Structure and Function of the Acidic Ribosomal Stalk Proteins

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Abstract: The acidic L7/L12 (prokaryotes) and P1/P2 (eukaryotes) proteins are the only ribosomal components that occur in more than one, specifically four, copies in the translational machinery. These ribosomal proteins are the only ones that do not directly interact with ribosomal RNA but bind to the particles *via* a protein, L10 and P0, respectively. They constitute a morphologically distinct feature on the large subunit, the stalk protuberance. Since a long time proteins L7/L12 have been implicated in translation factor binding and in the stimulation of the factor-dependent GTP-hydrolysis. Recent studies reproduced such activities with the isolated components and L7/L12 can therefore in retrospect be regarded as the first GTPase activating proteins identified.

GTP-hydrolysis induces a drastic conformational change in elongation factor (EF) Tu, which enables it to dissociate from the ribosome after having successfully delivered aminoacylated tRNA into the A-site. It is also used as a driving force for translocation, mediated by EF-G. The *in vitro* stimulation of translation-uncoupled EF-G-dependent GTP-hydrolysis seems to be an intrinsic property of the ribosome that is dependent on L7/L12, reaches a maximum with four copies of the proteins per particle, and reflects the *in vivo* hydrolysis rate during translation. It is much larger than the analogous activity observed for EF-Tu, which is correlated with the *in vitro* polypeptide synthesis rate. Therefore, at least certain stimulatory activities of L7/L12 are controlled by the ribosomal environment, which in the case of EF-Tu senses the successful codon-anticodon pairing. Present knowledge is consistent with a picture in which proteins L7/L12 constitute a 'landing platform' for the factors and after rearrangements induce GTP-hydrolysis. The molecular mechanism of the GTPase activation is unknown.

While sequence comparisons show a large diversity in the stalk proteins across the kingdoms, a conserved functional domain organization and conserved designs of their genetic units are discernible. Consistently, stalk transplantation experiments suggest that coevolution took place to maintain functional L7/L12 – EF-G and P-protein – EF-2 couples.

The acidic proteins are organized into three distinct functional parts: An N-terminal domain is responsible for oligomerization and ribosome association, a C-terminal domain is implicated in translation factor interactions, and a hinge region allows a flexible relative orientation of the latter two portions. The bacterial L7/L12 proteins have long been portrayed as highly elongated dimers displaying globular C-terminal domains, helical N-termini, and unstructured hinges. Conversely, recent crystal structures depict a compact hetero-tetrameric assembly with the hinge region adopting either an α-helical or an open conformation. Two different dimerization modes can be discerned in these structures. Models suggest that dimerization *via* one association mode can lead to elongated dimeric complexes with one helical and one unstructured hinge. The physiological role of the other dimerization mode is unclear and is in apparent contradiction to distances measured by fluorescence resonance energy transfer. The discrepancies between the crystal structures and results from other physico-chemical methods may partly be a consequence of the dynamic functions of the proteins, necessitating a high flexibility.

INTRODUCTION

Ribosomes from all three kingdoms of life contain in their large subunits acidic proteins, comprising a morphologically distinct feature, the stalk protuberance [1]. In *Escherichia coli* there are four copies of these L7 and L12 proteins per particle [2], which are identical in sequence and only distinguished by acetylation of their N-termini (L7) or lack thereof (L12) [3, 4]. Depending on the culture conditions, the two polypeptides exist in varying ratios in the 50S subunits and seem to be functionally interchangeable [5, 6]. In the following they will therefore be jointly referred to as L12. The archaeal and eukaryotic analogs are termed L12 and P1/P2, respectively. In order to distinguish proteins with the same name from different kingdoms the prefixes, '*a*', and '*e*' are used here for archaeal and eukaryotic L-proteins, respectively. Names without prefixes designate bacterial proteins. The two classes of eukaryotic P-proteins, their

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names referring to their post-translational phosphorylation, are based on different sequences [7, 8] and in some organisms comprise subgroups with various numbers of members [8 and references therein]. These archaeal and eukaryotic stalk proteins are only distantly related to the bacterial counterparts [9] but again display, collectively, a fourfold redundancy on their large subunits. It is noteworthy that L12 and the eukaryotic P1/P2 proteins are the only macromolecular components that are present in multiple exemplars in the ribosomes. Furthermore, the L12 class constitutes the only r-proteins that are not in direct contact with rRNA [10, 11] but bind to the ribosome *via* r-protein L10 (P0 in eukaryotes) [12, 13].

Since their first isolation from *E. coli* ribosomes [14, 15] the acidic stalk proteins have received special attention. Particularly, the observation that these components play a central role in the ribosome-mediated stimulation of elongation factor (EF)-dependent GTP-hydrolysis [16] has nurtured these inquiries. The importance of the stalk is also attested by drastic changes in its morphology during the translational cycle, recently visualized by electron microscopy (EM) [17-19].

Herein, we give a reconciliation of the results of over three decades of research on bacterial L12 and try to relate them to findings on the eukaryotic P-proteins. A special emphasis is on the studies aimed at exploring the proteins' three-dimensional structures. Because the stalk [1] is not seen in the recent crystal structures of a 50S ribosomal subunit [10] and is poorly defined in that of the entire 70S ribosomes [20; Noller, H.F., personal communication], perhaps due to a high flexibility or loss during purification, the elucidation of isolated stalk component structures and their complexes with other ribosomal elements is crucial. Furthermore, it is gratifying that recently stimulation of the GTPase activity of EF-G by isolated r-protein L12 was observed [21], thereby opening a new way to study this interaction.

L12 AND RIBOSOMAL ENERGETICS

Role of GTP-Hydrolysis in Translation

Several of the translation factors are multi domain G-proteins [22]. Their negligible intrinsic GTPase activity is stimulated by interaction with parts of the ribosome after it reached defined functional states. These processes order the sequential events of the translational cycle. While the role of GTP-hydrolysis for the function of initiation factor (IF) 2 is unclear [22], it signals successful codon-anticodon pairing in the case of EF-Tu and, through conformational changes, triggers release of the tRNA from the factor and of the discharged factor from the ribosome [23]. EF-G exploits GTP-hydrolysis to generate a driving force for translocation [24]. Release factor (RF) 3 seems to be responsible for detaching the decoding RF1 and 2 from the ribosome by *in situ* GDP-GTP exchange. GTP-hydrolysis may then be used to dissociate the factor itself from the ribosome [25].

Stimulation of GTP-Hydrolysis by L12

Because *E. coli* L12 is easily extracted from ribosomes by ethanol/salt treatment, the effects of its removal on translational processes have long been noted [16, 26, 27]. While such functional analyses initially concentrated on bacterial systems, it now seems clear that the acidic r-proteins fulfill analogous functions across the kingdoms [28-30]. Noted first by Kischa *et al.*, the severely impaired GTPase activity of EF-G with L12-depleted ribosomes could be rescued by addition of the purified protein [16]. Similar effects were subsequently seen for EF-Tu [31]. During initiation, L12 seems to stimulate the IF-2-associated GTPase activity [32]. Together the results are consistent with L12 being a prime component of the ribosomal GTPase activating center [33]. Consistently, the proteins lie in the vicinity of an rRNA region neighboring the L11 binding site [34]. Since affinity-labeled EF-G•GTP reacted preferentially with L11 [35], this region became known as the GTPase-associated domain, something borne out by direct mapping of EF-G on the ribosome [36].

EF-G shows ribosome-dependent translation-uncoupled GTP-hydrolysis, which reaches a maximum with four equivalents of L12 [37]. This reconstituted hydrolysis rate *in vitro* matches the rate of *in vivo* protein synthesis. Conversely, the EF-Tu-dependent *in vitro* rates as well as poly-Phe synthesis are two to three orders of magnitude lower [37]. Therefore, while L12 seems to be able to optimally stimulate EF-G activity *in vitro*, additional signals are necessary to bring EF-Tu and peptide bond formation up to pace [38; Möller, W., in preparation].

INTERACTIONS OF L12 WITH TRANSLATION FACTORS

Interactions on the Ribosome

Based on the above investigations it has been suggested that L12 is involved in direct interactions with initiation [39], elongation [16], and release factors [40]. Further evidence for contacts between L12 and translation factors comes from several lines of inquiry: Addition of purified L12 restored the EF-Tu-dependent Phe-tRNA binding activity of ribosomal cores [31]. Changes in the proteolysis pattern of L12 were observed upon docking of EF-G [41] and EF-Tu [42]. Mutations in RF1, eliciting a temperature sensitive phenotype and increased misreading of stop codons, were found to be compensated by a L12 mutation in *E. coli* [43]. Other L12 mutants showed impairments in the EF-G and EF-Tu functions [44]. More directly, EM images of bacterial ribosomes in complex with either EF-Tu [45] or EF-G [46] showed a bridge between the L12 stalk and the respective factor. The EM structure of a complex of eukaryotic 80S ribosomes and *e*EF-2, the eukaryotic analog of EF-G, similarly displayed an extensive contact between the factor and the stalk [47] suggesting an evolutionary conservation of the protuberance as a factor interaction site. Very convincing evidence for this conserved interaction comes from the recent grafting of eukaryotic P-complexes onto *E. coli* ribosomal cores [48]. The ensuing hybrids specifically responded to the pertaining translocase, EF-G in the case of

an [L10•$(L12)_4$]-stalk and *e*EF-2 in case of a [P0•$(P1)_2(P2)_2$]-stalk. Similar transplantation experiments detected that even within the eukaryotic kingdom, elongation factors seem to interact more vividly with their own stalk proteins than with those from related species [49].

It should be noted that the L12 region may not be the only association site for translation factors and may only be transiently occupied during the entry of the factors. A highly conserved rRNA region neighboring the stalk, the sarcin-ricin domain, has been shown to directly bind to elongation factors [50, 51]. Proteins L6, L11, and L14, all located at the base of the stalk, are likewise implicated in the binding of the factors. A model for EF-G interacting with this latter region has recently been proposed based on the crystal structure of an archaeal 50S subunit [52]. However, a deletion of domains 4 and 5 in EF-G still allowed factor binding and ribosome-dependent GTP-hydrolysis but removed the EF-G-associated footprint on the sarcin-ricin loop [53], contrasting the notion of the latter being the prime elicitor of the GTPase activity. In another recent study, ribosomes depleted of L11 were investigated *via* cryo-EM [54]. The placement of L11 suggested that the protein formed a contact to domain 5 of EF-G. However, in the structure of the *Thermus thermophilus* 70S ribosome [20] a dimer of L12 has been tentatively placed into the electron density [20; Noller, H.F., personal communication], and a C-terminal domain (CTD; see *Domain Organization and Structures*) comes to lie in the immediate vicinity of the implicated L11 region. Because a L12 structure has not been positioned in the EM maps, the identified bridge could be as well to L12 or to L11 and L12 at the same time. As another possibility, L12 could be necessary for supporting a L11 conformation that can interact with the factor. Consistently, L11 and L12 are found as immediate neighbors in other cryo-EM reconstructions [55] and in crosslinking studies [56]. Taken together, it seems that L12 remains one of the most consistent candidates implicated in primary factor binding and stimulation of GTP-hydrolysis.

In some EM reconstructions, the stalk is seen to adopt a transient bifurcated structure upon EF-G•GTP binding [17, 18]. After GTP-hydrolysis, a contact of the G-domain of the factor to a reeled-in CTD of L12 was suggested [17]. These interpretations are in disagreement with the above deletion studies on EF-G, which imply that L12-contacts are build up before GTP-hydrolysis occurs [53].

Interactions in Isolation

Only very recently has it been possible to demonstrate an association of the acidic r-proteins and elongation factors in isolation, strongly supporting the view of L12 (P1/P2) as a factor binding site. Using surface plasmon resonance, both P1 and P2 from yeast were shown to form complexes with *e*EF-2 with dissimilar dissociation constants in the micromolar to nanomolar range [57]. The interactions were accompanied by a conformational change in the factor and were modulated by P-protein phosphorylation. For the bacterial L12 – EF-G system similar evidence came from fluorescence resonance energy transfer (FRET) studies and the demonstration of enhanced GTP turnover following interaction [21], although the latter activity was still lower than what is seen with entire ribosomes and a control with other proteins replacing L12 was not conducted. While isolated L12 also interacted with EF-Tu no GTPase activation was observed, indicating that here additional ribosomal constituents or events are required [58]. The different answers of EF-G and EF-Tu toward exposure to purified L12 indicate that the observed effects are specific and not a fortuitous consequence of, *e.g.*, the inherent chaperoning properties of the factors which are also coupled to GTP-hydrolysis [59, 60].

Interaction of L12 with EF-Tu

For guanine nucleotide exchange, EF-Tu requires interaction with a stimulatory factor, EF-Ts. The EF-Tu•EF-Ts interaction has been elucidated at atomic detail [61, 62]. Because it resembles contacts of L12 to the G-domain of EF-Tu in EM reconstructions [45], Rodnina and coworkers have investigated sequence and structure homologies between the L12 and EF-Ts proteins [63] (Fig. **1A**). Conserved features in the N-terminal domain (NTD) of EF-Ts and the C-terminal portion of L12, whose structure is also known [64], were discerned. In particular, the invariant Lys9, Arg12, and Lys23 of EF-Ts, which make critical contacts to helix D of EF-Tu, are conserved as Lys70, Arg73, and Lys84 in L12. An excellent superposition could be found for the three-dimensional fold of the corresponding helical hairpins (Fig. **1A**). Cross-reactivity of monoclonal anti-L12 antibodies supported the presence of a region of similarity in the two proteins [65]. Most importantly, the regions provided a canyon for helix D of EF-Tu with similar topography and charge distribution in both ligand proteins [63]. The analysis suggests that L12 uses a region of its CTD (Fig. **1B**) to interact with EF-Tu in a manner similar to EF-Ts. It supports the action of L12 as a primary factor binding site for EF-Tu and is consistent with L12 facilitating the release of the γ-phosphate after GTP-hydrolysis [63].

Molecular Mechanism of GAP Activity

In retrospect, the above results show that L12 likely was the first GTPase activating protein (GAP) identified. It will be interesting to see whether its mode of action agrees with one of the presently known GAP mechanisms [21]. Until recently, an attractive hypothesis was that the single Arg residue, which is conserved in bacteria in the CTD [21] and in archaea and eukarya in the NTD [30], might serve in analogy to the Ras-GAPs as an 'Arg-finger' [66], complementing the active site environment of the G-protein [67]. However, this model has been revoked for archaea [30] and drawn into question in bacteria by mutational analyses [21]. The mechanism may therefore resemble that observed for the regulators of G-protein signaling [68] or of ARF-GAP [69], which remodel the active site by direct contacts or allostery, respectively.

OTHER EFFECTS OF L12 ON TRANSLATION

In addition and maybe interrelated to the above role in factor interaction, mutations in *E. coli* L12 have been

isolated which affected the level of misreading [44, 70, 71] and suggested that the protein is also involved in the control of the translation accuracy. Similar effects had previously been seen in certain buffer systems upon removal of L12 [27]. Furthermore, the eukaryotic acidic P-proteins may serve subtle regulatory purposes, influencing the expression level of certain mRNAs [8].

Because of their strong implication in several important functions of the ribosome, L12-like proteins are a notable exception to the paradigm that rRNAs comprise the functional components of the particles while the proteins serve merely structural roles [72-76]. The root of this paradigm lies in RNA having catalytic properties [77, 78] and in the belief of an RNA world [79]. Coevolution of short peptides and primitive RNAs would offer an alternative view on the nature of the proto-ribosome and the evolution of protein synthesis [80].

PHYLOGENETIC COMPARISONS

While the bacterial L12 proteins comprise a highly homologous assembly, the archaeal variants are related to the eukaryotic representatives and there is little similarity discernible between the bacterial and archaeal/eukaryotic groups [7, 9, 30, 81-83]. It has been generally observed that the information-processing machineries of archaea are more related to eukarya than bacteria [30] and interestingly, the first sign of these relationships in ribosomes came from sequence comparisons of r-proteins [81, 84, 85] rather than rRNA. On the other hand, the domain organization, featuring a functionally separable NTD, a connecting hinge region, and a CTD (see *Domain Organization and Structures*), seems to have been preserved in all species [28]. Recent analyses, which suggest that also the functionalities of these domains have been maintained throughout evolution [28-30], obliterate earlier suggestions of domain rearrangements [7, 83, 86-89]. Furthermore, in bacteria and archaea the genetic organization in the corresponding operons (L11-L1-L10-L12) is the same [90-92].

Experiences with hybrid ribosomes demonstrated that P1/P2 from yeast are active in complementing *E. coli* ribosome cores [93] and, *vice versa*, *E. coli* L12 can complement yeast cores [33, 94]. However, archaeal/bacterial hybrids were found inactive which may be partly attributable to the halophilicity of the species tested [95, 96]. Within the eukaryotic system a more subtle diversification has been documented for the P1/P2•P0 interaction: While a common rRNA association mode for P0 proteins could be demonstrated by complementation studies, yeast ribosomes with heterologous P0 proteins bound different subgroups of P1 and P2 proteins [49]. Together with the above-described transplantations of entire rat P-complexes ($P0•(P1)_2(P2)_2$) onto *E. coli* ribosomes [48] and the mixing of stalk components among different eukaryotes [49], the studies suggest that the rRNA association mode of L10 (P0) is conserved within and across kingdoms, that the interaction of L12 (P1/P2) with L10 (P0) was somewhat diversified but maintained some common features, and that the elongation factor/acidic protein interactions changed drastically once eukaryotes/archaea and bacteria diverged from their common ancestor. Consistently, it has been insinuated that the NTDs, responsible for ribosome binding (see *Domain Organization and Structures*), exhibit a conserved three-dimensional fold [30] despite the lack of obvious sequence relatedness, as was demonstrated for other pairs of bacterial/eukaryotic proteins [97].

Along with the sequence divergence one can also discern functional differences between the kingdoms. P1/P2 function is modulated by phosphorylation [57, 98]. The N-terminal acetylation in bacterial proteins is generally regarded to be without functional significance [5, 6] and seems to be a rare feature in bacteria. However, in *E. coli* the reconstituted factor-dependent GTPase activity, starting from CsCl-purified 50S cores, is higher with L12 than with L7 [38], possibly hinting at a specific role in a few species. While the acidic proteins are freely exchangeable in eukaryotes between ribosomes and non-phosphorylated cytoplasmic pools [28, 99], they are permanently associated with the bacterial particles [100].

L10-like proteins from archaea and eukarya are considerably larger than the equivalents from bacteria [7] and incorporate a portion homologous to the *a*L12/P-protein CTDs [92]. In *Sulfolobus* the last 33 amino acids of *a*L10 are identical to those in *a*L12 [92]. Possibly, therefore, the pentameric complexes in archaea and eukarya harbor five factor binding sites. In agreement, P1/P2 can be knocked out in yeast and the cells are still viable with a remaining copy of P0, while removal of all P-proteins is lethal [99]. However, P0 does not harbor a fully redundant P1/P2-like activity, since cellular expression profiles changed in response to P1/P2 deletion, suggesting an active role of the latter proteins in the selective translation of mRNAs [8]. Similar deletion studies have not been conducted in bacteria.

DOMAIN ORGANIZATION AND STRUCTURES

Functional Domains

Based on biochemical and genetic investigations the bacterial L12 proteins have been ascribed three functional domains: the NTD, the CTD, and an intervening hinge region [101]. The NTD is responsible for the self-association of the protein [28, 102] and for the interaction of the ensuing complexes with L10 [28, 103] (see below). Association through the NTDs has also been shown for the corresponding eukaryotic P1/P2 [29]. The NTD was interpreted as α-helical with the dimerization module possibly designed as an antiparallel four helix bundle [104-106]. Oligomerization through the NTDs has recently been directly visualized in crystal structures of L12 complexes from *Thermotoga maritima* [107].

The globular CTD is implicated in translation factor binding [28, 103, 108-110]. One model suggests that, making initial contact with the CTD, the translation factors can subsequently interact with the GTPase activation and peptidyl transferase domains through flexible attachment of the CTD to the bulk subunit *via* the hinge [30, 111, 112]. The CTD from *E. coli* L12 has been the first component of the ribosome elucidated at close to atomic resolution by x-ray

crystallography [64]. A recent crystal structure of a full-length L12 molecule [107] corroborated the globular structure of the domain that consists of a three-stranded antiparallel β-sheet decorated with three α-helices. It resembles the RNA recognition motif (RRM) of a group of RNA binding proteins although no interaction with rRNA or extra-ribosomal RNA could be demonstrated [10, 11]. A continuous surface of invariant residues, encompassing parts of two CTDs in the crystals of the C-terminal fragments, was interpreted as a likely interaction site with the translation factors [110]. Contrary to this interpretation, L12 dimers could be crosslinked in different orientations [113] and L12 mutants with only one CTD functionally complemented ribosomal cores [114]. In addition, the two CTDs within a dimer can be widely separated [115] due to the motional freedom furnished by the hinge [116]. An independent arrangement of the head groups is also seen in the crystal structures of the entire molecules [107] and likewise an electron microscopically observed bifurcated stalk was interpreted as a L12 dimer with separated CTDs [17, 18].

The linker domain between the terminal portions is indispensable for L12 functions [117] and is seen as a flexible element of the protein [116, 118-120]. This picture is supported by EM images of the entire 50S particles [121]. Here the percentage of particles with a stalk is consistently 50%, suggesting two conformational states, one with an extended hinge, the other with a hinge folded inwards [37]. NMR spectra implied that the elasticity partly stems from cis-trans isomerizations of Pro peptide bonds [104]. Conversely, the conformational diversity observed in L12 crystal structures suggested that the hinge can undergo a helix-coil transition [107]. The flexibility of the hinge may allow L12 to adopt different global conformations and may be the reason for the independent movements of the CTDs and the association with different areas on the ribosome [113, 114, 122, 123; see *Diversity in the Ribosomal Location*]. Certain deletion mutants in the hinge region showed increased error rates [71] and decreased the overall translation rate *in vivo* [117]. In addition, hinge deletions led to defective binding of elongation factors and interfered with stimulation of GTP-hydrolysis [124]. A lengthening of the linker region by up to 14 residues affected the L12 functions less drastically [120]. Considering the importance of the hinge and its correct length it is surprising that its exact sequence does not seem to matter [120]. Particularly striking in the latter context is the observation that in bacteria the hinge is almost entirely composed of only four residues, Ala, Val, Pro, and Gly [83]. Possibly the restricted sequence of the hinge in L12 proteins has been selected to facilitate the above mentioned helix-coil transitions during translation.

Oligomerization and Shape

For bacterial ribosomes, immuno-EM has shown that there is a single extended L12 region encompassing the stalk [1, 125], suggesting a local crowding or association of the four peptide copies on the 50S subunit [126]. Subsequently, crosslinking studies with the *E. coli* protein detected dimers [127]. Small-angle X-ray scattering saw *E. coli* L12 in solution as a tightly associated and highly elongated dimer (~180Å in length) [127] in agreement with results from sedimentation and viscosity measurements [128, 129]. Sedimentation equilibrium studies also indicated dimers for the isolated acidic r-proteins from the other lines of decent [130] and the dimeric state of L12-like proteins in solution has now been widely accepted [102, 127, 131]. Symmetrical and staggered arrangements of the constituents have been proposed [56, 104, 115, 122, 132, 133]. The model most propagated has been that of a symmetrical, parallel dimer in which the CTDs are well separated from the NTDs [104]. There are indications from NMR that in *E. coli* the L12 dimers are maintained on the ribosome [116].

Interestingly, under certain conditions L12 or analogs have also been observed as tetramers [134, 135]. Especially the acidic r-proteins from some extremophiles frequently displayed a tetrameric arrangement [136]. The *T. maritima* homolog could be crosslinked up to the tetramer level [137]. A potential pitfall in these investigations is the formation of L10•$(L12)_4$ complexes *via* residual L10 in the preparations, which may mimic a tetrameric state for the isolated L12 component. Nevertheless, in light of the repeated observations of L12 tetramers and recent x-ray crystallographic results [107], the picture of a static, elongated L12 dimer should be regarded with some caution. Other r-proteins, *e.g.* L18 and L25, have been ascribed an elongated shape based on similar solution studies [127, 138] but subsequently NMR and crystal structures of the proteins alone and in complexes [10, 139, 140] showed globular molecules. In the special case of L12, elongation and compaction may interchange during the translational cycle.

An Effort to Reconcile L12 Crystal Structures with that in Solution and on the Ribosome

Detailed atomic structures have so far only been reported for the bacterial L12-like proteins. While *E. coli* L12 has at various times been subjected to NMR investigations [104, 106], no full high-resolution three-dimensional solution structure has been determined. The N-terminal region of dimeric L12 indicated an antiparallel four helix bundle followed by an unstructured hinge to separate the CTDs from the N-terminal part. NMR results agreed with various other methods, indicating a high α-helix content and a flexible hinge [131, 141, 142].

Recent crystal structures of *T. maritima* L12 revealed a number of surprising features [107] (Fig. **2**). The molecules associated into tetramers in the crystallographic asymmetric units composed of two full-length molecules and two N-terminal fragments (Fig. **2E**), in contrast to the preferred dimer state of isolated *E. coli* L12. Two full-length molecules were held together by a four helix bundle that encompassed the hinge region (called the 'core dimer' of the structures; Fig. **2C**). Both full-length molecules were furthermore associated with an N-terminal L12 fragment *via* a five helix bundle that incorporated the hinge of only one partner ('peripheral dimers'; Fig. **2D**). The hinge regions of the fragments adopted elongated, unstructured conformations (Fig. **2B**). One possible interpretation would be to view the peripheral dimers as the preferred solution state of L12 and the core dimerization as a way in which they could associate on the ribosome. Alternatively, tetramerization could be due

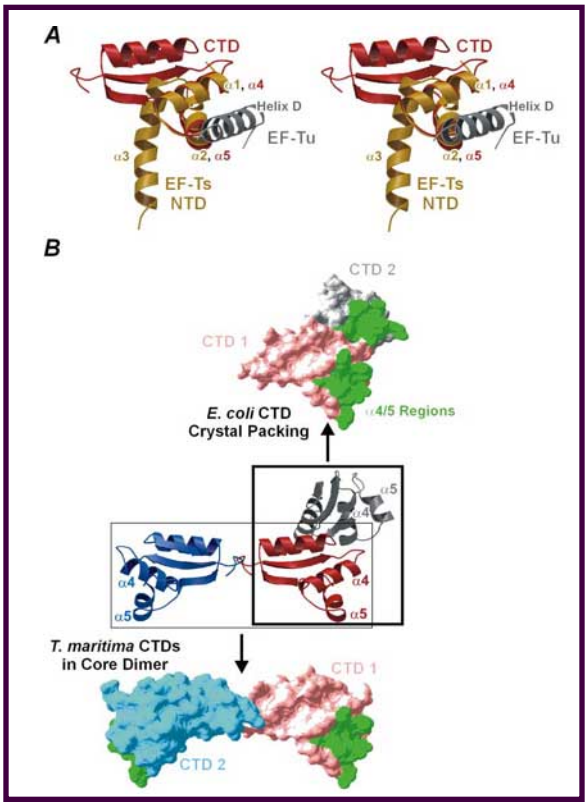

Fig. (1). (A) Superposition of an L12 CTD (red) and the N-terminal three helices of EF-Ts (gold) in complex with helix D of EF-Tu (gray). The superposition defines a presumed interaction motif for EF-Tu on L12 (helices 4 and 5) (B) Mapping of conserved residues of the presumed interaction motif (α4/5 regions) on the surface of L12 CTDs. The upper panel shows two CTDs as associated in the crystal packing of the *E. coli* C-terminal fragment (red and gray; [64]) where the two interaction surfaces (green) are close to each other. The lower panel shows the arrangement of the CTDs (red and blue) as observed in the *T. maritima* core dimers where the interaction surfaces (green) are remote. Using the same color coding, the central panel gives a ribbon diagram of the relative orientations of the CTDs as used in the other portions of the figure. The orientation of the red CTD serves as a reference and is fixed in all parts of the figure.

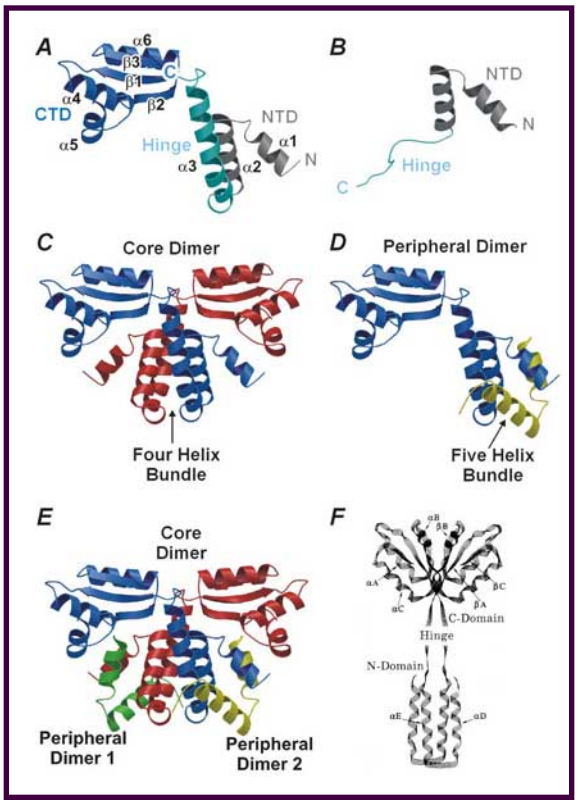

Fig. (2). (A) Full-length monomer of L12 with α-helical hinge as seen in two crystal structures. The three functional domains are identified by color coding and the secondary structure elements are labeled. (B) N-terminal fragments of the structures with unfolded hinge and the same color coding as in (A). (C) The core dimer of the structures. (D) One of the peripheral dimers of the structures. (E) Hetero-tetrameric assembly as seen in two different crystal environments. (F) Proposed structure from NMR studies. Reproduced from [105] with permission.

to molecular crowing in the crystals, although the same structure was observed in two crystal forms. As another possibility, one of the contacts in the crystals may be replaced on the ribosome by equivalent interactions with L10 [107; see *Complexes with L10/P0*].

In order to decide which of the two dimerization modes may be the physiological one, one can recruit the rich collection of biochemical and biophysical findings. The core dimerization portrays the molecules with a compact shape, in contrast to most observations in solution, as discussed above. It was equally unexpected of the hinge region to form a major part of the interface as seen in the central dimers and, as pointed out by Liljas and coworkers [143], the accepted flexibility of the hinge seems incompatible with the observed strong interactions within a dimer.

It has been known for a long time that oxidation of the methionine residues in the NTD of *E. coli* L12 leads to disintegration of the dimers [144]. When *E. coli* L12 is modeled according to the *T. maritima* structure, these methionines map in their majority into the peripheral dimer interface (Fig. **3A**). Oxidation may lead to a disruption of the hydrophobic contacts.

After appropriate derivatization, distances within a dimer and between dimers marked by residues Met1, Lys29, and Lys51 of L12, and within the pentameric complex, using in addition Cys70 of L10, have been determined by FRET measurements and crosslinking [145-147] (Table **1**).

Table 1. Distances in L12 and L10•$(L12)_4$ Complexes

A. FRET Distances (Å) Between Met1 or Lys51 of L12 and Cys70 of L10

Residues	Dimer 1 – L10	Dimer 2 – L10
Met1(L12) – Cys70(L10)	42	58
Lys51(L12) – Cys70(L10)	45	55

B. FRET Distances (Å) Measured Between Two L12 Dimers on the Ribosome.

Residues	Distance
Met1-Met1	40
Lys51-Lys51	70
Met1-Lys51	60

C. Distances (Å) Within L12 Dimers (Crystal [T. Maritima], FRET and Crosslinking [E. coli])

Residues	Core Dimer	Peripheral Dimer	FRET	Crosslinking
Met1-Met1	46	16	33	-
Lys51-Lys51	17	-	45	-
Met1-Lys51	32	-	41	-
Lys29-Lys51	27	-	-	10

Comparison to the corresponding distances of the Cα-atoms in the crystal structures shows some disagreements with both crystalline dimer modes (Table **1**). While FRET distances between the L12 N-termini match both dimer organizations quite poorly, the intradimeric Met1-Lys51 FRET distance and the intradimeric Lys29-Lys51 distance estimated from crosslinking are in general agreement with the core dimer. However, the Lys51-Lys51 FRET distance within a dimer is incompatible with the core dimer fold. Unfortunately, the N-terminal fragments of the peripheral dimers are not long enough to allow a thorough comparison with the situation in these constructs. For the same reason, the interdimeric distances determined by FRET between the two peripheral dimers cannot be seen in the crystals. In particular, an interdimeric Lys51-Lys51 distance of 70Å (Table **1**) on the ribosome indicates that the two dimers fan out from their L10-bound NTD bases toward their CTD positions in a way not observed in the present crystal structure of L12 alone. A partial explanation for the poor matches between crystallographic and FRET distances may also be the uncertainty in the conformations of the side chains that support the chromophores and crosslinkers. Furthermore, the FRET distances are weighted averages of all possible conformers.

Taken together, while the tetrameric crystal structures may be related to the observations of L12 tetramers in solution, a dimer is the most likely form for the isolated and

L10-bound proteins. Presently, a model based on the peripheral dimer conformation seems to be most consistent with the known facts [143]. In any case, the available structures clearly demonstrate that dimerization involves the entanglement of the N-terminal α-helices. Significantly, both dimerization modes involve the hinge, but to a different extent: the peripheral dimers leave one hinge free and unstructured. The crystal structures clearly demonstrate the ability of the hinge to adopt both α-helical and extended conformations. It has been observed that the helical content of L12 molecules increases significantly in the presence of ~50% ethanol and 1M NH_4Cl [148]. The effect is presumably not simply due to a detachment of L12 from the ribosome under these conditions because NMR studies, which detected a disordered hinge, were also performed on isolated L12. Rather, the results argue for a functionally relevant helix-coil transition. The high ammonium sulfate concentration in the crystallization trials [137] could have acted as a stabilizer of a structured hinge, which could be achieved during translation by contacts within L12 dimers (peripheral dimerization) or to other molecules (core dimerization).

COMPLEXES WITH L10/P0

In order to associate with the large ribosomal subunit, the four copies of L12 [2] bind to a single molecule of L10 [12]. Because of its high stability, part of the complex was visible as a unique spot on denaturing two-dimensional gels and was for some time considered a separate polypeptide, L8. Furthermore, studies on L12 and L10 showed quite early a strong interdependency between them in the elongation factor dependent GTPase reactions [149]. It is assumed that two dimers of L12 bind to L10. The L10 sequence lacks an internal symmetry and it will be interesting to see how the two implicit dimer binding sites are distinguished. In agreement with two unequal dimer binding sites on L10, it has been observed that there are a strong and a weak binding site of L12 on the ribosome [150]. Recent L10 deletion analyses by Traut's group showed that the C-terminal 20 amino acids of L10 are important for both dimer binding sites while removal of the last 10 amino acids affected only one site [151]. The L10 C-terminus therefore plays a crucial role either by directly contacting the L12 dimers or by organizing the binding sites.

Investigations of the eukaryotic stalk suggested a P0•$(P1)_2(P2)_2$ organization [152, 153]. The complex is less stable than the bacterial equivalent and cannot be extracted as a whole by ethanol/NH_4Cl treatment [154, 155]. Interestingly, it has recently been shown that the mammalian P1 stalk proteins bind to the L10-equivalent, P0, while the P2 proteins are attached to the complex only through P1 [156]. Possibly these different association modes of P1 and P2 are related to the different binding modes for the two bacterial L12 dimers to L10.

In bacteria it is known that hydrophobic interactions are responsible for tying L12 to L10 [132]. Consistently, a universally conserved phenylalanine residue is exposed in the loop between the NTD and the hinge and may be a main latching point for L10 [107] (Fig. **3B**). Similar types of interactions are implicated in the archaeal [28, 30] and eukaryotic [157] complexes.

Based on the available data, several models can be envisioned for L10-L12 complexes. They are mostly distinguished by the assumed oligomeric state of the bound L12 molecules. L10 may contact a peripheral L12 dimer by replacing the other full-length L12 molecule of the crystal structures, stabilizing the helical hinge of one molecule and making almost exclusive interactions with only the full-length molecule. Alternatively, such an L12 dimer could be complexed by L10 through the N-terminal five helix bundle, with contacts to both L12 monomers. Third, as originally favored by Wahl *et al.* [107] based on conservation patterns and distribution of residues on L12 implicated in L10 interaction, L10 may replace the peripheral fragments in contacting the core dimer. And lastly, L10 could bind the tetrameric assemblies through the N-terminal helical bundles.

DIVERSITY IN THE RIBOSOMAL LOCATION

It has been shown that the L10•$(L12)_4$ complex binds cooperatively with L11 to the GTPase center within domain II of *E. coli* 23S rRNA [12, 34, 158 -161]. The binding characteristics are conserved in the homologous eukaryotic system comprised of r-protein *e*L12 (corresponding to bacterial L11), the P-complex and the 28S rRNA GTPase domain [49, 162]. rRNA binding of the complexes occurs through the L10 (prokaryotes) or P0 (eukaryotes) components. In agreement with one accepted location of L12, the crystal structures of 50S subunits [10, 163] accounted for all density in the core of the particles by rRNA and other proteins, leaving the invisible stalk as a site for L12. Density for the stalk region was still weak in the structure of the entire *T. thermophilus* ribosome [20; Noller, H. F., personal communication] but a tentative placement of one L12 dimer has been undertaken. The quality of the 5.5Å maps did not allow tracing of the still unknown L10 structure (Noller, H. F., personal communication).

Complicating the picture, FRET and crosslinking studies with L12 labeled at various positions have detected different whereabouts of the protein [56, 115, 122, 123, 164], possibly correlated with different stages of translation. Besides to L10, *E. coli* L12 can be crosslinked to L11 and, more weakly, to L2 and L5, and to the small subunit components S2, S3, S7, S14, and S18 [56, 164]. While the crosslinks to the GTPase center region (L10 and L11) and the neighboring L5 are comprehensible from the structure of the ribosomal particles [10, 20, 163], L2 is located on the opposite end of the subunit (Fig. **4**) and would be difficult to touch even by L12 with an extended hinge. Assuming a fully expanded hinge of about 20 residues, the CTD could be at most 70Å away from the NTD. The root-mean-square end-to-end distance for a random coil of 20 residues would be only about 17Å. Still, the diversity in the observed crosslinks portrays the CTDs visiting the base of the L12 stalk, the peptidyl transferase domain, and the head of the 30S subunit [164] and is indicative of a high flexibility of the L12 molecules on the ribosome (Fig. **4**). Therefore, the stalk may be at least transiently composed of only a single dimer while

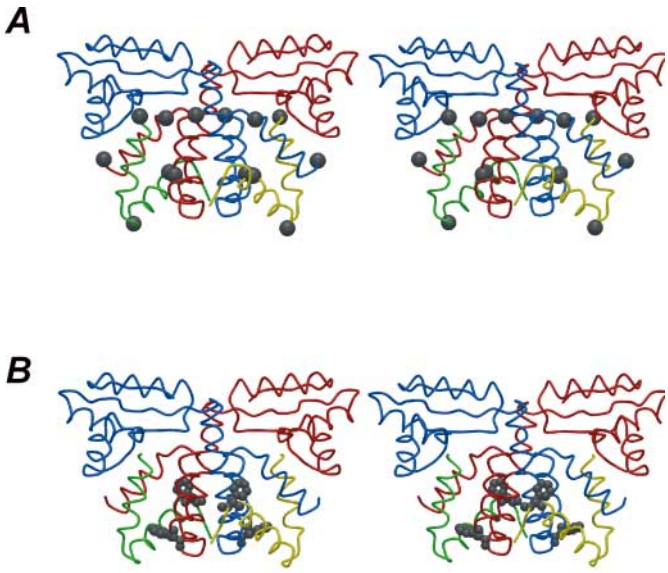

Fig. (3). (A) Mapping of *E. coli* L12 Met residues (gray spheres) on the structure of *T. maritima* L12 (color coded as before). (B) Position of the conserved Phe residues (gray balls) in the structure of *T. maritima* L12 implicated in the interaction with L10.

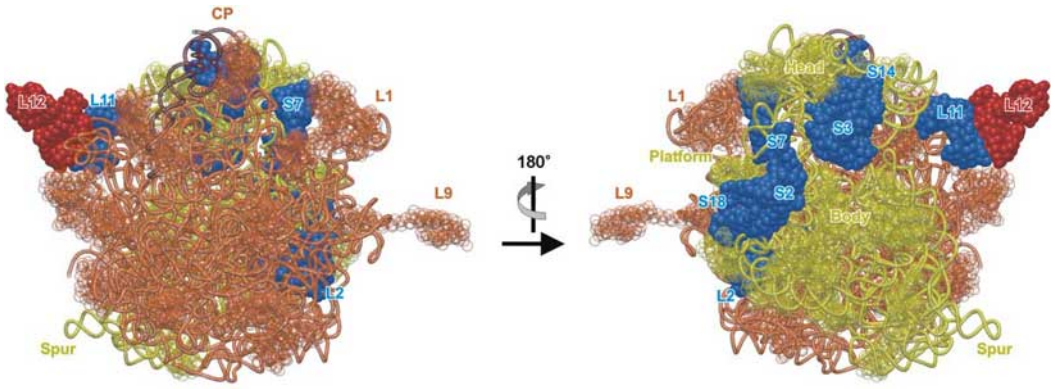

Fig. (4). Views from two opposite directions on the *Thermus* 70S ribosome (coordinates from [20]) to display r-proteins (blue) with which L12 (red) could be crosslinked. Small subunit components – yellow, large subunit components– brown, 5S rRNA – dark brown. Cα-positions of r-proteins are shown as semitransparent balls, rRNAs as semitransparent tubes. Relevant proteins are labeled. Some morphological landmarks are indicated. CP – central protuberance.

another may take over dynamic functions during translation [16, 111, 165]. In agreement, some researchers suggested that the stalk comprises a low-affinity binding site for only a single L12 dimer [145, 150]. These conclusions are corroborated by a recent mapping of the *E. coli* L12 CTDs on the 70S particles by cryo-EM [55]. These studies saw the domain at four different positions, in agreement with the diverse crosslinks. These positions were not visited all at the same time, consistent with a dynamic role in translation. Three of the sites are in a distance of 80 – 90Å from the stalk position of L12, which, as pointed out by the authors, could be covered by the combined length of the CTD and an extended hinge.

EXTRARIBOSOMAL FUNCTIONS AND RELATED PROTEINS

r-proteins are now well acknowledged for their structural and possibly functional roles within the ribosome. However, it is less appreciated that some of these proteins serve crucial extra-ribosomal purposes as well [166, 167]. L12 alone does not seem to take over known functions outside the ribosomal framework. For P2-like proteins it has been observed that they can serve as intracellular iron binders [168] although a physiological relevance has not been proven. Isolated L10 and the L10•$(L12)_4$ complex are involved in the translational regulation of their own operon [166, 169] as also known for other bacterial r-protein operons [170, 171]. Usually a rRNA-binding member of the operon is recruited to associate with a cognate element on the mRNA [172]. The association may then directly or indirectly sequester mRNA sequences important for ribosome entry or initiation of translation. Furthermore the L10-analog P0 has been shown to harbor an apurinic/apyrimidinic endonuclease activity functioning in DNA repair [173-175].

The L12 fold, especially that of the CTD, seems to be a popular motif for diverse cellular functions. It has already been pointed out that the CTD structure is related to the RRM fold of RNA binding proteins. The completely unrelated sequence of the ovomucoid proteinase inhibitor has been found to adopt an almost identical conformation, stabilized by three disulfide bridges [176, 177]. Another curiosity, a bacterial 3α-hydroxysteroid dehydrogenase from *Comamonas testosteroni* is homologous to a fusion of bacterial L10 and L12 genes [178, 179]. There is no indication, however, that the ribosomal stalk proteins harbor any of the latter inhibitory or enzymatic functions. Some of the latter observations may indicate that r-proteins, in this case the stalk proteins, may represent a primordial pool of folds which were incorporated by genetic mechanisms into other proteins with a subsequent diversification in their functions.

Finally, it should be mentioned that proteins P1/P2 are strongly autoimmunogenic. They are important players in the ethiology and pathogenesis of systemic *lupus erythematosus* as judged from the high anti-P1/P2 antibody titers in the serum of 10% of these patients [180].

PROLOGUE

The intensive research on L12 and the related archaeal/eukaryotic proteins has produced numerous seemingly conflicting observations. The most prevalent unresolved questions include the relevance of dimer *vs.* tetramer formation, possible differences in the structures of isolated and ribosome-bound L12 complexes, the atomic details of the L10•$(L12)_4$ interaction, the mode of interaction of L12 with elongation factors, the mechanism of stimulation of the GTPase activity of supernatant factors, and the dynamic functions of L12.

L12 has once been compared to a 'gate keeper' at the entrance to the ribosome but the questions remain for what exactly and how. The protein is able to directly or indirectly offer a water molecule to GTP held tightly by the translation factor GTPases. L12 is also an exceptional motile protein. X-ray crystallography and biochemical experiments tell that this acidic Ala/Gly-rich protein can undergo a transition from a state with a flexible hinge in the middle to one in which the hinge curls up into an assembly of α-helices. The situation reminds a little of poly-Ala that also builds up α-helices that become marginally unstable on addition of charged side chains like Glu [181]. In general, transient interactions between proteins, between proteins and RNA, and between RNAs as they occur on the ribosome remain crucial issues. How they are steered is an important question for the future. Obviously, a structure of an L10•$(L12)_4$ complex is urgently needed to shed light on a deluge of observations in the field. It has been shown two decades ago that such complexes can be crystallized and yield useful diffraction data [182].

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ABBREVIATIONS

a = Archaeal

CTD = C-terminal domain

DNA = Deoxyribonculeic acid

e = Eukaryotic

EF = Elongation factor

FRET = Fluorescence resonance energy transfer

GAP = GTPase activating protein

GDP = Guanine nucleotide diphosphate

GTP = Guanine nucleotide triphosphate

IF = Initiation factor

L = Ribosomal protein from the large subunit

NMR = Nuclear magnetic resonance

NTD = N-terminal domain

P = Phosphorylated ribosomal proteins

r = Ribosomal

RNA = Ribonucleic acid

RF = Release factor

S = Ribosomal protein from the small subunit

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