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Atomic Force Microscopy captures folded ribosome bound nascent chains

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Abstract

Direct visualization of co-translational folding of nascent polypeptide chains is challenging. Here we present, for the first time, AFM images of large protein constructs based on the membrane binding domain of ankyrin-R, complexed with the ribosome. The characteristic “horse-shoe” shape of ankyrin-R emerging from the ribosome was captured.

Ribosomes are molecular machines responsible for the synthesis of proteins. Prokaryotic ribosomes (70S) consist of a large (50S) and a small (30S) subunit that are composed of complexes of RNAs and proteins. The translation process has been the subject of intense research¹ that has recently included novel single-molecule experimental approaches^{2–4}. The newly translated nascent polypeptide chain (NC) emerges from the ribosome through a long (~10 nm) and narrow (~1–2 nm) exit tunnel situated in the 50S subunit⁵ and needs to fold correctly to be functional. Recent studies using cryo-EM^{6, 7} and NMR⁸ performed on stalled ribosome nascent chain (RNC) complexes suggest that the folding process is concomitant with translation⁹ and that the ribosome is actively involved in co-translational folding of the NC¹⁰. This complex process is believed to be assisted by a number of auxiliary proteins such as trigger factor and various other chaperones^{11, 12}. Here, we used atomic force microscopy (AFM) to directly visualize this process of co-translational folding using a large (132 kDa) polypeptide. For this study we chose to use the membrane binding domain of ankyrin-R¹³. A 12 ANK repeat stack from membrane binding domain of ankyrin-R was shown in X-ray crystallography study to produce a characteristic solenoid-like structure¹⁴. Also, AFM images of 24 ANK repeats of the related protein, ankyrin-B, captured a characteristic “horse-shoe” shape¹⁵ predicted for ankyrin-R¹⁴. Our hypothesis was that ankyrin-R would produce this characteristic horse-shoe pattern when visualized by AFM¹⁵ if the structure folds into its native conformation still bound to the ribosome. Our protein construct (I27₂–AnkR–I27–SecM consists of an N-terminal purification tag (*Strep*-tag), two I27 domains of titin followed by the membrane binding domain of ankyrin-R (24-ANK repeats) with its spectrin binding domain (residues 1–86), followed by a single I27 domain, and the C-terminal SecM^{150–166} “stall sequence” (Fig. S1a, see ESI[†]). SecM stall sequence is commonly used to obtain stable RNCs both *in vitro* and *in vivo*^{16–18}. The C-terminal I27

domain is useful for the current study because it partially fills the exit tunnel and it serves as a part of the linker (together with the included part of spectrin binding domain) to ankyrin-R that physically separates the horseshoe shaped membrane binding domain from ribosomal structure. CD spectra of a similar construct I27₂-AnkR-I27₂ were consistent with a strong α -helical profile of related protein - ankyrin-B¹⁵ (Fig. S1d, see ESI[†]) suggesting, that ankyrin-R folds correctly when flanked by I27 domains. We obtained I27₂-AnkR-I27-SecM-based RNCs (Fig. 1) using the *in vitro* protein synthesis system that is free of trigger factors or any other chaperones. Using an *in vivo* *E. coli* expression system, we produced isolated I27₂-AnkR-I27-SecM polypeptides, which were released from the ribosomal structures using a benzonase/lysozyme based extraction method before purification. In both cases, we confirmed the presence of I27₂-AnkR-I27-SecM protein by SDS-PAGE (Fig. S1b, see ESI[†]). Isolated polypeptides and RNCs were purified using *Strep*-tag spin columns. This purification procedure produced polysomes with bound nascent chains as well as individual RNCs¹⁹ (Fig. 1). Both RNCs and free polypeptides were deposited on a freshly cleaved mica surface and imaged in air by AFM. Ribosomes interact with the surface and overcrowd the substrate immediately after the sample solution is introduced, preventing the successful imaging over time. To overcome these problems, substrates were briefly exposed to the sample and immediately dried for imaging in air with tapping mode. This method proved to be suitable for studies on ribosome structure²⁰ proteins and protein complexes^{21, 22}. Particles present on each imaged surface covered with RNC sample (Fig. 1) were analyzed by means of height to avoid possible artefacts that come from the broadening of the tip over time affecting measurements of parameters such as particle volume. Stability of tips during performed measurements and the size of ribosome complexes prevented the strong influence of tip convolution effect on height measurements²³. As shown on example obtained for one image in figure S2a (see ESI[†]), inspection of the overall particle's height distributions (their position and width) on analyzed surfaces revealed the presence of four distributions with heights centered at around 11 nm, 8.5 nm, 4 nm and 2 nm, that can be attributed to ribosomes (70S), ribosomal subunits (50S, 30S), and proteins present in the reaction mixture, respectively^{20, 24, 25}. Analogically, a control sample was prepared on mica from the *in vitro* reaction mixture lacking plasmid (Fig. S3c, see ESI[†]). Similarly four distributions of particles with different proportions were observed in this case (Fig. S2a, see ESI[†]). In the control sample, there were fewer particles with heights around 11 nm ($n=97$, 16.6% of all particles vs $n=649$, 51.1% of all particles; Fig. S2a, see ESI[†]) but a similar number of particles with heights around 2 nm. These distributions of the particles reflect the effectiveness of sample purification. Fractions eluted from the column should contain only *Strep*-tagged nascent chains and thus, ideally, only ribosome bound nascent chains. However, due to nonspecific interactions with column, we also observe particles in eluted fraction of the control sample (Fig. S2a, see ESI[†]). Concentration of these particles is approximately five times lower (5.5 ± 2.2 ; number of repetitions $n=4$) than in RNC sample (Fig. S1c, see ESI[†]). From comparison of the height distributions of particles in control sample and RNC sample, we can estimate that presence of the 50S and 30S subunits on the surface covered with RNC-eluted sample can be explained partially by particles that bound non-specifically to the column. Also, disruption of the ribosomes during preparation of sample for imaging (drying in particular) is most likely increasing the number of ribosomal subunits on the surface. The proportion of particles with height around 2 nm is almost identical in the control and RNC covered samples, and thus can be attributed to structures non-specifically bound to the *Strep*-Tag spin column and treated as "background". Based on mean height $\pm$ s.d. for each obtained distribution of 70S, we have estimated that, on 32 analyzed $1.5 \mu\text{m}^2$ surfaces, we observe $n=2880$ of ribosomes with the average height of 11.7 ± 1 nm. A small fraction of ribosomes (1.1% of all analyzed 70S) displays a striking horseshoe shaped structural feature (e.g. structures 1 - 5 in figure 1) resembling the released I27₂-AnkR-I27-SecM proteins (compare with Fig. S3, see ESI[†]). This result is not surprising when taking into consideration that previously published RNC purification protocols have

shown the amounts of purified RNCs not exceeding 10% for much shorter polypeptides and these values seem to decrease for larger structures²⁶. However, there is a positive relationship (Fig. S2b, see ESI[†]) between the total number of observed 70S ribosomes and a number of observed RNCs on analyzed surfaces, providing additional information about effectiveness of each sample preparation and its influence on final RNCs yield. In addition it is possible that during the drying process the NCs might detach from ribosomes further decreasing the yield of RNCs. However, it is also possible that the true yield is greater than 1% but that the RNCs are not spread sufficiently on the mica surface to be captured by AFM. Based on visual inspection performed for all of these ribosomes, we have selected 31 particles displaying striking “horse-shoe” shaped feature attributed to the presence of I27–AnkR-I27-SecM ribosome-bound nascent chain for further dimension analysis. AFM images of released I27₂–AnkR-I27-SecM proteins captured examples of the characteristic horse-shoe shape (Fig. S3, see ESI[†]) similar to the AFM-imaged shape of ankyrin-B¹⁵ and consistent with the predicted structure of ankyrin-R¹⁴. These results suggest that ankyrin-R is correctly folded and that dehydration on mica has not significantly altered its shape. The small variations of the location of the horse-shoe shape relative to the ribosome observed in figure 2 can be explained by the flexibility of the rather unstructured polypeptide linker connecting ankyrin R to the ribosome. The size of the horse-shoe structure – end-to-end distance: 17.2 ± 2.6 nm (mean $\pm$ s.d., the number of observations $n=25$; Fig. S3, see ESI[†]) is consistent with the predicted value from the crystal structure¹⁴ of 12 nm for ankyrin-R and AFM-measured value of 12.9 ± 1.2 nm for ankyrin-B¹⁵. The small difference in length can be accounted for by considering the size of flanking I27 domains²⁷ and their position relative to horse-shoe shaped ankyrin-R. A detailed 3D image analysis of nascent chains bound to ribosomes (Fig. 2) determined the size of the horse-shoe shaped structure (Fig. 2c) to be 17.9 ± 1.8 nm ($n=31$) consistent with the data obtained for the released polypeptide. The “N-terminal arm” of the horse-shoe shape (19 nm; average 17.8 ± 1.9 nm; $n=31$) is longer than the “C-terminal arm” (13 nm; average 13.6 ± 1.6 nm; $n=31$). Also, its width in this case (Fig 2c) is greater than the width of the “N-terminal arm”. These observations are consistent with the design of our construct that carries two I27 modules (length 3.4 nm module²⁷) at the N-terminus of ankyrin-R and only a single I27 module at the C-terminus (Fig. S1a, see ESI[†]). Figure 2c also reveals that the horse-shoe shape is connected to the ribosome by a structure that represents a part of spectrin binding domain and one I27 domain (Fig 2b). The spectrin binding domain is believed to be unstructured¹⁴, and the C-terminal I27 module is also expected to be partially unfolded because a C-terminal α -strand, that is critical for I27 folding²⁸, is partially buried in the exit tunnel. Consistent with these predictions, the height of this connecting structure in AFM images is significantly smaller than that of the rest of the protein (e.g. 0.8 nm vs. 2.2 nm, Figure 2c). These structural details visualized using AFM support that we captured nascent chains of ankyrin-R construct emerging from the ribosome exit tunnel. Taken together, these results strongly suggest that ankyrin-R, connected to the ribosomal exit tunnel via I27 and SecM peptides, is folded correctly in the absence of trigger factor and other chaperones.

In conclusion, The AFM images presented here are the first to visualize at submolecular resolution correctly folded large nascent polypeptide chains. AFM imaging of co-translational folding of polypeptides might be a useful tool to characterize large proteins synthesis, especially with the advent of fast AFM²⁹, which may actually allow visualizing of co-translational folding in real time. However, this type of experiment in solution would be possible only if the problem of overcrowding of the surface by ribosomes would be addressed. This would have to involve surface modifications enabling binding of the ribosomes in a monolayer and changing kinetics of the observed expression. At the same time, the approach presented here enables visualization of polysomes with attached nascent chains adsorbed from the bulk solution that is closer to the native conditions, even though the sample has to be dried before the experiment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. AFM images of ribosome bound nascent chains of I27₂-AnkR-I27-SecM. (a) 2D Height images of a typical RNC sample. (b) Magnified and reoriented 3D images of identified RNC structures featuring characteristic horse-shoe shape of AnkR (marked with an arrow).

Fig.2.

Analysis of the geometry of AFM determined 3D structure of ribosome bound nascent chain of I272–AnkR-I27-SecM. (a) Shows a magnified image of the structure 1 from Figure 1b. (b) A cartoon representation of the horse-shoe shaped ankyrin-R attached to the ribosome *via* the spectrin binding domain and I27 module (based on crystallographic data). (c) Dimensions of the “C-terminal arm” (C, 13 nm, blue), “N-terminal arm” (N, 19 nm, green), and the end-to-end distance (16 nm, red) of I272–AnkR- determined by AFM.