The Ribosomal Stalk Binds to Translation Factors IF2, EF-Tu, EF-G and RF3 via a Conserved Region of the L12 C-terminal Domain

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Efficient protein synthesis in bacteria requires initiation factor 2 (IF2), elongation factors Tu (EF-Tu) and G (EF-G), and release factor 3 (RF3), each of which catalyzes a major step of translation in a GTP-dependent fashion. Previous reports have suggested that recruitment of factors to the ribosome and subsequent GTP hydrolysis involve the dimeric protein L12, which forms a flexible “stalk” on the ribosome. Using heteronuclear NMR spectroscopy we demonstrate that L12 binds directly to the factors IF2, EF-Tu, EF-G, and RF3 from Escherichia coli, and map the region of L12 involved in these interactions. Factor-dependent chemical shift changes show that all four factors bind to the same region of the C-terminal domain of L12. This region includes three strictly conserved residues, K70, L80, and E82, and a set of highly conserved residues, including V66, A67, V68 and G79. Upon factor binding, all NMR signals from the C-terminal domain become broadened beyond detection, while those from the N-terminal domain are virtually unaffected, implying that the C-terminal domain binds to the factor, while the N-terminal domain dimer retains its rotational freedom mediated by the flexible hinge between the two domains. Factor-dependent variations in linewidths further reveal that L12 binds to each factor with a dissociation constant in the millimolar range in solution. These results indicate that the L12-factor complexes will be highly populated on the ribosome, because of the high local concentration of ribosome-bound factor with respect to L12.

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Introduction

Ribosomes are molecular machines, whose flexible components enable the dynamic process of protein synthesis that includes movement along the mRNA, tRNA translocation and peptidyl transfer.¹ In the past few years, structural studies by X-ray crystallography and cryo-electron microscopy (cryo-EM) have revolutionized our understanding of ribosome function at atomic resolution.²-⁹ As increasingly more structural information on the ribosome becomes available, the focus of the field is shifting towards understanding the detailed motions necessary to accomplish translation.¹⁰-¹⁴

As a general rule, G-protein translation factors promote the transitions between different structural states of the ribosome, and these processes often involve GTP hydrolysis in a ribosome-dependent manner. Protein L12 (also denoted L7/L12) is the main component of the prominent “stalk” of the 50S ribosomal subunit in bacteria, and has been implicated as a key player in factor recruitment and factor-dependent GTP hydrolysis,¹⁵-¹⁷ as outlined further below. Functionally analogous proteins are present in eukaryotes and archaea, but these do not
show any amino acid sequence homology to L12.\textsuperscript{18–20} L12 forms a symmetric dimer mediated by its N-terminal domains (NTD), to which each C-terminal domain (CTD) is connected flexibly via an unstructured hinge segment.\textsuperscript{21–23} High-resolution structures are available for the isolated CTD of\textit{Escherichia coli} L12,\textsuperscript{24} as well as for the intact \textit{E. coli} L12 dimer.\textsuperscript{21} L12 is the only multi-copy protein of \textit{Thermotoga maritima} was determined, which re-
vealed that the L12 NTD dimers bind to helix α8 of L10. Apparently, the length of α8 determines the number of L12 dimers that bind to the ribosome in a given organism; helix α8 is longer in \textit{Thermotoga} L10 and contains one additional (third) binding site for a dimer of L12, compared with the case in \textit{E. coli}.\textsuperscript{16} Notably, the L12 dimers are highly disordered in all high-resolution crystal structures of the 70 S or 50 S particles.\textsuperscript{2,6,7,25} However, from cryo-EM reconstructions of the ribosome in various functional states, it appears that the stalk acquires different conformations during translation. Modeling of the L10–(L12<sub>NTD</sub>)\textsubscript{4} complex into the electron densities of different cryo-EM structures suggests that the orientation of helix α8 varies between functional states.\textsuperscript{16} Furthermore, a body of structural and biochemical data has shown that parts of the hinge region and the entire CTD are mobile in ribosome-bound L12,\textsuperscript{2,22,25,26–28} and that its conformation and flexibility are altered dramatically upon binding of the translation factors EF-G\textsuperscript{10,23,29,30} or EF-Tu\textsuperscript{31,32} to the ribosome. Evidence from chemical cross-linking\textsuperscript{33} and immuno-EM\textsuperscript{34} studies shows that the CTD contacts different parts of the ribosome, including the 30 S neck and head regions, as well as a position close to the EF-G binding site near L11.\textsuperscript{35} Thus, there is ample evidence that L12 can attain different conformational states during different steps of translation.

In bacteria translation involves four main G-proteins that catalyze different steps. These are: initiation factor 2 (IF2), which is required for initiation; elongation factor Tu (EF-Tu), which carries amino acid-charged tRNAs to the ribosome; elongation factor G (EF-G), which catalyzes tRNA translocation; and release factor 3 (RF3), which releases the class I release factors after removal of the nascent peptide.\textsuperscript{1} All of these factors possess the well-conserved G-domain, which binds and hydro-
lyzes GTP to carry out their functions, and the succeeding β-barrel domain (domain Ii). It has been shown that L12 alone can stimulate a low level of GTP hydrolysis by EF-G.\textsuperscript{36} Furthermore, ribosomes depleted of L12 are impaired in the GTPase activation of both EF-Tu and EF-G.\textsuperscript{15} The molecular mechanism of this GTPase activation is not fully understood; it has been suggested that L12 increases the catalytic rate of GTP hydrolysis,\textsuperscript{36} or increases the rate of binding EF-G to the ribosome rather than affecting the GTP hydrolysis step \textit{per se},\textsuperscript{11} or controls the release of inorganic pyrophosphate after GTP hydrolysis.\textsuperscript{17} A recent cryo-EM reconstruction of EF-G bound to the ribosome suggests a direct interac-
tion of the CTD of L12 with the G’ domain of EF-G.\textsuperscript{37} There is indirect evidence for interactions between L12 and EF-Tu as well. It has been shown that specific mutations in the L12 CTD affect the binding of the EF-Tu–GTP–aminoacyl-tRNA ternary complex to the ribosome.\textsuperscript{38} These data, together with the structural homology between the L12 CTD and EF-
Ts, have been used to generate a model for the structure of the EF-Tu–L12 complex,\textsuperscript{39} based on the crystal structure of the EF-Tu–EF-Ts complex.\textsuperscript{39} It is an open question whether L12 also stimulates the GTPase activity of IF2 and RF3. A recent cryo-EM structure of the initiation complex shows one L12 CTD intercalated between L11 and the G-domain of IF2.\textsuperscript{9} Because IF2, EF-Tu, EF-G, and RF3 have two homologous domains and occupy overlapping sites on the ribosome,\textsuperscript{9,31,32,40–42} it is expected that ribo-
some-bound L12 can interact with all of these factors.

Here, we demonstrate that L12 binds to IF2, EF-
Tu, EF-G, and RF3, through a strictly conserved region of its CTD. Estimated affinities between the factors and L12 in solution indicate that the corresponding complexes will be highly populated on the ribosome.

**Results**

Using heteronuclear NMR spectroscopy, we investigated the transient interactions between L12 and the translation factors IF2, EF-Tu, EF-G, and RF3, as well as bovine serum albumin (BSA), which served as a negative control. Separate titration series were performed for each of IF2, EF-Tu, EF-G, RF3 and BSA, where the unlabeled protein was titrated into a solution of 15N-labeled L12, and a 1H-15N spectrum was acquired at each titration point. The chemical shift of any amino acid residue is exquisitely sensitive to the magnetic environment of the nuclear spin, and the linewidth depends on the size of the molecular system. Changes in chemical shifts and linewidths provide residue-specific information on changes in the local environ-
ment and on the size of the molecular complex, because each backbone N-H amide group in L12 corresponds to a single cross-peak in the 1H,15N HSQC spectrum. Thus, these data map out which parts of L12 interact with each factor. The response was very similar in all titration series, and followed the general features described in detail below for the interaction between EF-G and L12. The HSQC spectra obtained in the presence of IF-2, EF-Tu, RF3 and BSA are available as Supplementary Data (Figures S1–S4), and the results are summarized below.
Changes in the $^1$H-$^{15}$N HSQC of L12 upon addition of EF-G

Figure 1(a)–(d) show $^1$H-$^{15}$N HSQC spectra of L12, acquired in the presence of various concentrations of EF-G. The spectral changes include both subtle and dramatic effects. At 0.4–0.8 molar equivalents of EF-G (with respect to the L12 dimer), a subset of cross-peaks in the $^1$H-$^{15}$N HSQC of L12 shows gradual changes in chemical shifts from those obtained in the absence of EF-G (Figure 1(a)–(c)). These cross-peaks correspond to residues V66–V68, K70, L80, and E82, which are located on a continuous surface region of the L12-CTD. The chemical shift changes become accentuated with increasing concentrations of EF-G. Concomitantly, all cross-peaks from the CTD become increasingly broadened, and eventually most cross-peaks disappear from the spectrum at the point where 1.2 equivalents of EF-G, with respect to L12 dimer, have been added (Figure 1(d)). In contrast, the cross-peaks from the hinge region and the NTD remain virtually unaffected by the presence of EF-G. These differential changes in line broadening serve as an important verification of binding, as explained in detail below (see Binding affinities). The present results clearly establish that factor-bound and free L12 are in fast to intermediate exchange, because (i) the chemical shifts of the residues in the binding interface change gradually upon addition of factor, and (ii) the CTD cross-peaks disappear well before the addition of one equivalent of factor with respect to L12 monomer, which indicates that each L12 CTD is exchanging between free and factor-bound states within the time-scale of the HSQC experiment.

Interactions between translation factors and L12

Figure 2 presents close-up views of a representative set of residues in L12 that experience chemical shift changes upon addition of factors. In addition, Figure 2 includes one representative residue from each of the NTD and CTD (M17 and K108, respectively), whose chemical shifts do not change.

Figure 2(a) shows the chemical shift changes observed for residues K70 (left-hand panel) and L80 (middle panel) during titration with IF2. Comparisons with residues M17 and K108 (right-hand panel) demonstrate clearly that the observed chemical shift changes are significant, even though these are relatively small (∼0.04 ppm in the $^1$H dimension). Figure 3(a) summarizes the factor-dependent chemical shift changes in L12 upon addition of IF2. In the IF2–L12 complex, the largest chemical shift perturbations occur for residues K70, L80 and E82, with minor changes observed for the neighboring residues V66 and V68, and also for V20 in the NTD.

Figure 2(b) shows the behavior of residues V66 and E82 during titration with EF-Tu, and Figure 3(b) summarizes the data for all residues. The largest chemical shift changes are observed for residue E82, with smaller changes observed for V66 and V68. In
this dataset, there is a drift in pH with factor addition (presumably due to insufficient buffering capacity of the solutions) that results in a change in chemical shift across the entire sequence, as exemplified by residues M17 and K108 in the right-hand panel of Figure 2(b). The magnitude, but not the direction, of the chemical shift changes due to the drift in pH varies between residues, thus making this data set noisier than the others. However, the chemical shift changes associated with the pH drift do not mask those associated with factor binding, as evident from Figure 3(b).

Figure 2(c) shows expanded regions of the spectra displayed in Figure 1, highlighting the titrating chemical shifts of residues V67 and L80 upon addition of EF-G. Figure 3(c) summarizes the quantitative analysis of the data obtained for the interaction between EF-G and L12. Again, the largest shift changes in L12 induced by the addition of EF-G are observed for residues V66–V68, K70, L80, and E82.

Figure 2(d) shows the chemical shift titrations displayed by residues G79 and L80 upon addition of RF3 to L12. The largest chemical shift perturbations occur for residues L80 and E82, with minor changes observed for E27 and G79 (Figure 3(d)). In this case, the chemical shift change for E27 in the NTD is clearly significant, but most likely due to titration of a nearby charged group, e.g. the carboxylate side-chain, rather than to interactions with RF3. This issue is further addressed below.
Control experiment: titration of L12 with BSA

To verify that the identified interactions between the factors and L12 do not reflect promiscuous binding due to e.g. hydrophobic effects, we performed a negative control experiment by titrating L12 with BSA. Upon addition of BSA to L12, the largest chemical shift perturbation occurs for residue E27 in the NTD (Figure 3(e)), paralleling the observations for RF3. There was no significant chemical shift change in the CTD upon addition of BSA.

Non-specific chemical shift changes

In each titration series, minor chemical shift changes appear for a number of residues in the NTD (Figure 3). In the majority of cases, these changes are insignificant and are simply due to noise, but in a few cases the changes are significant. These aberrant results can be explained by minor changes in salt concentration, buffering capacity, or electrostatic screening due to altered concentrations of protein throughout the titration series. However, these caveats do not pose any problem when interpreting the data in terms of factor binding, because the linewidths serve as a separate control of factor interactions, as explained below.

Differential line broadening upon factor binding

Factor binding leads to uniform broadening (decrease in peak height) of all cross-peaks from the CTD, while the linewidths of the cross-peaks from the NTD and the hinge region remain unaffected (Figure 4). This demonstrates that the CTD attains the slow rotational correlation time characteristic of a large molecular mass complex. In contrast, the NTD, which is attached flexibly to the CTD via the hinge region, experiences virtually the same degree of rapid rotational averaging as in the free state. These results provide firm evidence that the CTD binds to the G-protein factors, whereas the NTD does not. Importantly, the differential line broadening thus serves as an independent verification of factor binding, and enables us to discriminate chemical shift changes that are due to factor binding from those that result from non-specific interactions or electrostatic effects due to titrating groups. Both in the absence and in the presence of factors, a single set of cross-peaks is observed from the two copies of the L12 dimer, demonstrating that at each titration point the two CTDs experience the same magnetic environment on average. This fact reflects fast exchange between the free and factor-bound states.

Figure 3. Factor-dependent chemical shift changes, $\delta \omega / \delta x$, plotted versus residue number. (a) IF2, (b) EF-Tu, (c) EF-G, (d) RF3, (e) BSA. The chemical shift change was evaluated as the slope, $\delta \omega / \delta x$, of a straight line fit to the data pairs $(\omega, x)$, where $\omega$ is the chemical shift and $x$ is the molar ratio of factor versus dimeric L12. Cross-peaks are color-coded according to the domain structure of L12: green, NTD; red, hinge region; blue, CTD. Chemical shift changes for $^1$H and $^{15}$N are represented by boxes and circles, respectively. Values for $^{15}$N were scaled by a factor of 0.2 to make them comparable to $^1$H values. Note that the vertical scale differs between panels.
and equal probability of factor binding for the two CTDs of the symmetric dimer. Thus, the present results do not indicate that two CTDs bind simultaneously to one factor molecule. Furthermore, it should be noted that addition of BSA to L12 does not result in any additional line broadening (Figure 4(e)) clearly indicating that the observed interactions between L12 and the translation factors are specific.

**Binding affinities**

The factor-dependent line broadening of L12 enables us to estimate the affinity between L12 and each G-protein factor. The linewidths of the cross-peaks from the free state contain contributions due to relaxation in the bound state. It should be noted that chemical exchange does not contribute to the linewidths for the large majority of residues, which do not exhibit chemical shift changes upon binding of the CTD to the factor. Using a two-state model (free and factor-bound states), approximate transverse relaxation rates were calculated for each of the concentrations used in the experimental titrations. Dissociation constants ($K_D$) were obtained by iteratively fitting Equations (1)–(4) to the experimental data for the CTD of L12, shown in Figure 5. These fits are constrained by the transverse relaxation rates calculated on the basis of the rotational diffusion correlation times of the free state ($\tau_{c,L12}$), which is known from experiment, and the factor-bound state ($\tau_{c,F}$), which is estimated from an empirical relation between $\tau_c$ and the molecular mass of the complex. The results are presented in Table 1. In each case, the factor-dependent linewidths of residues in the L12 CTD correspond to a $K_D$ value in the millimolar range. It should be noted that these $K_D$ values are rough estimates that depend on the estimated $\tau_{c,F}$ of the bound states, which are subject to systematic errors. The errors in $K_D$ reported in Table I are the standard asymptotic errors of the fits, which reflect the quality of the fits, but do not include systematic errors. Plausible sources of systematic errors in $\tau_{c,F}$ include significant inter-domain motions or rotational diffusion anisotropy. We evaluated the effects of systematic errors in $\tau_{c,F}$ on the fitted value of $K_D$: an error of ±20% in $\tau_{c,F}$ corresponds to an error of ±50% in $K_D$, with shorter correlation times yielding lower values of $K_D$ (i.e. higher affinities). Overall, the values of $K_D$ estimated here should be accurate to within one order of magnitude.

**Discussion**

The CTD of ribosome-bound L12 displays virtually the same degree of flexibility as in the free state. Thus, our present results on the L12-factor

![Figure 4] Cross-peak intensities of residues in L12 plotted versus residue number. Data are shown for varying molar ratios ($x$) of factor to L12 dimer, with symbols according to increasing value of $x$ in the order blue diamond, red square, yellow triangle, green circle, and purple cross. (a) IF2, $x=0$, 0.4, 0.8, 1.2, 1.6. (b) EF-Tu, $x=0$, 0.1, 0.2, 0.3, 0.4. (c) EF-G, $x=0$, 0.4, 0.8, 1.2, 1.6. (d) RF3, $x=0$, 0.4, 0.8, 1.1, 1.7. (e) BSA, $x=0$, 0.4, 0.8, 0.9, 1.3, 1.8, 2.2. Cross-peak intensities were evaluated as peak heights. Data were normalized using the N-terminal residue, which is considered unaffected by factor binding. The domain structure of L12 is indicated by the colored bar above the x-axis: green, NTD; red, hinge region; blue, CTD.
complexes in solution are expected to serve as valid models for the corresponding interactions on the ribosome. Previous reports have shown that the translational GTPases bind to overlapping sites near the base of the L12 stalk on the ribosome.9,31,32,40–42 Our present results show clearly that the translation factors also share an overlapping interaction surface on the CTD of L12. Figure 6 highlights in red the residues in the L12 CTD that exhibit significant chemical shift changes upon factor binding. Yellow color indicates those residues that are unaffected by factor addition, as well as a single residue (K65) for which data are missing due to spectral overlap. In each case, the chemical shift perturbations pinpoint the same helix-loop-helix motif (Figure 6, left-hand panels) in the CTD of L12. In general, the major effects are observed for residues L80 and E82, located at the N-terminal tip of helix 5, and for V66, V68 and K70 in the N-terminal half of helix 4. As evident from the right-hand panels of Figure 6, the identified residues define a continuous surface on the L12 CTD, except for the interaction with EF-Tu. Notably, the residues forming the identified binding surface are highly conserved (Supplementary Data Figure S5), and match partly with those (K65, V66, I69, K70, R73, and K84) identified by site-directed mutagenesis to interact with EF-Tu and EF-G.17,38 The apparent differences between the binding surfaces for the different factors may suggest that the detailed interactions vary slightly between the different L12-factor complexes. However, this conclusion cannot be reached at present, because differences among the factors in their amino acid composition of the binding surface can potentially generate different chemical shift changes on L12, even if the binding surface of the latter is identical in all four cases. The same argument also rationalizes the apparent discrepancies between the present results and those derived from site-directed mutagenesis.17,38 Furthermore, the mutagenesis studies do not detect the binding interface between

Table 1. Dissociation constants for the interactions between translation factors and L12

<table>
<thead>
<tr>
<th>Translation factor</th>
<th>$K_D$ (mM)</th>
</tr>
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<tbody>
<tr>
<td>IF-2</td>
<td>2.5±0.3</td>
</tr>
<tr>
<td>EF-Tu</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>EF-G</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>RF3</td>
<td>0.4±0.1</td>
</tr>
</tbody>
</table>

The reported errors in the fitted values of $K_D$ were evaluated as the standard asymptotic error of the fit. Systematic errors are not included; see the text for a detailed discussion of these.
Figure 6. Residues forming the factor-binding surface on the CTD of L12. Residues with significant chemical shift perturbations are colored red and labeled with residue number. The left-hand panels show ribbon diagrams of the backbone trace, while the right-hand panels show the contact surface. Binding surfaces are shown of (a) IF2, (b) EF-Tu, (c) EF-G, and (d) RF3. This Figure was prepared using Molmol.54
L12 and the factor directly, but rather report the effects on binding that a given mutation exerts, which may be indirect. Thus, despite different experimental conditions, the two methods confirm each other by identifying largely the same binding surface. The present NMR data further generalize these observations by demonstrating that all the major translational G-proteins interact with the CTD of L12 in essentially the same way.

Early studies recognized that the identified region on L12 was of potential functional importance.24 The fact that a conserved region of L12 interacts with four different translational GTPases suggests directly that the latter share a common, and possibly conserved, structural feature involved in the interaction. Recent cryo-EM results suggested that the CTD of L12 interacts with the G' domain of EF-G.37 In light of our results, the conclusion from the cryo-EM study is unexpected, because the G' domain is quite unique to EF-G. By contrast, all four translation factors share the G domain as well as domain II. Helix D of the G domain in EF-Tu is involved in binding EF-Ts, and is most likely to interact with L12, as demonstrated previously.58 Our present results identifying the same binding surface for four G-factors on L12 strengthen the view that only a conserved domain on the G-factors would interact with L12, which remains to be verified experimentally.

The present data yielded estimates of the binding affinities between L12 dimer and each factor, which range between 0.2 mM and 2.5 mM (Table 1). It should be noted that the estimated values of K_D are approximate because they were derived using a simplistic analysis, as discussed above. Previous studies have determined the dissociation constant for the EF-Tu–L12 complex to be 10 μM, which is in reasonable agreement with the value determined here, considering that the previous study was performed in the presence of GTP. The millimolar dissociation constants should be compared to the local concentration of factor once it is bound on the ribosome, which is of the order of at least 1 mM, suggesting that the L12-factor complexes will be highly populated on the ribosome. It seems unlikely that all copies of L12 interact with the G-protein factor that is bound to the ribosome. Indeed, the present identification of a single factor-binding site on the L12 CTD suggests strongly that a single CTD binds to the ribosome-bound factor at a given time. The other copies of L12 could potentially interact with G-protein factors not yet positioned in their final binding sites on the ribosome.

The present experiments were performed with samples prepared in polymix buffer, without added guanine nucleotides. Under these conditions, IF2 and EF-G are most likely free of nucleotides, whereas EF-Tu and RF3 contain bound GDP, as a consequence of their high affinities for GDP in the free (non-ribosome-bound) state, with K_D values in the nanomolar range.56 The present conditions thus represent the situations where the factors are in their resting states. To this extent, our results implicate a role for L12 in the recruitment of factors to the ribosome, prior to factor activation by GTP binding. Addressing the potential effects of L12 on GTP hydrolysis requires that the factors be in the GTP-bound state. It is conceivable that the interaction and affinity between L12 and a translational G-factor depend on whether the factor is bound to GTP or GDP. Comparative analyses of each factor in different states (free and nucleotide-bound) will be the subject of future studies.

In conclusion, we have demonstrated that the CTD of L12 binds directly to the G-protein translation factors IF2, EF-Tu, EF-G, and RF3 using a conserved binding surface. The novel observations presented here should provide important points of reference for model building of complexes between L12 and IF2, EF-Tu, EF-G, and RF3, as well as for detailed interpretation of three-dimensional reconstructions based on cryo-EM images.

Materials and Methods

NMR sample preparation

The 15N-labeled ribosomal protein L12 from E. coli was produced and purified as described.22 The NMR samples contained L12 in polymix buffer at pH 6.8. The concentration of the 15N-labeled L12 sample was approximately 0.5 mM, as measured by the Bradford assay.

Expression and purification of G-protein factors

His-tagged factors IF2, EF-Tu and EF-G were overexpressed in E. coli BL21(DE3). The plasmids carrying His-tagged IF2 and EF-Tu were a kind gift from A. C. Forster.7 The His-tagged proteins were purified on a Ni-NTA column using an imidazole elution gradient. The purity of EF-Tu was greater than 99% after this single purification step. EF-G and IF2 were further purified on a QFF column using a NaCl elution gradient. Protein solutions were concentrated using Ultra-spin concentrators (Amicon). E. coli RF3 cloned in the plasmid pOSEX3 was overexpressed in E. coli MKH13 by salt induction and purified as described.59 All factors were dialyzed against polymix buffer and stored at −80 °C. Due to their limited stability in the absence of GDP, both EF-Tu and RF3 were purified in the GDP-bound state.

NMR spectroscopy

All experiments were performed at 30.0(±0.1) °C on a Varian Unity Inova 600 MHz spectrometer equipped with an inverse broadband probehead and single-axis pulsed-field gradient capabilities.1H–15N HSQC reference spectra were typically recorded with spectral widths of 8000 Hz, sampled over 512 complex points in the 1H dimension, and 1650 Hz, sampled over 128 complex points in the 15N dimension. Chemical shift assignments of L12 were obtained as described.22,50,51 15N-labeled L12 was titrated with each of the proteins IF2, EF-Tu, EF-G, RF3 and BSA. Each titration series were typically included five additions of protein and a 1H–15N HSQC spectrum was acquired at each titration point. Spectra were thus obtained for samples with molar ratios...
of factor to L12-dimer of $x = 0, 0.4, 0.8, 1.2$ and $1.6$ for IF2; $0, 0.1, 0.2, 0.3$ and $0.4$ for EF-Tu; $0, 0.4, 0.8, 1.2$ and $1.8$ for EF-G; $0, 0.4, 0.8, 1.1$ and $1.7$ for RF3; and $0, 0.4, 0.9, 1.3, 1.8$ and $2.2$ for BSA.

**NMR data processing and analysis**

All data were processed using nmrPipe. The raw data were apodized using a shifted sine bell in both the $^1H$ and $^{15}N$ dimensions. The final size of each matrix was $2048 \times 256$ real points after zero filling and Fourier transformation. Cross-peak positions and intensities were evaluated using CCPN analysis, and linewidths were extracted using nmrPipe.

**Determination of dissociation constants**

The linewidths of the L12 CTD depend on the relative populations of free and factor-bound CTDs. Hence, these data report on the binding affinities between L12 and the factor in question. The dissociation constant is given by the equilibrium concentrations of factor-bound and free L12 dimer:

$$K_D = \frac{[L12][F]}{[L12:F]}$$  \hspace{1cm} (1)

in which $[L12]$ denotes the concentration of free L12 dimer, $[F]$ is the factor concentration and $[L12:F]$ is the concentration of factor-bound L12 dimer. Equation (1) is based on the assumption that a single factor molecule binds to each dimer of L12. Assuming further that factor binding involves a single CTD in each L12 dimer, the relative populations of free and factor-bound CTDs are given by:

$$p_F = \frac{2[L12] + [L12:F]}{2([L12] + [L12:F])}$$  \hspace{1cm} (2a)

$$p_B = \frac{[L12:F]}{2([L12] + [L12:F])}$$  \hspace{1cm} (2b)

Thus, $K_D$ can be expressed in terms of the relative populations of free and factor-bound L12 CTDs:

$$K_D = \frac{(2p_F - 1)[F]}{2p_B}$$  \hspace{1cm} (3)

and $[F]$ can be expressed in terms of $K_D$ and the total concentrations of L12 and factor. If the exchange rate between the bound state and the free state is fast, the resulting linewidths can be approximated as:

$$R_2 = A(p_F(\tau_{c,L12} + \tau_{c,F}) + p_F\tau_{c,L12})$$  \hspace{1cm} (4)

where $\tau_{c,L12}$ and $\tau_{c,F}$ are the correlation times for rotational diffusion of L12 and free translation factor, respectively. $A$ is a residue-specific scaling factor that takes into account local variations in the relaxation rate, which are caused by a range of factors, e.g. fast internal fluctuations, the density of surrounding $^1H$ spins, etc. Equation (4) is based on the assumption that the single non-bound CTD of the L12 dimer in the factor-bound state contributes in the same way to the linewidth (i.e. has the same relaxation properties) as each of the two CTDs in the free state. For each factor, the value of $\tau_{c,F}$ was approximated from an empirical relation, scaled to the present temperature and water viscosity using Stokes’ law. The molecular masses are: IF2, 97350 Da; EF-Tu, 43182 Da; EF-G, 77450 Da; and RF3, 59443 Da; yielding approximate values of $\tau_{c,F}$ of: IF2, 49 ns; EF-Tu, 22 ns; EF-G, 40 ns; and RF3, 30 ns. The rotational diffusion correlation time for the L12 CTD in the free state is $\tau_{c,L12} = 5.9$ ns. 

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**Supplementary Data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2006.10.025

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