes of digestion.

Among the 50S proteins exhibiting increased stability when comparing the 70S state to the isolated subunit, L22 is probably the most interesting as it becomes dramatically more stable when analyzed in intact ribosomes. MALDI-MS digestion data shows that in isolated 50 subunits, L22 is detected intact for only 10 minutes, but in 70S is intact after 500 minutes of digestion. Protein L22 is located in the polypeptide exit tunnel, with its globular domain surrounding the tunnel and its beta-hairpin section buried into the center of the 70S ribosome.

Assembly

We also examined these data to determine if any correlations exist between limited proteolysis stability and subunit assembly order. While the two 30S proteins that are differentially digested in 30S and 70S are proteins that bind late in the assembly of the 30S subunit, these are not the only small subunit ribosomal proteins that bind at the late assembly stage. For the 50S proteins that are differentially digested, clearly these effects are unrelated to assembly order as L19 is the only protein more stable in the 70S state that is found in the second reconstitution fraction for large subunit assembly.¹⁷ The other four ribosomal proteins are found in the initial reconstitution fraction, although only L22 is

essential for large subunit assembly and L5 is essential for binding 5S rRNA to the large subunit. Thus, these comparative limited proteolysis investigations, unlike other chemical probing experiments,²³ do not report information relating to ribosome subunit assembly order.

Comparison to Other Limited Proteolysis Studies

Among those ribosomal proteins found to exhibit limited proteolysis behavior which changed after subunits were assembled into intact ribosomes, only L1, L11 and L19 were analyzed in the work of Littlechild.²⁷ From intact ribosomes, those three proteins were found to yield large molecular weight fragments. In the present work, L1, L11 and L19 were found to be more stable within the assembled ribosome. Of particular note is that the large subunit proteins are significantly less stable in isolated 50S subunits, yet reflect a more stable organization in assembled 70S ribosomes. Among those tryptic fragments that could be assigned to a specific ribosomal protein, those detected for L19 generate large molecular weight fragments consistent with the results from Littlechild. As discussed previously,²⁹ differences in the limited proteolysis behavior of other ribosomal proteins between the results reported by Littlechild and those found here likely arises because the Littlechild data was obtained on 70S extracted ribosomal proteins, while those here were obtained directly from ribosomal subunits or 70S ribosomes.

Interestingly, all of the large subunit ribosomal proteins which were found in this work to increase in stability after ribosome assembly, were found to be susceptible to limited proteolysis in isolated 50S subunits in the work of Krufft and Wittman-Liebold,²⁸ except L22, which was not detected in that prior study. More specifically, those researchers found L1, L5 and L11 to yield small molecular weight fragments upon limited proteolysis with sites of cleavage by trypsin to be primarily internal sites of these three proteins. In this work, no fragments corresponding to cleavage by trypsin could be detected for those three proteins when they were analyzed within intact 70S ribosomes.

Comparison to Other Probing Methods

Mass spectrometry-based investigations of ribosome structure have also been conducted using H/D exchange²³ and chemical labeling.²⁴ As both of those studies focused on intact ribosome topology and organization, rather than differences arising during subunit assembly, few specific comparisons can be made at this point. The amidation approach by Reilly and co-workers does report on the number of unmodified lysine residues (and N-termini) that were accessible for labeling within intact ribosomes of *C. crescentus*. Because lysine is a potential site for cleavage by trypsin, that approach, if performed on *E. coli*, would provide an upper boundary on the number of possible cleavage sites to be seen in these limited proteolysis experiments. In addition, either labeling method could be utilized on isolated subunits, with the approach by Reilly and co-workers being most applicable to defining specific changes in overall amounts and locations of labeling upon ribosome assembly.

Although the data in this report were only obtained using trypsin, which is specific for only arginine and lysine residues, prior results from intact 70S ribosomes using a less specific enzyme, Proteinase K, revealed similar trends in ribosomal protein stability. Thus, while the use of alternative enzymes could provide additional information relating to protein stability, solvent accessibility and conformational changes to specific ribosomal proteins arising from subunit assembly, the results obtained here with trypsin do provide a representative view of those regions of the ribosomal subunits that exhibit the most significant differences in protein stability to limited proteolysis upon subunit assembly.

In summary, we have found that limited proteolysis results are affected by subunit assembly, with six specific *E. coli* ribosomal proteins becoming more stable to limited proteolysis when present in 70S ribosomes than in their individual subunits. Ribosomal protein S10 was the only protein found to be less stable when in the 70S complex than in an isolated subunit. The ribosomal proteins whose stability increases in the 70S state are consistent with prior structural and biochemical studies on these proteins, signifying that the limited proteolysis approach does not misidentify proteins that could be either solvent accessible or involved in interactions with other components of the ribosome besides rRNA. The advantages of using MALDI-MS as the detection step in these limited proteolysis experiments include the rapid analysis time, as no separation of ribosomal proteins is required prior to MALDI-MS, the relative sensitivity and broad detectability of the approach, and the capability to identify specific limited proteolysis fragments, which reveals the likely sites of proteolytic cleavage.

The present findings have established the appropriate benchmarks for application of limited proteolysis to other conformational assemblies of the ribosome, as well as other large multi-protein and/or ribonucleoprotein complexes. In particular, this approach can be directly extended to structural analysis of assembly intermediates. That application would require the appropriate isolation of assembly intermediates from appropriately assembled subunits or intact ribosomes, and we have found that SDG ultracentrifugation to be an effective and recommended isolation technique. The amount of information obtained will depend on the quality of the MALDI-MS data, and detection of (nearly) all of the constituent ribosomal proteins from such intermediates is necessary to accurately define limited proteolysis behavior at the level of individual ribosomal proteins. Interpretation of specific sites of limited proteolysis is facilitated by the use of more specific proteases, such as trypsin, and, at least for ribosomal subunits, trypsin provides a similar level of information on conformational flexibility as that obtained from less specific proteases such as Proteinase K.²⁹ Based on these findings, studies are currently underway in this lab to characterize structural differences between 50S assembly intermediates and the correctly folded 50S subunit.

MATERIALS AND METHODS

Materials

Tryptone and yeast extract were obtained from Sigma (St. Louis, MO). Sinapinic acid (SA) was obtained from Fluka (Milwaukee, WI). Acids and organic solvents were HPLC grade or better. Trypsin (sequencing grade) and RNase-free DNase were purchased from Promega (Madison, WI). Molecular-grade sucrose was obtained from Sigma Chemical (St. Louis, MO). The MALDI calibration standards were obtained from Bruker Daltonics (Billerica, MA).

Culturing

Escherichia coli (MRE 600) 70S ribosomes were cultured in-house following standard cell growth, harvesting and isolation procedures as described previously³⁷ with minor modifications. Briefly, cells were grown to mid-log phase, and then refrigerated for 30 min at 20 °C to produce run-off ribosomes. The cells were harvested via centrifugation and lysed using a French Press. After incubation with RNase-free DNase, the lysed cells were centrifuged and the ribosome-rich supernatant was retained. The crude ribosome solution was purified by layering over an equal amount of a 1.1-M sucrose cushion and centrifuged overnight at 39,000 rpm, 4 °C in a 50 Ti rotor with a Beckman-Coulter ultracentrifuge (Fullerton, CA). The purified ribosomes pellets were redissolved in ribosomal storage buffer (10 mM Tris-HCl (pH 7.6), 10.5 mM magnesium acetate, 50 mM NH₄Cl, 0.25 mM EDTA) and stored at -80 °C until further use.

Isolation of ribosomes and Ribosomal Subunits

Following culturing, 70S ribosomes were prepared by storing in 10 mM magnesium concentration buffer (as described above). The presence of 70S ribosomes was confirmed using an agarose gel to detect the presence of 16S and 23S ribosomal RNA.

30S and 50S subunits were isolated and purified using a 0–45% sucrose gradient. The sucrose gradients were made with 22.5% sucrose in dissociation buffer (10 mM Tris-HCl at pH 7.6, 1 mM magnesium acetate, 60 mM NH₄Cl, and 4 mM β-mercaptoethanol) by the freeze - thaw approach. Purified ribosomes were diluted with dissociation buffer and chilled at 4 °C overnight. Approximately 200 A₂₆₀ units of purified ribosomes were applied to the top of the gradient and centrifuged in an SW28 rotor at 4 °C for 17 h at 19000 rpm. A Teledyne-Isco (Lincoln, NE) density gradient fractionation system was used with the UV detector set to 260 nm. Fractions of 1.0 mL were collected over the entire gradient. The fractions corresponding to the 30S and 50S subunits were recombined, respectively, and pelleted in a 50 Ti rotor at 4 °C for 18 h at 48,000 rpm. The pellets were resuspended in ribosomal storage buffer and stored at –80 °C until needed.

Limited Proteolysis

Limited proteolysis of the 30S and 50S ribosomal subunits was accomplished using trypsin. For subunit digestions 400 µg of 30S or 50S were diluted with ribosomal storage buffer to a volume of 80 µL. Two 4.5 µL-aliquots were then removed. One aliquot was incubated at 37 °C with no enzyme added for 500 minutes and the other aliquot was directly prepared for MALDI-MS as described below (T=0). Trypsin was added to the remaining sample at an enzyme to substrate (subunit) ratio of 1:500 (w/w). Equal aliquots (4.5 µL each) were withdrawn from the reaction after 10 min, 30 min, 60 min, 125 min, 250 min and 500 min. All reactions were performed at 37 °C. Immediately after removal of each aliquot, 0.5 µL of 25% trifluoroacetic acid (TFA) was added to the aliquot to quench proteolysis. Aliquots were either analyzed immediately by MALDI-MS, or stored at –80 °C until analysis.

MALDI-MS

MALDI-MS was performed using a Bruker Reflex IV reflectron MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA) equipped with a nitrogen laser as previously described.^{38,39} All samples were analyzed under identical instrumental parameters: linear mode with a detector gain of 25x, positive ion polarity at an acceleration voltage of 20 kV, extraction plate voltage of 17.1 kV and lens voltage of 10.1 kV, and 300 accumulated laser shots at a laser power of 30%. All samples were analyzed under identical instrumental parameters.

Saturated sinapinic acid (SA) in 33% aqueous acetonitrile/0.1% trifluoroacetic acid (TFA) was used as the matrix. One µL of sample, which was prepared by mixing one µL of acidified ribosomal solution (around 800 nmol 50S) with nine µL of SA matrix, was spotted onto a MALDI target plate. The MALDI instrument was calibrated initially with Bruker Protein Standard I composed of insulin, ubiquitin I, cytochrome c, and myoglobin and then recalibrated internally with well-resolved ribosomal proteins L30 (*m/z* 6410.7), L29 (*m/z* 7273.5), L25 (*m/z* 10693.5), L18 (*m/z* 12769.3), L13 (*m/z* 16018.7), and L6 (*m/z* 18772.5).

For each digestion period, the sample was placed on four separate spots on the MALDI plate using the dried-droplet technique, and the results for each digestion period were determined by averaging the data from the four separate spots.

Modeling of Data

PyMol software⁴⁰ was used for modeling the crystal structures. The 30S subunit structures were modeled using *E. coli* PDB 2AVY, and the 50S subunit structures were modeled using *E. coli* PDB 2AW4.³⁶

Acknowledgments

This work was supported by GM58843 from the National Institutes of Health and the University of Cincinnati.

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Figure 1.

Digestion profiles of the 30S subunit and the 70S ribosome with trypsin. The plots are grouped based on the location of the ribosomal proteins and show the time each protein is digested in 30S and in 70S. Two proteins, S10 and S11, were found to have digestion times that varied from 30S to 70S.

Figure 2.

Digestion profiles of the 50S subunit and the 70S ribosome with trypsin. The plots are grouped based on the location of ribosomal proteins and show the time each protein is digested in 50S and in 70S. Proteins L4, L7/L12, L9, L20 and L23 were not detected in the isolated 50S subunits analyzed at time = 0. Sucrose density gradient ultracentrifugation confirmed sedimentation corresponding to 50S subunits with no evidence of subunit dissociation. Five proteins, L1, L5, L11, L19, and L22, were found to have digestion times that varied from 50S to 70S.

Figure 3. Ribbon view of the 30S subunit from *E. coli* (PDB 2AVY).³⁶ The ribosomal RNA is shown in dark blue, protein S7 in yellow, protein S10 in green, S11 in orange, and the remaining proteins in pink. Major structural features are identified.

Figure 4.

Surface presentation of the 30S ribosomal proteins whose limited proteolysis behavior is affected by subunit association. Color scheme: 16S rRNA is represented in dark blue; protein S7 in yellow, protein S10 in green, S11 in orange, and the remaining 30S proteins in pink. The 50S subunit is represented in brown. (a), (b), (c) and (d) are 90° rotations about the horizontal axis. The view in (d) is equivalent to the view in Figure 6d, and is provided for perspective on subunit assembly. The 30S subunit was modeled using PDB 2AVY and the 50S subunit was modeled using PDB 2AW4.³⁶

Figure 5. Ribbon view of the 50S subunit from *E. coli* (PDB 2AW4).³⁶ Protein L5 is shown in green, L11 in red, L19 in yellow and L22 in orange (L1 is not present in the crystal structure). The remaining 50S proteins are shown in pink, and the rRNAs are shown in dark blue. Major features are identified.

Figure 6.

Surface presentation of the 50S ribosomal proteins whose limited proteolysis behavior is affected by subunit association. Color scheme: 5S and 23S rRNAs are represented in dark blue; protein L5 in green, L11 in red, L19 in yellow and L22 in orange (L1 is not present in the crystal structure). The remaining 50S proteins are shown in pink. The 30S subunit is represented in brown. (a), (b), (c) and (d) are 90° rotations about the vertical axis. The view in (d) is equivalent to the view in Figure 4d, and is provided for perspective on subunit assembly. The 30S subunit was modeled using PDB 2AVY and the 50S subunit was modeled using PDB 2AW4.³⁶

Table 1

Limited proteolysis digestion fragments identified in subunit and intact ribosome analyses. Only fragments arising from those proteins whose stability differed between the subunits and intact ribosome are shown.

Proteins	Protein fragments detected from isolated subunits (30S or 50S)	Protein fragments detected from intact 70S ribosomes ²⁹
S10	n.d.	n.d.
S11 *	10-125/11-126/12-127/14-129	10-125/11-126/12-127/14-129
S11	12-129	12-129
L1 *	11-199	n.d.
L5	n.d.	n.d.
L11 *	4-133	n.d.
L11 *	3-133	n.d.
L19	6-105	n.d.
L19 *	2-109	2-109
L22	11-84	n.d.
L22	7-88	n.d.

* Protein fragments still detected after 500 min incubation with trypsin.

n.d. – no protein fragments identified.