

Nonnative Helical Motif in a Chaperone-Bound Protein Fragment

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ABSTRACT The effect of cotranslationally active chaperones on the conformation of incomplete protein chains is poorly understood. The secondary structure of a 77-residue chaperone-bound N-terminal protein fragment corresponding to the first five helices (A–E) of apomyoglobin (apoMb₁₋₇₇) is investigated here at the residue-specific level by multidimensional NMR. The substrate-binding domain of DnaK, DnaK- β , is employed as a chaperone model. By taking advantage of the improved spectral quality resulting from chaperone deuteration, we find that DnaK- β -bound apoMb₁₋₇₇ displays a region of nonnative helicity at residues away from the main chaperone binding site. The nonnative structural motif comprises portions of the native D and E helices and has similar characteristics to the reported nonnative DE helical region of acid-unfolded full-length apoMb. Upon incorporation of the missing C-terminal amino acids, a structural kink develops between residues 56 and 57, and two separate native D and E helices are generated. This work highlights, for the first time to our knowledge, the presence of a nonnative helical motif in a large chaperone-bound protein fragment under physiologically relevant solution conditions.

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Our understanding of how proteins achieve their native structure in buffered solutions has progressed considerably over the past few decades (1). However, we know very little about how proteins fold in the cell, where the presence of additional interacting and noninteracting molecules has the potential of affecting both mechanisms and yields.

A number of recent studies utilizing model systems have targeted the effect of physiologically relevant factors on protein folding, including vectorial chain elongation (2,3), ribosomal tethering (4), molecular chaperones (5), and molecular crowding (6). Biophysical investigations addressing cotranslational protein folding, however, are still scarce. We showed that the folding behavior of N-terminal fragments derived from apomyoglobin (apoMb) is fundamentally different from the *in vitro* refolding of the full-length protein. The N-terminal apoMb polypeptides misfold and self-associate under physiologically relevant conditions (2), suggesting that chaperones and/or the ribosome are needed to prevent the aggregation of apoMb in the cell during translation. Indeed, self-association of the protein fragments is prevented in the presence of DnaK- β , the substrate-binding domain of the cotranslationally active bacterial chaperone Hsp70 (5). More recent multidimensional NMR investigations revealed that misfolding-prone apoMb fragments are held in a globally unfolded conformation retaining some residual secondary structure (7). However, several important conformational details could not be elucidated. Given the incomplete level of information and the fact that related studies only address very small substrates (8,9), it is fair to state that the structural properties of chaperone-bound polypeptides and the effect of chaperones on protein folding (both during and after protein biosynthesis) are still poorly understood.

Here, we provide the first detailed description, to our knowledge, of the residual secondary structure of a chaperone-bound protein fragment whose length largely exceeds that of the known core residues directly in contact with the chaperone (10). Surprisingly, we detect nonnative helical secondary structure for some of the residues away from the chaperone binding site.

The substrate of interest is the 77-residue N-terminal apoMb fragment apoMb₁₋₇₇, which includes amino acids spanning the first five helices of the full-length native protein. ApoMb₁₋₇₇ misfolds and self-associates in isolation, resulting in barely detectable NMR signals (5). In the presence of chaperone, however, most of the expected NMR resonances are retrieved. We selected the substrate-binding domain of DnaK, DnaK- β (5), as model chaperone. At 1:1 molar ratios of apoMb₁₋₇₇:DnaK- β , the protein fragment is in a nearly completely chaperone-bound state (5). The moderate ¹H chemical shift dispersion of DnaK- β -bound apoMb₁₋₇₇ shows lack of an overall tertiary structure. Previously reported initial assignments and corresponding C ^{α} secondary chemical shifts for DnaK- β -bound apoMb₁₋₇₇ lacked information on a significant portion of the polypeptide (7). Practical challenges associated with sample conditions included: 1), mandatory use of low temperature (needed to prevent substrate aggregation); and 2), spin-diffusion across the macromolecular complex. The above challenges led to broad lineshapes, which prevented detailed conformational analysis and hindered the assignment process.

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Here, we resort to deuteration of the DnaK- β chaperone, which partially solves the spin diffusion problem and leads to improved NMR spectral quality. Most importantly, chaperone deuteration enables elucidating novel key structural details on the chaperone-bound substrate.

Despite its improved spectral features, the ^{13}C , ^{15}N -apoMb $_{1-77}$ - ^2H -DnaK- β substrate-chaperone complex defies substrate backbone assignments via the classical suite of triple-resonance experiments. Even TROSY-type strategies at 800 MHz did not lead to higher quality data. Therefore, we resorted to a combination of HNCA and a series of ^1H , ^{15}N HSQC-detected pH titrations to maximize the number of assigned resonances. The HNCA and the starting spectrum of the pH titrations were acquired under the conditions noted in Fig. 1. The pH was then gradually decreased, and multiple spectra were collected at several progressively lower pH values, down to pH 2.5. At the pH titration endpoint, both the chaperone and the fragment are unfolded and no longer bound. Assignments at low pH (S. Rajagopalan, C. Chow, E. C. Fulmer, D. Fedyukina, Y.-J. Eun, and S. Cavagnero, unpublished), where NMR resonances are significantly more intense, were done via conventional NMR methods and then back-traced to higher pH to assign the chaperone-bound apoMb $_{1-77}$ resonances at pH 5.8. The backbone assignments resulting from pH titration were confirmed by the detectable connectivities and partial assignments from the pH 5.8 HNCA, which also provided C^α chemical shifts for the assigned residues.

The above strategy led to backbone H^{N} , N , and C^α assignments for most of the detectable resonances, corresponding to 46 out of 77 residues (12), as shown in Fig. 1. The unassigned residues belong to either amino acids 26–35, previously identified as composing the core of the chaperone-binding

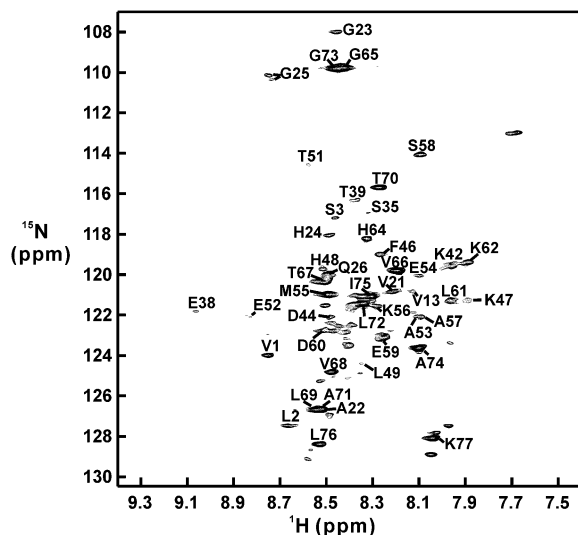


FIGURE 1 ^1H , ^{15}N -HSQC NMR spectrum of apoMb $_{1-77}$ in the presence of deuterated DnaK- β chaperone. Both species were 100 μM in 10 mM sodium acetate at pH 5.8. Data were acquired on a Varian INOVA 600 MHz NMR spectrometer at 4°C. The assigned resonances are labeled.

site (10), or the highly nonpolar residues belonging to the native A helix. The former set of resonances are likely broadened beyond detection because of lack of local mobility at the binding site core of the 23.5 kDa complex at 4°C (see also Supplementary Material). The amino acids belonging to the native A helix are likely exchange-broadened since they are nearly-undetectable at 4°C even in the acid-unfolded state of apoMb $_{1-77}$.

Secondary chemical shifts for the assigned C^α nuclei (Fig. 2 A) were calculated by subtracting residue-specific random coil chemical shifts (13) from the experimentally determined chemical shifts of the relevant nuclei. Backbone chemical shifts are very sensitive to electronic environment and are effective reporters of secondary structure at atomic resolution (13). The helices of full-length native apoMb show large and positive C^α secondary chemical shifts ((14); Fig. 2 B, lower panel).

Remarkably, chaperone-bound apoMb $_{1-77}$ displays non-native helicity across the region comprising the native D and E helices. In addition, the detectable helical profile of chaperone-bound apoMb $_{1-77}$ is similar to that of acid-unfolded apoMb ((15); Fig. 2 B, top panel) except for the approximately twofold larger helicity in the DE region. Either the lower temperature and/or differences in the carboxyl group protonation state of the four acidic residues (Glu-52, Glu-54, Glu-59 and Asp-60) of the apoMb $_{1-77}$ DE region are likely responsible for the increased helicity at pH 5.8.

The short peptide (T51-K63) corresponding to the DE portion of the sequence was reported to be partially helical in

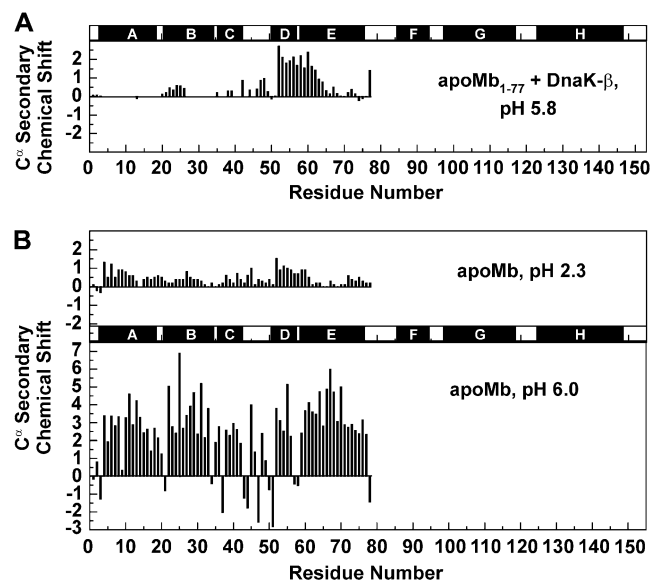


FIGURE 2 (A) C^α secondary chemical shifts of apoMb $_{1-77}$ N-terminal fragment in the presence of deuterated DnaK- β (1:1 molar ratio) at pH 5.8 and 4°C. Chemical shifts were obtained from resonance assignments performed in the presence of deuterated chaperone. NMR experimental details are provided in Supplementary Material. (B) Known C^α secondary chemical shifts of the 1-77 region of acid-unfolded (pH 2.3, 25°C) (15) and native (pH 6.0, 35°C) (14) full-length apoMb.

isolation (16). In addition, the presence of the DE helical motif away from the chaperone binding site and the fact that this motif is also present at low pH in the absence of chaperone suggest that the nonnative structure results from a local intrinsic conformational trend of the DE sequence in the context of apoMb₁₋₇₇.

Fig. 3 provides a schematic representation of the chaperone-bound apoMb₁₋₇₇ conformation supported by this work and a view of apoMb₁₋₇₇ under different experimental conditions. At pH 5.8, apoMb₁₋₇₇ has a tendency to self-associate and aggregate, which can be averted either by a decrease in pH or by complex formation with DnaK- β (5). In both the chaperone-bound and low pH cases, a globally unfolded conformation lacking significant stable tertiary contacts and comprising the nonnative DE residual helical region is generated. We propose that the chaperone-bound state of N-terminal protein fragments may effectively serve as an unfolded state under physiologically relevant conditions, before protein synthesis is complete. The presence of a chaperone-bound complex is important primarily because this state enables the full-length protein to undergo folding and become bioactive within the same medium that ensures minimal aggregation via chaperone-binding while protein synthesis is in progress.

In summary, this work identifies, for the first time to our knowledge, the presence of nonnative helical conformation in a chaperone-bound substrate lacking the C-terminal region. Our previous studies with a small peptide substrate corresponding to the apoMb₁₋₇₇ binding site core (7) revealed that the peptide undergoes local helix unwinding and switches to a more extended conformation upon chaperone binding. Therefore, chaperone-bound apoMb₁₋₇₇ is characterized by two types of nonnative motifs: helical and extended. On incorporation of the missing C-terminal residues, apoMb acquires the ability to reach its folded state, and native D and E helices develop.

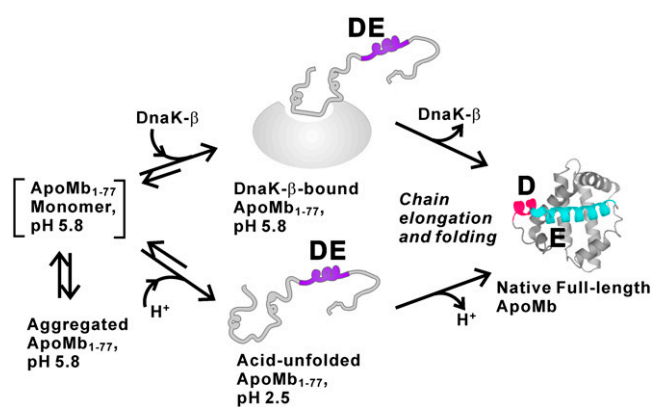


FIGURE 3 Proposed cartoon representation of apoMb₁₋₇₇ conformations populated under different conditions. The species enclosed in square brackets may be metastable and is not detectable in solution. The residual nonnative helicity of DnaK- β -bound apoMb₁₋₇₇ is shown in purple. Upon substrate chain elongation and folding, the DE helical region is replaced by individual D (red) and E (blue) helices.

SUPPLEMENTARY MATERIAL

To view all of the supplemental files associated with this article, visit www.biophysj.org.

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