Lecture

TRANSITIONS IN RIBOSOME ASSEMBLY

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ABSTRACT

The recent crystallographic structures of the ribosomal subunits have revealed the picture of the final product of a complex assembly process that condenses the rRNA and the ribosomal proteins into active ribosomes. The folding of rRNA is the rate-limiting step of this complicate assembly pathway. Some ribosomal proteins are required for facilitating the structural rearrangements of rRNA and avoiding the kinetic traps that frequently impede RNA folding. It is thought that the long basic r-protein extensions that penetrate deeply into the subunit cores play a key role in this process through disorder-order transitions and/or co-folding mechanisms. A current view is that such structural transitions may facilitate the proper rRNA folding. In this paper, the structures of the free and bound forms of proteins that have been experimentally found to be essential for the first steps of ribosome assembly have been compared. It is shown that the extensions of L3, L4, L20 and L22, have different structural and dynamics properties that probably correlate with different functions. This study also suggests that the specific coil-helix transition that occurs in a phylogenetically conserved cluster of basic residues of the L20 extension is strictly required for the large subunit assembly in eubacteria. In contrast, in showing that the ordering of the inner loop of L4 upon rRNA binding is not required for the assembly function of L4, this study indicates that different categories of disorder-order transitions are associated with different biological functions.

INTRODUCTION

In addition to provide a considerable amount of structural data about the mechanisms of translation, the recent crystallographic structures of ribosomal particles (1-5) have brought to the scientific community a picture that could represent ”the origin of the world”. Similar to the Courbet’s painting, the spectacle of the most puzzling 3D labyrinth that nature has invented produces a kind of metaphysic vertigo that recurrently asks “how this so complex macromolecular structure assembles spontaneously in the cells?” Indeed, although it could seem a huge time relatively to the millisecond scale folding of a polypeptide chain into an active protein, the minute required for ribosome assembly *in vivo* is negligible relatively to the months or years that human brain needs for conceptualising its folding pathway. Perhaps the most fascinating issue of following the 23 S rRNA path within the 50 S large particle, is to realize that, at the end of a long travel along about 3000 nucleotides, and a visit of a large gallery of RNA motifs and folds, the exit is at close vicinity of the starting point. Adding the ribosomal proteins to this macromolecular daedal makes ribosomal assembly one of the most tricky problem of molecular biology. In contrast to other large molecular assemblies such as virus or flagels (6), ribosomal particles lack of an apparent symmetry. Symmetry greatly helps to rationalize how individual components are ordered into complex spatial arrangements. Also, while the shape of B-DNA double helix helps to organise its supramolecular assemblies into regular arrays (7), RNA double helices display a large conformational diversity that prevent a regular organisation. Fortunately, many biochemical, genetical and structural data may be put together for trying to understand how ribosomal proteins and rRNA co-fold into functional ribosomal particles. The purpose of the present paper is to try to elucidate how the extensions of the ribosomal proteins (L3, L4, L20 and L22) that are essential for the first steps of the eubacterial large subunit assembly, participate to the rRNA folding pathway.

RIBOSOME ASSEMBLY

The two bacterial subunits 30S and 50S assemble into a functional 70S particle that consists roughly two-thirds RNA and one third protein (8,9). The small subunit contains 16S RNA and 21 “S” ribosomal proteins. The large subunit 50 S is composed of two RNA, 23 S (2904 nt) and 5 S RNA (120 nt) and 33 proteins (10). All the information required for the *in vitro* assembly is contained

within rRNA and ribosomal protein sequences. During the course of the assembly, a set of RNA conformational changes and protein binding leads to particles of increasing compactness. The process is highly interactive and the binding of many proteins depends on prior binding of other proteins. Similar basic principles have been found from the assembly of the 30S and 50S subunits. Primary binding proteins bind directly and independently to rRNA. Intermediate structures of increasing compactness have been identified *in vitro*. Since similar intermediates have been observed *in vivo*, it is thought that they reveal important aspects of RNA folding and protein binding during the assembly pathway. *In vivo*, the early assembly reactions already start with a small number of r-proteins shortly after the onset of rRNA synthesis. Thus, ribosomal assembly that is coupled with transcription only takes one minute. However, ribosome assembly *in vitro* takes several hours with the need of several steps of incubation at high temperature (11).

Assembly of 30 S that is simpler and faster than 50S is now well documented (8, 9, 12, 13). Incubation of 16 S RNA and a complete set of proteins at low temperature produces a 21 S reconstitution intermediate. It contains 16 S RNA and first and second binding proteins. Heating to 42°C induce a conformational change that result in a 26 S particle RI*. Addition the third binding proteins leads to the formation of the 30 S particle. The assembly landscape of the 30 S subunit proceeds through a global rate-limiting conformational change and traverses a landscape dotted with various local conformational transitions (12).

The assembly of the 50 S large particle is much more complex. An assembly map has been elaborated for the 50 S particle of *E. coli* ribosome (14,15). Three reconstitution intermediates have been found. $RI_{50}(1)$ 33 S, $RI_{50}*(1)$ 41 S, $RI_{50}(2)$ 48 S and 50 S. 22 proteins are incorporated into the first intermediate $RI_{50}(1)$. During the assembly gradient, five proteins essential for the early assembly reaction ($RI_{50}*(1)$) bind exclusively near the 5'-end of the 23S RNA. Among them, L4, L20, L22 and L24 that bind on the first rRNA domains are essential. L24 that binds near the 5'-end and L3 that binds at the 3'-end of the 23S RNA, are considered as initiator proteins since they bind independently to other r-proteins. The existence of two major protein assembly centres (L24 and L3) located at the ends of the 23S rRNA (I+II and V+VI) has been more recently confirmed by reconstitution experiments using separate transcripts of the six major structural domains of 23S RNA (16). This study indicates that the two centres assemble independently of each other around protein L24 and L3. Then five primary binding proteins L3, L4, L20, L22 and L24 play an essential role on the assembly of the first reconstitution intermediate. While post-transcriptional modifications of the 23 S RNA are not essential for the assembly (17), a recent work has pointed

the importance of transient structures during post-transcriptional refolding of the pre-23S S RNA for the large subunit assembly (18).

The three dimensional structures of the ribosomal particles (1-5) are in good agreement with these biochemical data. A detailed analysis of the proteins of the large subunit of *H. marismortui* has provided interesting structural insights on 23S assembly. (i) Larger protein/RNA interface seems to correlate with proteins that bind early in the course of assembly. (ii) Component buried in the interior must bind the assembling ribosome earlier.

DO RIBOSOMAL PROTEIN EXTENSIONS PLAY A ROLE IN SUBUNIT ASSEMBLY?

One of the most surprising features of ribosomal proteins found in the crystal structures of large and small ribosome subunits is the finding that almost half of the proteins have globular bodies with extensions (1). Globular domains are found on the particle's exterior while the extensions penetrate deeply into the subunit core and are intertwined with rRNA helices (Figure 1). These extensions often lack obvious tertiary structure and in many regions are also devoid of significant secondary structure. As a consequence, most of the proteins that contain extensions do not crystallise at the free state. When their crystallisation is possible, the extensions are generally not visible in the electron density map since they are disordered. Although the biological role of the extensions is still unclear, it has been postulated that they could participate to ribosome assembly on the basis of the crystal structures of the ribosome subunits. The detailed analysis of the ribosomal proteins of the large particle of *H. marismortui* has brought many structural insights that support this hypothesis (19). First, the extensions are basic and flexible, a property that make them candidate for assembling RNA segment during rRNA folding.

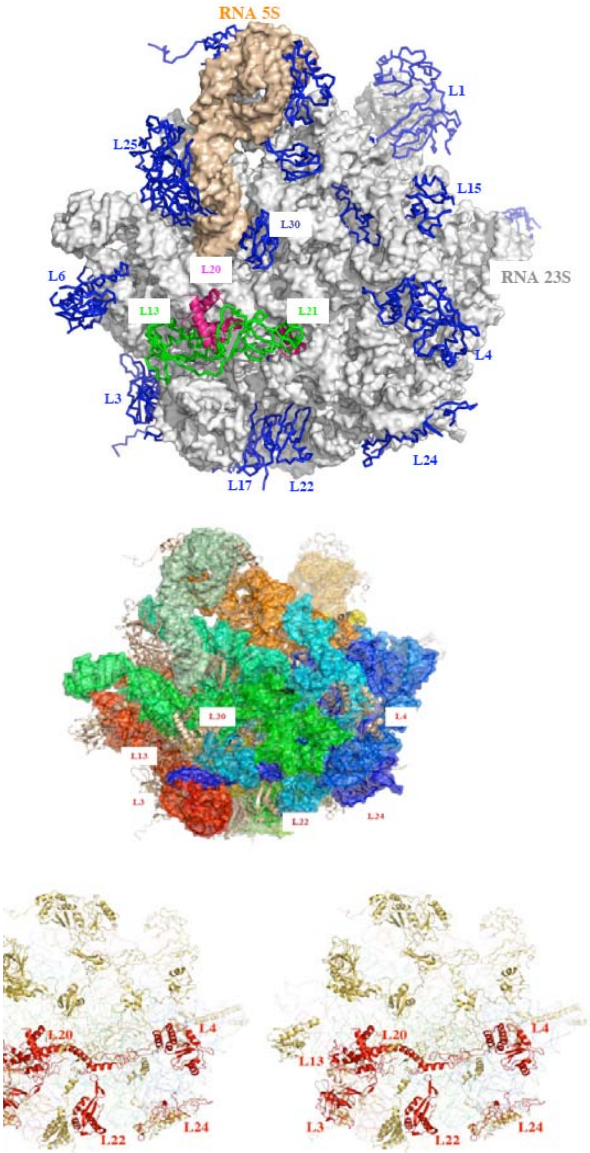

Figure 1.

a) The large subunit of *T. thermophilus* (4). The ribosomal proteins are depicted with blue ribbons, the 23 S RNA is represented in grey and the 16 S RNA is

represented in pale pink. L20 protein is represented with pink ribbons; its neighbours L13 and L21 are depicted in green.

b) Same view of the large subunit with a colour scheme that puts in light the path and the domains of the 23 S RNA. 23 S RNA is coloured from blue to red from the 5'-end to the 3'-end.

c) Stereo view of the large subunit showing the distribution of the six ribosomal proteins (L3, L4, L13, L20, L22 and L24) essential for the formation of the first reconstitution intermediate *in vitro*.

In both subunits, these extensions have a distinctive amino acid composition and they differ from the globular domains mainly in glycine (13.7 % vs. 8%), arginine (15,9 % vs. 7.5 %) and lysine (12,7 % vs. 5.1 %) (19,20). The basic nature of the extensions enables them to neutralize the highly negatively charged RNA backbone. The higher glycine contain is supposed to increase their flexibility and to avoid steric clashes in tightly packed RNA regions (19). Second, it has been noted that extensions that represent only 18 % of the proteins are responsible for 44 % of the total RNA surface buried by protein interaction. Because they make many contacts with rRNA and often interact with more than one domain of the RNA, it is thought that one role might be the stabilization of the proper RNA tertiary structure. Third, the finding of extensions in proteins essential (L3, L4, L22 and L20) for the formation of the first intermediate RI_{50} (1) in *in vitro* (14,15) reconstitution experiments has suggested that they may participate to ribosome assembly. Another possible role of the absence of secondary structure in the extensions is that it could allow interactions in major grooves of RNA double helices that are not wide enough to accommodate larger elements of protein secondary structures such as α-helices (19, 20).

This hypothesis fits well with current views on protein/RNA interactions in which induced fit or co-folding are required for the assembly (21,22). Many other examples of order-disorder transitions have been observed in protein/RNA interactions and growing evidences have shown that intrinsically unstructured proteins (IUPs) participate to many assembly and regulation functions (23-26). Following this view, co-folding or disorder/order transition in r-proteins extensions would help to avoid the kinetic traps that frequently impede the correct RNA folding during the course of ribosome assembly. The examination of subunit crystal structures also suggests that the globular domains of the assembly proteins bind first to rRNA (19, 20). Then, the extensions would bind additional segments in different domains, thus contributing to approach and seal distant rRNA regions.

However, steric considerations require that proteins that contain extensions bind 23 S RNA at a stage prior to the formation of significant tertiary structure. Otherwise, the extensions would not have access to their binding sites. Therefore, the binding of extensions must occur before final assembly of the surrounding parts of the subunit.

However, a deeper analysis suggests that the picture may be more complex. Recent genetic, biochemical and structural data have indeed shown that r-protein extensions are not systematically involved in the subunit assembly. First, the observation that many proteins that possess extensions are not essential for ribosome assembly indicates that they are not strictly correlated with an assembly function. It is also important to note that in 30S subunit, none of the primary binding proteins has the extended basic tails. Rather, they appear to be typical globular proteins (20). Moreover, in the 50S particle, although the assembly initiator protein L24 is devoid of secondary and tertiary structure, it does not have an extension that penetrates in the ribosome core. L24 is bound at the ribosome surface similar to the other globular domains of other ribosomal proteins.

What about proteins essential for ribosome assembly? The effect of the deletion of the extensions of L4 and L22, two primary binding proteins that are essential for the 50 S subunit assembly has been tested *in vivo* (27). Both proteins bind initially to domain I and are essential for the formation of the first reconstitution intermediate RI_{50}*(1). Surprisingly, this study has shown that the extended loops of L4 and L22 are not only dispensable for assembly into 50 S ribosomal particle but also for the proper assembly of proteins that bind later in 50 S assembly pathway (27). These experiments provide a clear demonstration that the globular domains of these two proteins are sufficient to initiate the assembly of the large 50 S particles. In consequence, this finding does not support the general concept that extensions of ribosomal proteins play a role in ribosome assembly. Another study has also shown that C-terminal tails of S9 and S13 are not essential for ribosome functions (28). However, these two protein are not essential for the early steps of the 30 S subunit assembly.

Among the proteins that are essential for the large subunit assembly, L20 is, to our knowledge, the sole example in which the extension is strictly required for correct folding of the 23 S RNA into an active ribosome subunit. L20 that is one of the most basic proteins of the eubacteria is a primary binding protein that belongs to the five proteins essential for first reconstitution steps *in vitro* (29,30). L20 can also replace the assembly initiator protein L24 for the initiation of assembly at permissive temperature (31). L20 has been also shown to be essential *in vivo*, as a deletion within its gene is lethal (32). Moreover, deletion experiments have shown that the N-ter extension is strictly required for normal assembly (32).

Within the eubacterial 50 S subunit, L20 is bound at the interface of two RNA domains and interacts with the helix H40/41 on one side and the helix H25 on the other side (Figure 2). Its globular C-ter domain interacts with L21 and L13 at the surface of the ribosome (Figure 1). Its spectacularly long α-helical extension penetrates deeply into the subunit core and seals the approach of two domains of the 23 S RNA (Figure 1c, 2). Biochemical data have shown that L20 extension is required for the assembly *in vivo* of the large ribosome subunit. What does distinguish the extension of L20 from the ones of L4 and L22 that could explain its specific function in ribosome assembly?

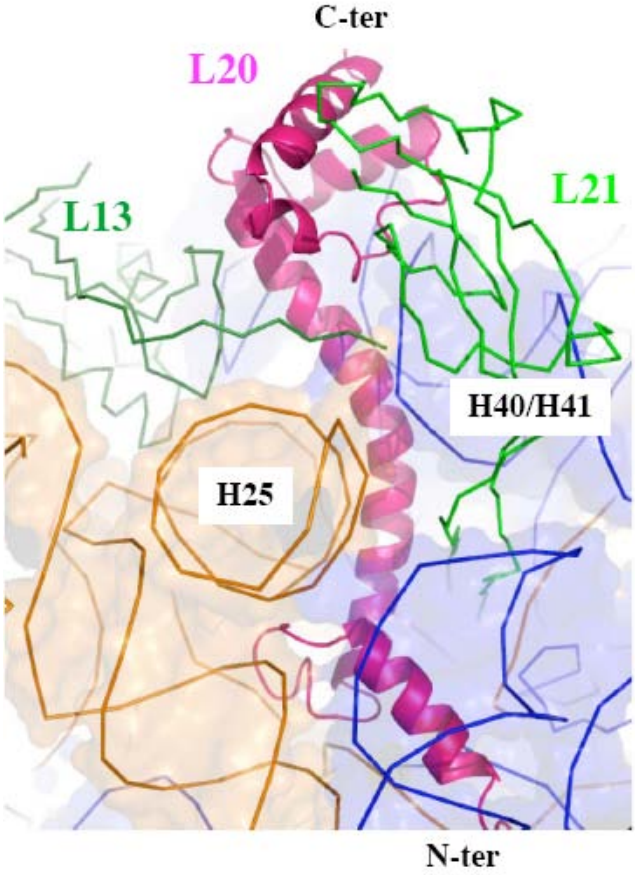

Figure 2. The path of L20 within the core of the large subunit of *T. thermophilus* (4) (as a representative structure for eubacteria).

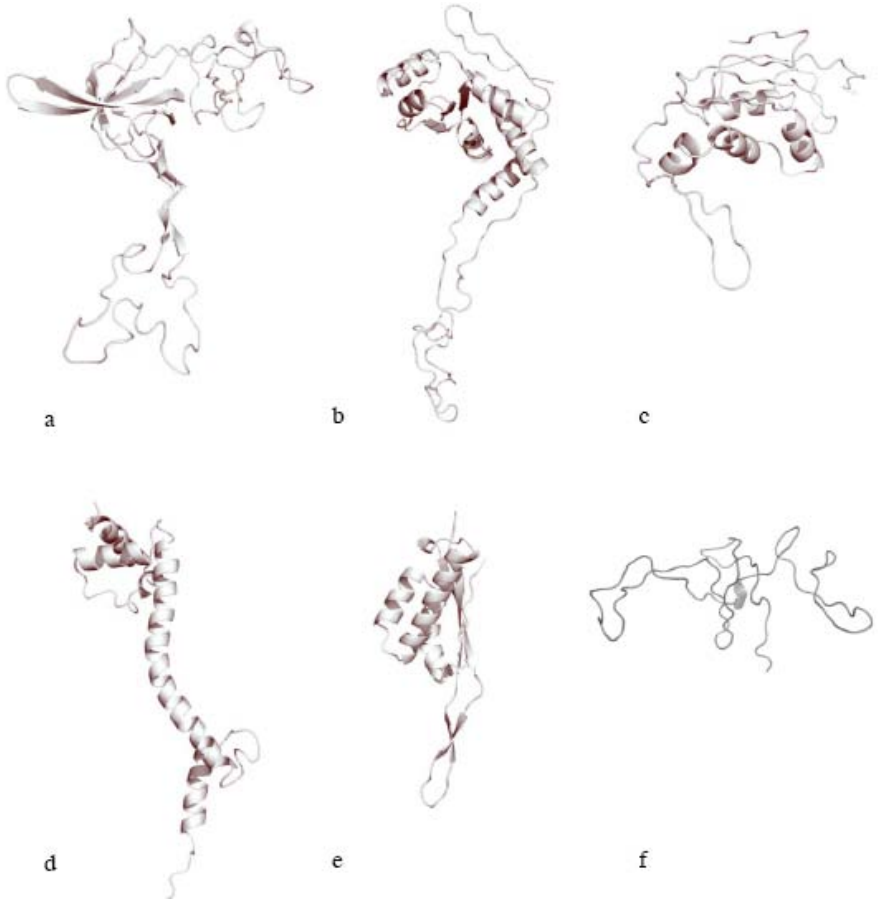

Figure 3. Comparison of the six proteins that are essential for the early steps of the assembly of the 50 S subunit of the eubacterial ribosome. a. L3. b. L4. c. L13. d. L20. e. L22. f. L24.

In contrast to the inner loops of L24 and L22, the extension of L20 (60 aas) is structured into a spectacularly long α-helix α2 and a smaller N-terminal α1 helix that penetrate deeply into the ribosome core (Figure 2). As shown in figure 3, the extensions of L4 and L22 adopt a different secondary structure within the ribosome. Although it is ordered through its interaction with the rRNA, the inner loop of L4 (68 aas, arg45-lys103) is totally devoid of secondary structure (Figure 1c, 3). The inner loop of L22 (23 aas, glu78-ser101) is smaller and is folded into a β-hairpin. Interestingly, the extension of L3, another protein essential for the formation of the first reconstitution intermediate, is similar to that of L4 and

consists of a loop devoid of secondary structure. However, to our knowledge, deletion experiments have not been yet performed to test the role of L3 extension in ribosome assembly. This analysis suggests that the specific role of L20 extension may be related to its particular structural and dynamics properties. Another difference between these four proteins is the structure of the globular domain. L20 is unique in having a globular domain entirely made of α-helices.

SPECIAL STRUCTURAL AND DYNAMICS PROPERTIES OF L20 EXTENSIONS

The picture of the protein extensions within the crystal structure of the ribosome particles provides a view of the final product of the assembly. These data are therefore insufficient to have a complete view of the molecular mechanisms of rRNA folding. Thus, the comparison of the free and bound forms of ribosomal proteins may provide useful insights on the molecular events occurring during rRNA-protein binding. They can help to understand how induced fit or co-folding with their RNA target may assist the subunit assembly. Indeed, key steps of the rRNA folding process may involve structural rearrangements upon rRNA/protein binding (21,22).

Crystal structures of the free forms are available for four of the proteins (L4, L13, L22 and L20) essential for the early steps of assembly. The crystallographic structure of L4 shows that its inner loop is totally disordered in the crystal. Thus, the comparison of the free and bound forms suggests that a co-folding mechanism takes place during its binding on its rRNA target (33). However, deletion experiments have shown that the L4 extension is not required for the proper ribosome assembly (see above, 27). It can be therefore deduced that the structural transition that orders the L4 inner loop within the large particle does not play an essential role in the 23 S rRNA folding. On the other hand, the β-hairpin extension of L22 adopts a similar conformation in the free and bound states (34). However, here also, the extension is not required for the assembly. This provides another example in which an extension that is structured into a defined secondary structure in the free and the bound state is not required for assembly.

An NMR study has proposed that the extension of L20 is totally unstructured in the absence of rRNA (35). Disorder prediction programs such as PondR (36) also show that the extension displays many unstructured regions (Figure 4).

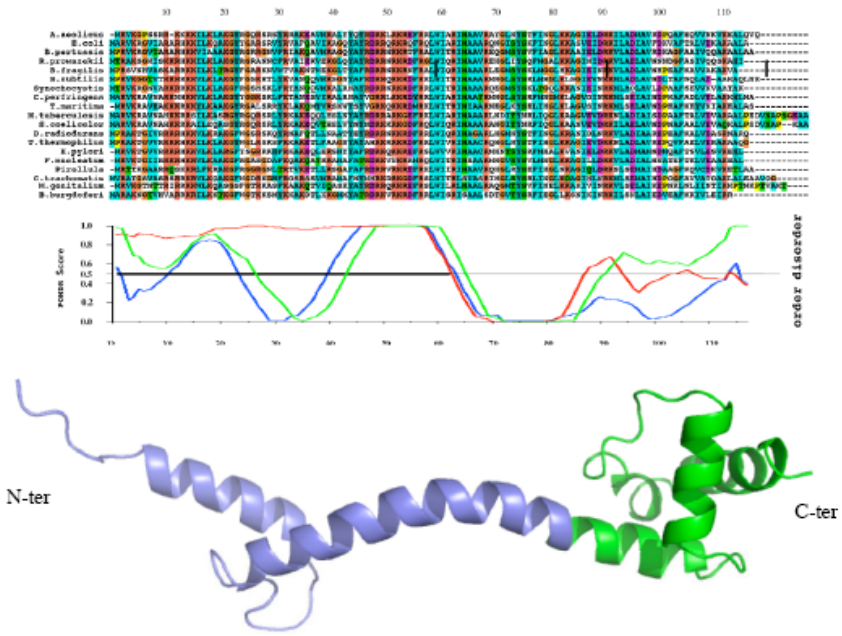

Figure 4. Sequence alignment of the ribosomal protein L20 of representative species of eubacteria and disorder prediction calculated with the program PondR for three species. Red: *A. aeolicus*. Blue; *E. coli*. Green: *D. radiodurans.*

However, the crystal structure of the free L20 of *Aquifex aeolicus* has revealed a unique picture of the dynamic of the protein since two folding states of the protein have been trapped into the unit cell (37). This structure has shown for the first time the coexistence of a folded and a partially unfolded form of a protein in a crystal structure. Having them together in identical physico-chemical conditions allowed an unbiased structural comparison of two folding states of a protein. In the partially unfolded form, both C-ter globular domain and the N-ter extension display unstructured regions (Figure 5). Also, a region of the extension that corresponds to a cluster of phylogenetically basic residues (arg 48 – arg 57) has undergone a helix-coil transition. In the corresponding region of the folded form, the side chains of the basic amino acids point on the same side of the α-helix along three helix turns (Figure 5). This has indicated that the electrostatic repulsion between the positively charged side chains is responsible for the instability of this helical segment. Indeed, the spatial arrangement of the side chains along the same side of three α-helix turns generates a very high density of positive charge.

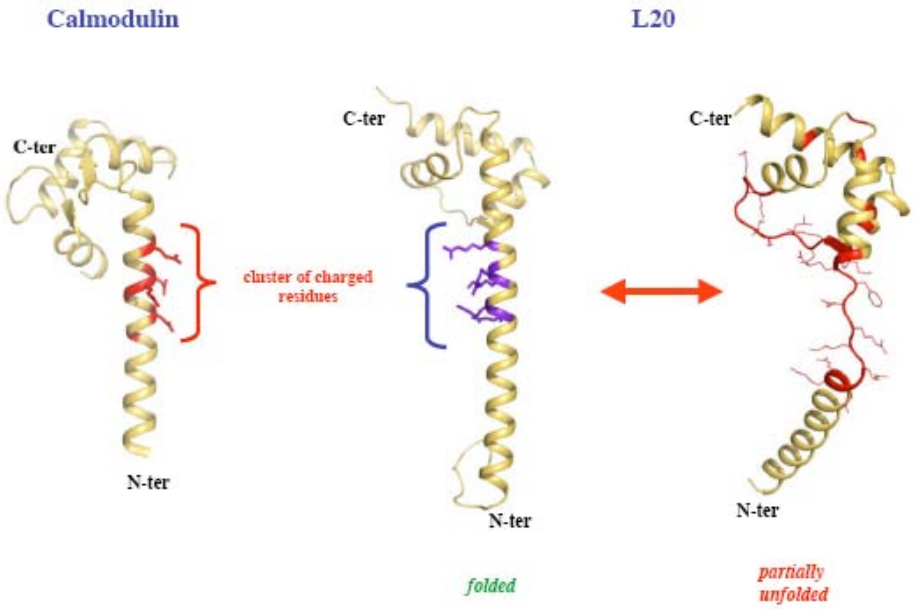

Figure 5. Comparison of the folded (middle) and the partially unfolded (right) forms of L20 in the crystal structure of the free L20 (37). The basic residues that point on the same side of the helix α2 of the folded form are depicted in purple. The calmodulin (left) display a similar distribution of negatively charged residues (red) along its α-helical linker.

A similar organisation of negatively charged residues is found in the α-helical linker of calmodulin (38) (Figure 5). In calmodulin, this region is also characterized by a high instability that confers to the protein the ability to fit to many different binding partners (38). Thus, this study has shown L20 is specifically unfolded in an evolutionary conserved cluster of basic amino acids. It seems likely that the particular distribution of the charged residues along the α-helix is responsible for its higher flexibility that may have a functional role in ribosomal assembly. In addition, the comparison of the two forms has revealed that a switch mechanism allows a structural communication between the C-terminal domain and the N-terminal extension (Figure 6). Two transient salt bridges that involve two conserved basic amino acids (arg 90 and lys 91) at the interface of the two domains “stabilize” the partially unfolded form. Their formation seem to be related with the partial unfolding of the extension. The disruption of these salt bridges upon complete protein folding generates a dramatic reorganisation of the surface electrostatic potential (Figure 6 and 7).

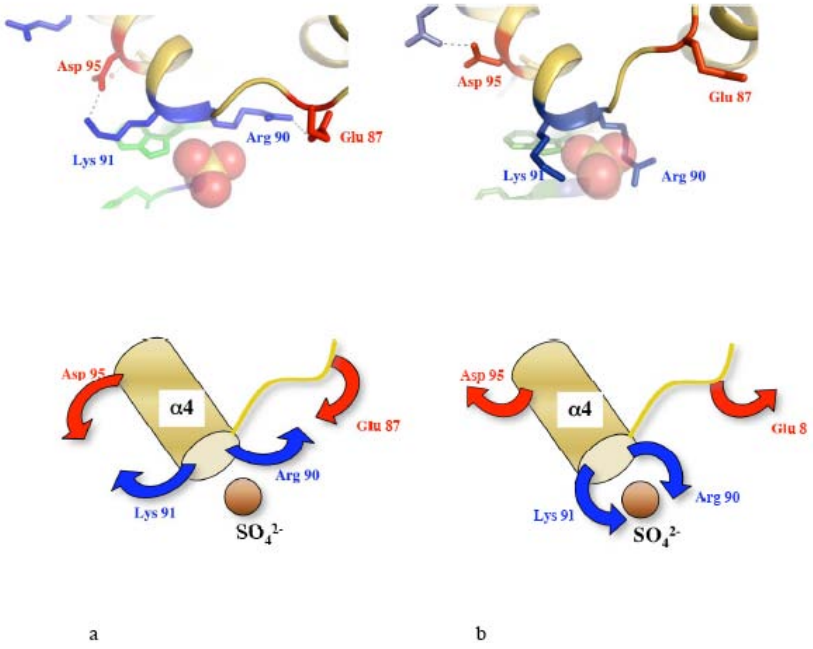

Figure 6. Structural rearrangements at the interface of the globular domain and the extension of L20 of *A. aeolicus*. a. The partially unfolded form is stabilised by the formation of two salt bridges that link two evolutionary conserved basic residues arg 90 and lys 91 to asp 95 and glu 87 located within the globular domain. b. In the folded form, the two salt bridges are disrupted and the side chains of arg 90 and lys 91 embrace a sulfate ion. The sulfate ion occupy an position identical to a phosphate group of the H40/41 junction within the 50 S subunit of the eubacterial ribosome.

Thus, the two forms have different binding properties and affinities for RNA. It has been speculated that the distinct structural and dynamics features of the two folding states play defined roles during the different steps of the folding pathway of the 23S RNA (37). Why is the coil helix transition that occurs in L20 extension essential for the early steps of 23 S RNA folding? Several hypotheses may be proposed.

i) The specific coil helix transition in L20 extension may help to avoid a kinetic trap during the folding of the helix H40/41 and H25. Alternatively, the co-folding process would stabilize a state of higher energy RNA binding site. This idea is supported by a recent NMR study that shows that the rRNA target of L20 adopts a different structure in the free and bound form (39). Thus, L20/RNA co-folding process would

lower the energy required for the structural rearrangement of the RNA site required for the subsequent steps of 23 S RNA folding. Indeed, it is now admitted that the rate limiting steps for RNA folding pathways are RNA conformational changes

ii) Another possibility could be that the coil helix transition would be required for bringing distant rRNA segments into close proximity during the course of assembly (Figure 7). Indeed, at the beginning of the folding process rRNA is more flexible and less compact. Such a fishing mechanism has been proposed in endocytosis (37,40). Indeed, the size of an unstructured peptidic segment that is longer than an α-helical one can interact with more distant partners.

iii) The transient unfolding of the long α-helix may be required for fitting into the extremely narrow groove of the H40/41 helix junction? The resulting structure would shield the electrostatic repulsion between the phosphate groups that delineate the extremely narrow groove of the L20 binding site of the H40/41 junction

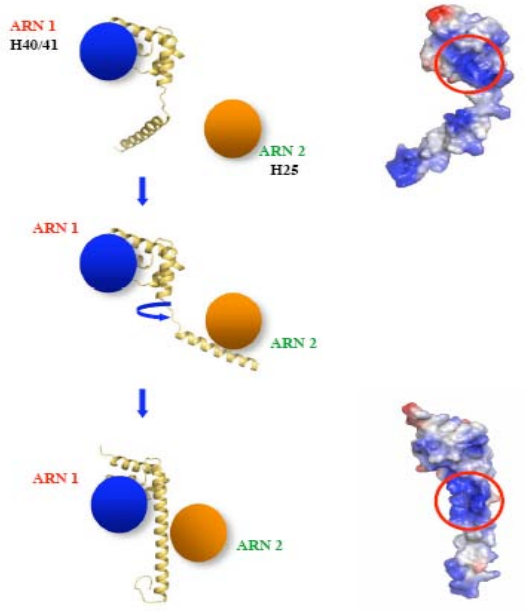

Figure 7. Fishing mechanism that can help to bring into proximity RNA segments coming from different rRNA domains. The two forms of L20 display very different electrostatic surface potential and affinity for RNA (top: partially unfolded, bottom: folded).

CONCLUSION

The ribosomal proteins L3, L4, L13, L20 and L22 that are essential for the early steps of the large subunit assembly have extensions with different structural and dynamics properties. Available biochemical and structural data suggest that the coil-helix transition observed in the long basic extension of L20 plays a specific and essential role in the 23 S RNA folding. This helix coil transition occurs in a discrete region whose flexibility is specifically tuned by phylogenetically conserved distribution of basic residues. This reveals that well-defined regions of structural “disorder” in protein may have specific functions. A similar helix-coil transition that occurs in the linker of calmodulin plays an essential role in the recognition of its multiple structurally distinct binding partners (38).

In contrast, the co-folding process that leads from a disordered loop to an ordered one upon L4/rRNA association seems to be not essential for ribosome assembly. It is interesting to suggest that such kind of transition may have a less specific role than coil-helix transitions during rRNA folding. One could imagine that the ordering of a disordered loop may be required for shielding the negatively charged RNA backbone. Thus, the deletion of this loop could be compensated by the binding of cations. It would be interesting to know what would be the phenotype associated with the deletion of the L3 extension. A prediction would be that the deletion of L3 extension that shares structural similarities with L4 would not affect ribosome assembly.

FUTURE PROSPECTS

A question that seems until today unsolved is why L20 that is an essential protein in eubacteria is not present in archea and eukaryotes. How do archea and eukaryotes compensate the absence of L20 during the early steps of the large subunit assembly? A careful structural comparison of the large subunit structure of *H. marismortui* and the ones of eubacteria would provide some structural insights. Interestingly, H25, one of RNA binding site of L20, is longer in archea. It would be then interesting to introduce the missing RNA part within eubacterial ribosome that relieve the essential character of L20.

Another question about L20 is who bind first? The extension or the globular domain? A current view is that that the globular domains of r-proteins bind first to rRNA. However, knowing that the subunit assembly is co-transcriptionally

dictated by the rRNA synthesis, it is possible that in the case of L20, the extension would bind first. Indeed, the binding site of L20 extension is the helix H25 which is well in 5' relatively to the H40/41 helix junction recognized by the globular domain.

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