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Probing Side-Chain Dynamics of a Ribosome-Bound Nascent Chain Using Methyl NMR Spectroscopy

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Cotranslational folding of a fledgling nascent polypeptide chain is a vectorial process, initiated by the structural information encoded in the N-terminal region of the amino acid sequence that is attached to the ribosome while the remaining C-terminal part is either sequestered in the ribosomal tunnel or yet to be translated. Such a folding process is distinct from the refolding of an isolated protein for which both the N- and C-termini are available to participate in even the earliest steps of the folding process.[1] To explore the nature of cotranslational folding at atomic resolution, we have recently demonstrated that solution state NMR spectroscopy can be used to investigate the structure and dynamics of a ribosome-bound nascent chain (RNC).[2] Despite the requirement for quantities of RNCs that are larger by several orders of magnitude than those required by many other biophysical and biochemical methods,[3] our approach provides a unique opportunity to compare atomic resolution folding initiated while the nascent chain is attached to the ribosome with that occurring *in vitro* as initiated from the denatured state.[4]

Crucial to the success of our efforts,[2] in addition to a sophisticated large-scale sample preparation scheme, has been the recording of SOFAST-HMQC spectra[5] at high magnetic fields; this approach enables the acquisition of sufficient spectral information from a uniformly ^{15}N-labeled RNC sample at a concentration of <10 μM prior to the release of the nascent chain from the ribosome. As the molecular weight of the ribosome is in excess of 2.5 MDa, ^{1}H$-^{13}$C methyl NMR spectroscopy of protein side chains[5] appears an attractive complementary approach to obtaining ^{1}H$-^{15}$N backbone amide correlations as a result of the sensitive enhancement provided by fast methyl group rotation and the equivalence of the three protons. Indeed, a number of near-megadalton protein complexes have now been successfully characterized by methyl NMR spectroscopy at the side-chain level.[6] Moreover, the ^{1}H$-^{13}$C correlations of methyl groups reflect the degree of tertiary structure formation and hence provide a powerful means of defining the details of the folding process.

Here we describe the use of ^{1}H$-^{13}$C methyl SOFAST-HMQC[4] spectroscopy to probe the structure and dynamics of the side chains of the Ig_2-RNC construct we are engaged in studying (the nascent chain consists of two immunoglobulin (Ig) domains, repeats 5 and 6 of the F-actin cross-linker filamin from *Dictyostelium discoideum* (ddFLN), and is designated as ddFLN5 and ddFLN6, respectively, hereafter). Within 3.5 h of acquisition time using a 900 MHz spectrometer equipped with a cryogenic probe, we obtained a ^{1}H$-^{13}$C correlation spectrum with remarkable spectral quality despite the complexity of the system. In line with our previous observations in the main chain spectra,[2] the side-chain spectrum of the ribosome-bound ddFLN5 exhibits a large number of well-resolved resonances that correspond to the folded ddFLN5 along with additional crosspeaks that are intense and poorly dispersed; the latter can be attributed to the methyl-containing residues located in the disordered and truncated ddFLN6 that acts as a linker sequence (Figure 1). The observed chemical shifts of the methyl groups of the ribosome-bound ddFLN5 are found to be virtually identical to those of its isolated counterpart,[7] $\Delta\delta(^{1}\mathrm{H}) = 0.003 \pm 0.014$ ppm, indicating that its overall structure is closely similar. More than one-third ($n = 22$) of the expected 56 ^{1}H$-^{13}$C correlations of the methyl groups in the ribosome-bound ddFLN5 can be resolved by recording only 16 complex data points along the indirect (^{13}C) dimension with a narrow sweep width of less than 15 ppm to focus on the methyl region. The overall signal-to-noise ratio (*S*/*N*) of the resolved methyl signals is ca. 2.5 times higher than that of the backbone amide resonances when identical acquisition parameters are used[8] (see Supporting Information, SI). Side-chain methyl NMR spectroscopy hence affords an opportunity to obtain crucial spectral information from ribosome-bound nascent chains and also from dynamic regions of intact ribosomes that was otherwise inaccessible via main-chain resonances[9] (Figure S2).

Similar to previous observations of the backbone amide resonances,[2,4] the resolved methyl group resonances reveal some degree of differential line broadening (Figure 1a). To ascertain whether or not this effect originates from interactions with the ribosome, we released the nascent chain by incubating the Ig_2-RNC with puromycin.[2] This led to a marginal increase in the overall *S*/*N* ratio without affecting the median value of the observed *S*/*N* ratio. Notably, however, the eight broadest methyl resonances exhibited a considerable increase in their peak heights, resulting in an increase of the average *S*/*N* ratio from 8.9 ± 2.5 to 12.9 ± 3.4, i.e., a reduction in the dynamic range of the side-chain resonances of the nascent chain as a result of its detachment from the ribosome (Figures 1b and S1).

Structural mapping of the sites that exhibit differential line broadening at the side-chain level reveals that the most affected methyl groups cluster in the interior of the domain structure, particularly those of I674, V717, V729, and I738, while most of the unaffected residues correspond to surface side chains (Figure 1c). This result is in clear contrast to our previous finding that those backbone amide groups that undergo marked line broadening when associated with the ribosome are predominantly loop residues colocalized in close proximity to the N-terminus of ddFLN5, a finding we attributed as the result of transient interactions between ddFLN5 and the ribosome surface (Figure 1d).[2,4]

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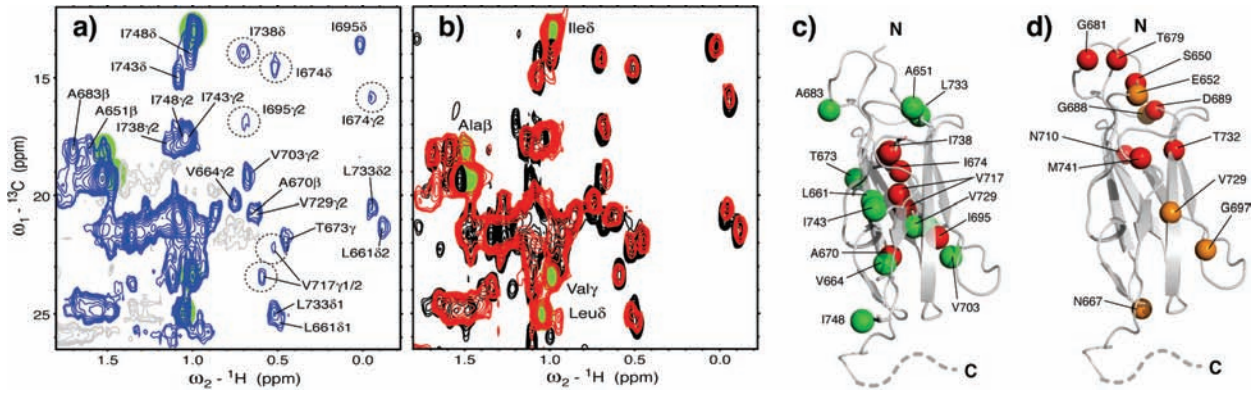

Figure 1. (a) Methyl region of the {^{1}H−^{13}C} SOFAST-HMQC spectrum of the Ig_2-RNC and (b) that of puromycin-released Ig_2 (red) overlaid on the spectrum of isolated ddFLN5 (black). Crosspeaks that show larger line widths in the Ig_2-RNC spectrum in comparison with that of the puromycin-released Ig_2 are indicated with dashed circles and labeled with corresponding atom identities in (a). Additional crosspeaks in the nascent chain spectra are tentatively assigned to amino acid types and indicated in filled green circles. Structural mapping of observed line broadening in side-chain methyl groups (c) and backbone amide groups (d) onto the crystal structure of ddFLN5 (PDB ID: 1QFH).[12] The methyl carbons whose corresponding resonances are broadened or unaffected are shown as red or green spheres, respectively, labeled with their residue identities in (c). (d) Similarly, the nitrogen atoms of those that exhibit significant line broadening or are beyond detection are shown as orange and red spheres, respectively. The N- and C-termini are indicated as is the flexible C-terminal linker that connects the ddFLN5 to the ribosome shown by a dashed gray line.

The difference between the residues that show main chain broadening and those that show side-chain effects illustrates the complementarity between the two structural probes. Of all the residues that have been subject to line-width analysis, V729 is the only residue that shows resolved crosspeaks in both the backbone amide and side-chain methyl NMR spectra, enabling pairwise comparison between the corresponding backbone and side-chain dynamics; such correspondence for other residues is lacking largely due to spectral overlap. In the case of V729, the backbone amide exhibits moderate line broadening (just over the threshold of 1 SD)[2] while the corresponding methyl resonance is not detectably perturbed in comparison with neighboring crosspeaks (V729γ2 in Figure 1a). The previously observed changes in backbone dynamics of the ribosome-bound ddFLN5[2] have been primarily associated with polar groups and glycines in the loop region with V729 and M741 being the only two methyl-containing residues. Also located in the proximity of the N-terminus of ddFLN5, the methyl groups of A651 (flanked by S650 and E652 both of which exhibit amide proton line-broadening) and those of A683 and L733, however, do not exhibit obvious line broadening like their closer neighbors.

While the exact origin of the effect of ribosome attachment on the observed line-broadening of the methyl groups in the interior of the folded ddFLN5 is unknown, it is notable that, in the native ddFLN5 structure, six out of the eight broadened methyl groups are within 3.5 Å of at least one aromatic side chain, while none of the unaffected methyl groups has such close aromatic neighbors.[12] This differential methyl line broadening is likely to arise from the altered ring current effects stemming from these nearby aromatic neighbors within the hydrophobic core of ddFLN5. With an estimated correlation time of 3−5 μs[10] and a mass difference of ca. 200-fold relative to isolated ddFLN5, the attachment to the 2.5 MDa ribosome via the unstructured C-terminal ddFLN6 is likely to generate some degree of entropic force (entropic elasticity) similar to the phenomena observed during mechanical unfolding of proteins at the single molecule level.[11] Such subtle although significant mechanical perturbation imposed by the ribosomal attachment could therefore lead to pronounced fluctuations (breathing motions) within the folded ddFLN5 in response to the "external force", resulting in changes in the aromatic ring-flipping giving rise to line broadening of neighboring methyl groups.

In summary, we have shown that methyl NMR spectroscopy provides improved sensitivity to enable quantitative analysis of resolved side-chain resonances of a ribosome-bound nascent chain. The results presented here provide dynamical information regarding the motional coupling between the nascent chain and the ribosome that is complementary to the information about the dynamics that results from the analysis of the main-chain resonances. The information extracted from the side-chain methyl resonances will be crucial for enabling a detailed structural description of the way a nascent chain folds in a cotranslational manner on the ribosome.

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Supporting Information Available: Detailed description of experimental procedures and additional NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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