Developing NMR methods for macromolecular machines: Measurement of residual dipolar couplings to probe dynamical regions of the ribosome

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We describe an NMR approach based on the measurement of residual dipolar couplings (RDCs) to probe the structural and motional properties of the dynamical regions of the ribosome. Alignment of intact 70S ribosomes in filamentous bacteriophage enabled measurement of RDCs in the stalk protein bL12, and this direct structural information was used to refine a 3D structure of its C-terminal domain (CTD). Orientational constraints on the CTD alignment arising from the semiflexible linker were further probed by a paramagnetic alignment strategy, and provided direct experimental validation of a structural ensemble previously derived from SAXS and NMR relaxation measurements. Our results demonstrate the prospect of better characterising dynamical and functional regions of more challenging macromolecular machines and systems, for example ribosome–nascent chain complexes.

NMR | ribosome | residual dipolar coulings | bL12 | lanthanide binding tag

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In recent years, X-ray crystallography and cryo-electron microscopy (cryo-EM) have elucidated high-resolution structures of ribosome particles, revealing intricate mechanistic details of their function during the translation process (1, 2). In parallel, NMR-based observations of nascent polypeptide chains emerging from the ribosome are providing unique structural and mechanistic insights into co-translational folding processes (3, 4). In order to further develop solution-state NMR as a technique for structural studies of dynamic regions within large complexes, we have explored the measurement of residual dipolar couplings (RDCs) within intact ribosomes, focusing in particular on the mobile bL12 protein from the GTPase-associated region (GAR) of the prokaryotic 70S ribosome. RDCs have been used to characterise other macromolecular machines and assemblies, including HIV-1 capsid protein, bacterial Enzyme I, and the 20S proteasome (5–7). These developments are particularly relevant as macromolecular complexes tend to exhibit a wide variety of functional motions that are challenging to characterise by standard methods.

The GAR is a highly conserved region of both prokaryotic and eukaryotic ribosomes involved in the recruitment and stimulation of the GTPase activity of auxiliary factors associated with the key steps of translation (8). The prokaryotic GAR includes helices 42–44 and 95 of the 23S rRNA, and ribosomal proteins bL10, bL11 and bL12 (Figure 1a). bL12 is unique among the ribosomal proteins in being present in multiple copies – four copies (two dimers) on the E. coli ribosome (9) – and has a mobile C-terminal domain (CTD) connected to the ribosome-bound N-terminal domain (NTD) via a disordered linker. This flexibility, which has been proposed to facilitate the recruitment of translation factors from the cellular space to the 30S/50S interface (8, 10, 11), has precluded structure determination by cryo-EM and X-ray crystallography. Solution-state NMR spectroscopy is therefore uniquely suited to the characterisation of this region. Indeed, ^{1}H,^{15}N-correlation spectra of 70S ribosomes are found to contain resonances arising almost exclusively from the bL12 stalk. Previous studies have found that the structure of the CTD on the ribosome is indistinguishable from that in isolation, and that only two of the four copies are mobile on the NMR timescale (12–14).

In this work, we continue our NMR investigations of bL12 by measurement of residual dipolar couplings (RDCs) for intact 70S ribosomes. RDCs can be measured under weakly anisotropic solution conditions and report on both the local structure of the protein and the overall orientation of domains with respect to a laboratory frame of reference (15, 16). As such, they are useful sources of distance-independent structural information that are highly complementary to chemical shifts, scalar couplings and NOEs. Anisotropy can be induced by a variety of steric and electrostatic methods including liquid crystals, bicelles, bacteriophage and stretched polyacrylamide gels (17).

We tested the alignment of ribosome samples in Pf1 bacteriophage and $C_{12}E_5$ PEG/hexanol mixtures (18). PEG/hexanol mixtures induced precipitation and inhomogeneous alignment (SI methods), but strong alignment was obtained in Pf1 bacteriophage, which was therefore used for further measurments (Fig. S1). Amide RDCs (D_{NH}) were determined for both isolated and ribosome-bound bL12 by measurement of ^{15}N frequency differences between amide res-

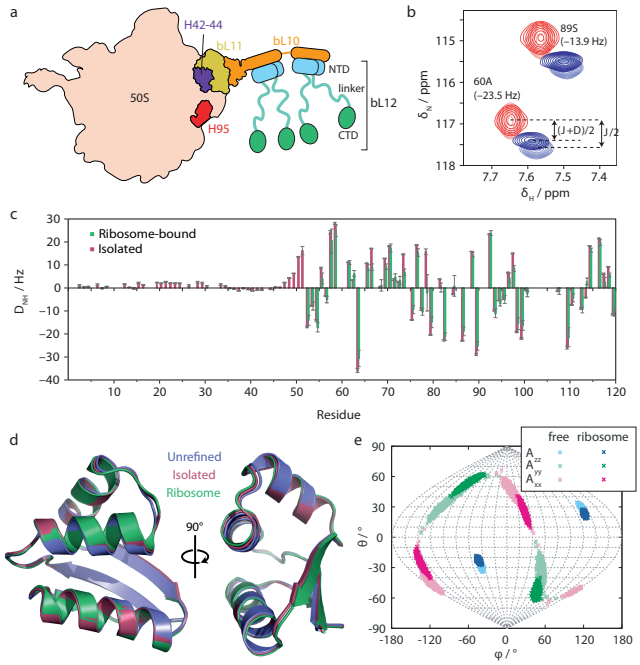

Fig. 1. (a) Schematic illustration of a bacterial ribosome 50S subunit illustrating the position and composition of the stalk region. (b) Excerpt from ^{1}H,^{15}N HSQC (red) and TROSY (blue) spectra of the ribosome-bound bL12 CTD in isotropic and aligned conditions (light and dark colouring, respectively), acquired at 700 MHz and 298 K. (c) Amide RDCs measured for ribosome-bound and isolated bL12. (d) Refinement of the bL12 template structure (PDB code:1rqu) using observed RDCs for isolated and ribosome-bound bL12. (e) Sanson-Flamsteed projection of the principal axes of the alignment tensor calculated for free and ribosome-bound bL12.

onances in ^{1}H,^{15}N HSQC and TROSY spectra (Fig. 1b) (19). We found that this approach provided the greatest precision when compared with alternative methods such as the in-phase/anti-phase approach (Fig. S2). Measurements of additional couplings to C' and Cα atoms were also explored, but the sensitivity was not sufficient to obtain precise results. The integrity of ribosomal samples was monitored with ^{1}H stimulated-echo diffusion measurements, acquired in an interleaved fashion with HSQC and TROSY experiments (Fig. S3).

Despite the 2.4 MDa molecular-weight of the 70S ribosome, and the low sample concentration (10 µM), RDCs could be obtained for the entire CTD of ribosomal bL12 (bL12$_{\mathrm{ribo}}$), with an RMS uncertainty ($\langle\sigma_D^2\rangle^{1/2}$) of 1.8 Hz relative to a range of ±30 Hz (Fig. 1c and SI methods). Notably, despite the multiple copies of bL12 on the ribosome, only a single set of splittings could be resolved, indicating that their conformations and alignment were effectively averaged over the ms chemical-shift timescale. RDCs in the CTD of isolated bL12 (bL12$_{\mathrm{free}}$) were of a similar magnitude, but alignment of the isolated NTD (whose resonances are unobservable in the ribosome-bound form) appeared to be significantly weaker, with RDC values of only a few Hz (Figure 1b). These small RDCs are probably due to weaker interaction between the NTD and the phage (both negatively charged) (20), leading to correspondingly weaker alignment.

The measured RDCs for the CTD were fitted by singular-value-decomposition (SVD) to a template structure (PDB code: 1rqu (21)) to determine the alignment tensor, A (22). The corresponding back-calculated RDCs showed good agreement with observed RDCs for both bL12$_{\mathrm{ribo}}$ and bL12$_{\mathrm{free}}$ (Q factors of 0.30 and 0.25 respectively, Table S1 and Fig. S4), although for both datasets the RMS deviation (RMSD) between measured and back-calculated RDCs was larger than the RMS measurement uncertainty (Table S1). Thus, while the structures of both bL12$_{\mathrm{ribo}}$ and bL12$_{\mathrm{free}}$ CTD are clearly similar to the template structure, some additional information appears to be present. This finding suggests the possibility of using the RDC data to derive improved structures for both bL12$_{\mathrm{ribo}}$ and bL12$_{\mathrm{free}}$.

As N-H RDCs could only be measured in a single alignment medium, structure refinement was restricted to secondary-structure elements (SI methods). This procedure resulted in small changes for both bL12$_{\mathrm{ribo}}$ and bL12$_{\mathrm{free}}$ structures (Fig. 1d), with Cα RMSDs (relative to the template structure) of 0.35 Å and 0.52 Å respectively, while Q factors improved slightly to 0.24 and 0.18 respectively (Table S2). Although not dramatic, this calculation is nevertheless an important step towards the use of RDCs for structural refinement in more complex systems, such as disordered RNCs.

Following refinement, we used the resulting structures to refit alignment tensors to the observed RDCs and so compare the preferred orientations of free and ribosome-bound bL12. The orientations of the principal axes of the alignment tensors can be visualised in a Sanson-Flamsteed projection (Fig. 1e), where numerical uncertainties were calculated using a Monte Carlo approach (23). We observe a small but significant difference in the orientations of the A_{xx} and A_{yy} axes (corresponding to a 5D angle between the alignment tensor components of 13.8 ± 7.6º (24)). This result indicates that the orientation of the bL12$_{\mathrm{ribo}}$ CTD is influenced by the presence of the core ribosome particle, therefore implying that the bL12 linker region is not completely flexible.

To further investigate the properties of the linker, we introduced the surface mutation S89C in the CTD of bL12 in order to incorporate a lanthanide-binding tag (LBT), Tm-DOTA-M8-4R4S (25) and induce paramagnetic alignment of the domain (Fig. 2a). The other domains within the bL12 dimer will therefore only exhibit alignment if there is propagation of the alignment from the tagged CTD via the linker(s). The S89C mutation did not perturb the structure of the domain or dimer formation (as judged by analysis of chemical shift perturbations and diffusion, Fig S5). Two samples were prepared using mixed ^{15}N/LBT labelling schemes to distinguish between direct and indirect domain alignment (Fig. 2a). The directly tagged CTD showed strong alignment (maximum observed RDCs of ca. 20 Hz, Fig. 2b and Table S3) with RDCs that fitted well to the template structure ($Q = 0.15$). Alignment of the NTD, and of the untagged CTD (separated by two linkers from the LBT-CTD), were weaker but non-zero (Fig. 2b and Table S3), indicating that alignment is propagated through the linker region.

The alignment strengths of the different domains can be quantified from the magnitude of alignment tensors determined by SVD fitting of observed RDCs (Fig. 2c). The ratio of alignment magnitudes between adjacent domains then de-

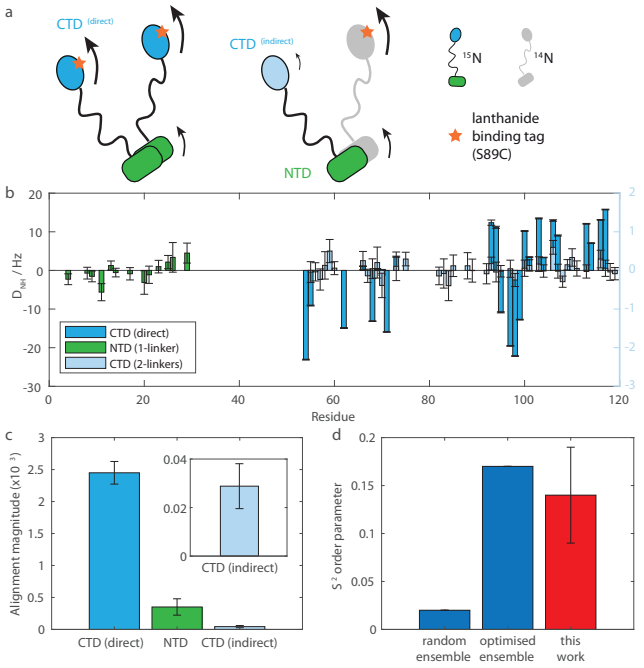

Fig. 2. (a) Mixed ^{15}N/lanthanide-binding-tag labelling schemes for measurement of paramagnetic alignment of domains. (b) Amide RDCs measured for directly and indirectly paramagnetically aligned bL12 domains. (c) Magnitude of fitted alignment tensors for directly and indirectly aligned bL12 domains. (d) bL12 linker order parameters determined experimentally (red) and calculated from random and SAXS/NMR-optimised ensembles (blue) (26).

fines an order parameter, $S^2 = 0.14 \pm 0.05$, describing the degree of coupling through the linker region. This measurement can be compared with previous predictions based upon: (i) a random ensemble with a fully flexible linker, and (ii) an optimised ensemble refined using SAXS and ^{15}N nuclear spin relaxation measurements (Fig. 2d) (26). Our measurements are consistent with the latter ensemble, an interesting feature of which is the anti-correlated extension and retraction of the two CTDs, which potentially might assist in the efficient recruitment of elongation factors to the ribosome.

In summary, we have shown that RDCs can be successfully measured by solution-state NMR spectroscopy for flexible regions of the 2.4 MDa 70S ribosome. The analysis of these measurements indicated a constraining effect of the ribosome on the alignment of the bL12 CTDs, a conclusion that was verified by independent measurements using specific paramagnetic alignment of domains. Importantly, this study has laid the foundation for detailed characterization of modified bL12 variants on the ribosome and also for more detailed structural investigations into the co-translational folding of ribosome–nascent chain complexes (3, 4).

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