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The Chaperonin GroEL Binds a Polypeptide in an α-Helical Conformation†

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ABSTRACT: Chaperones facilitate folding and assembly of nascent polypeptides in vivo and prevent aggregation in refolding assays in vitro. A given chaperone acts on a number of different proteins. Thus, chaperones must recognize features present in incompletely folded polypeptide chains and not strictly dependent on primary structural information. We have used transferred nuclear Overhauser effects to demonstrate that the *Escherichia coli* chaperonin GroEL binds to a peptide corresponding to the N-terminal α-helix in rhodanese, a mitochondrial protein whose in vitro refolding is facilitated by addition of GroEL, GroES, and ATP. Furthermore, the peptide, which is unstructured when free in aqueous solution, adopts an α-helical conformation upon binding to GroEL. Modification of the peptide to reduce its intrinsic propensity to take up α-helical structure lowered its affinity for GroEL, but, nonetheless, it could be bound and took up a helical conformation when bound. We propose that GroEL interacts with sequences in an incompletely folded chain that have the potential to adopt an amphipathic α-helix and that the chaperonin binding site promotes formation of a helix.

Folding, assembly, and targeting of nascent chains in vivo require the assistance of factors collectively known as molecular chaperones (Ellis & van der Vies, 1991). Moreover, each chaperone interacts with a number of polypeptides. Key questions about the roles of chaperones that are as yet unanswered are (a) what are the structural features that are recognized by chaperones and must be characteristic of incompletely folded nascent proteins? and (b) what is the mechanism by which chaperones facilitate folding and assembly? In the present study, we have addressed these questions by examining the interaction of GroEL, an *Escherichia coli* chaperonin, with a peptide derived from rhodanese, a protein whose in vitro refolding is greatly enhanced by addition of GroEL with GroES and ATP[1] (Mendoza et al., 1991). We have used the transferred nuclear Overhauser effect (trNOE) method (Clore & Gronenborn, 1982, 1983), which enables demonstration of binding and determination of the conformation of a bound ligand.

GroEL is a member of the class of chaperones known as chaperonin 60s (cpn60s) (Georgopoulos & Ang, 1990). It is a large protein composed of 14 60-kDa subunits arranged in two stacked rings of 7-fold symmetry. It is essential for growth in *E. coli*, and its expression is induced by stress. Functionally, GroEL has been implicated in phage assembly, in protein export, and in DNA replication. GroEL with GroES and ATP has been shown to facilitate efficient refolding in vitro of cyanobacterial RuBisCO (Goloubinoff et al., 1989) and of mitochondrial citrate synthase (Buchner et al., 1991) and rhodanese (Mendoza et al., 1991), which otherwise would irreversibly aggregate under the refolding conditions. GroEL may promote folding by binding intermediates bearing exposed hydrophobic surfaces, but no data have yet been obtained that would shed light on how GroEL recognizes unfolded proteins. We hypothesized that in order for GroEL to bind proteins with widely different sequences, hydrophobic surfaces must be presented in a local conformation that could fit into a binding

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[1] Abbreviations: NMR, nuclear magnetic resonance; 1D NMR, one-dimensional NMR; 2D NMR, two-dimensional NMR; NOE, nuclear Overhauser effect; trNOE, transferred NOE; NOESY, two-dimensional NOE spectroscopy; CD, circular dichroism; ATP, adenosine triphosphate; TFE, 2,2,2-trifluoroethanol; PAL, peptide amide linkage; Fmoc, 9-fluorenylmethoxycarbonyl; TOCSY, two-dimensional total correlation spectroscopy; FID, free induction decay; Tris, tris(hydroxymethyl)aminomethane; BisTris, bis[2-hydroxyethyl]aminotris[hydroxymethyl]methane.

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pocket or groove on GroEL (Landry & Gierasch, 1991). Amphipathic helices are a suitable structural motif for GroEL binding because they are stabilized in interfacial environments, can be formed by sequences in most proteins, and often persist to take part in a protein's native structure. Furthermore, we suggested that the binding motifs for GroEL would exist near the N-terminus of the polypeptide so that the chaperone could function during synthesis. We thus selected a sequence from the protein rhodanese that is near the N-terminus and helical in the native protein as a likely candidate for a GroEL-interactive region. In this report, we show by one- and two-dimensional NMR (1D and 2D NMR) and trNOEs that a 13 amino acid peptide (Ac-STKWLAESVRAGK-NH_2) corresponding to this N-terminal α-helix of rhodanese indeed is bound by GroEL and adopts an α-helical conformation when bound.

Materials and Methods

Peptides were synthesized by standard protocols (Atherton & Sheppard, 1989) on a PAL linker attached to a polystyrene support (Milligen) in a Milligen 9050 continuous-flow peptide synthesizer using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry and acetylated off-line with acetic anhydride prior to cleavage to yield the carboxy-terminal amide. Purification was accomplished by reversed-phase HPLC on a Vydac C18 column by use of an acetonitrile/water gradient with 0.1% trifluoroacetic acid. Purity and identity of the peptide product were checked by amino acid analysis, sequencing, and mass spectrometry.

GroEL was purified from overproducing strain OF225 containing plasmid pOF12 (Fayet et al., 1986) as follows: a soluble lysate of cultures grown at 37 °C to late log phase was prepared by sonication in Tris–saline (50 mM Tris-HCl and 125 mM NaCl, pH 7.5) at 4 °C. Subsequent purification steps were performed at room temperature unless otherwise noted. The lysate was fractionated by ammonium sulfate precipitation; the 1.6–2.2 M cut was resuspended in Tris–saline and heated in 15-mL aliquots for 30 min at 50 °C; insoluble proteins were removed by centrifugation; the supernatant, containing GroEL and GroES, was made 1 mM in ATP and 2.5 mM in Mg^{2+} and applied to a Sephacryl S-400HR gel-filtration column equilibrated in the same buffer solution containing Mg-ATP and 1 mM NaN_3; fractions containing the GroEL/ES complex were pooled, and the proteins were precipitated by addition of an equal volume of saturated ammonium sulfate solution; GroEL and GroES (comprising greater than 95% of the protein) were separated from each other and from remaining proteins by ion-exchange chromatography on a Mono-Q FPLC column (Pharmacia) equilibrated with 20 mM BisTris-HCl and 50 mM KCl, pH 6.0, by using a KCl gradient.

GroEL was concentrated to ca. 1.5 mM monomer by centrifugation in a Centricon-30 ultrafiltration device (Amicon) prior to dilution into one of two identical samples of the peptide; [native peptide] = 2.8 mM or [proline variant] = 3.2 mM. NMR spectra were recorded on a Varian VXR500 NMR spectrometer at 25 °C. Resonances were assigned by using total correlation (TOCSY) and nuclear Overhauser effect (NOESY) 2D spectra, which were obtained with standard pulse schemes and processed with the program FTNMR (Hare Research, Inc., Woodinville, WA) on a Sun4/260 workstation. For NOESY spectra, a total of 256 FIDs were acquired with sweep width 6000 Hz, 2048 points, and 32 scans per FID. The mixing time was either 300 or 50 ms in order to optimize detection of NOEs in the free peptide (short correlation time, slow cross-relaxation) or in the GroEL-bound peptide (long correlation time, fast cross-relaxation). The data were symmetrized for clarity; all of the cross peaks shown were present in unsymmetrized spectra. For 1D spectra, FIDs were acquired with 32 000 points and 64 scans. CD spectra were recorded on an AVIV Model 60DS CD spectrometer, [peptide] = 23 μM.

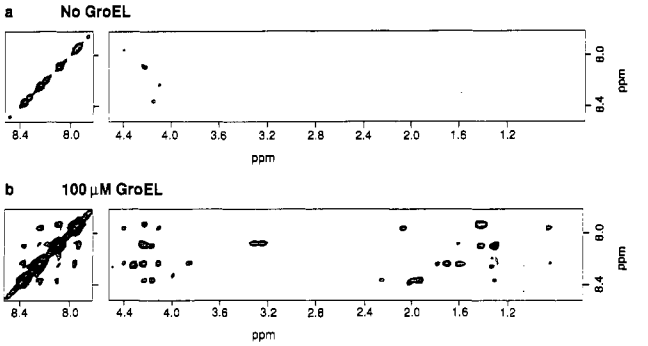

FIGURE 1: NOESY spectra of Ac-STKWLAESVRAGK-NH_2 in 40 mM potassium phosphate buffer, pH 6.1, at 25 °C (a) and with GroEL (b). Mixing time in (a) was 300 ms and in (b) 50 ms. The appearance of numerous intramolecular trNOE cross peaks in the presence of GroEL reflects efficient cross-relaxation in the bound state. [Note: Diagonal peaks in (b) appear larger because of line broadening; in fact, intensities of diagonal peaks are greater in (a).]

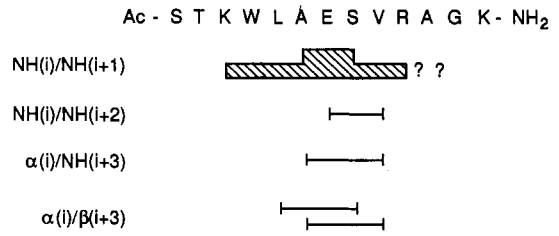

FIGURE 2: Summary of interresidue NOESY cross peaks indicative of helix in the peptide bound to GroEL. Cross peaks show that the specified protons approach within approximately 4 Å of each other (corresponding to the practical limit of NOE detection in this system). The hatched blocks indicate two levels of NOE magnitude. Question marks indicate interactions that could not be observed due to overlapping resonances. Lines connect residues participating in medium-range interactions and are strong evidence for a helical conformation. For example, the distance $\alpha(i)/\beta(i+3)$ in an α-helix is 2.5–4.4 Å; in an extended chain, this distance is greater than 10 Å. Additional trNOEs were observed but are not presented because they reflect distances that do not constrain the conformation of the polypeptide backbone.

Results

The room-temperature NOESY spectrum of the rhodanese peptide (Figure 1a) contains very few cross peaks, as a result of both its lack of a strongly preferred conformation and its short correlation time. The NOEs that are observed [mostly $H^{\alpha}(i)/NH(i+1)$] are typical of an ensemble of conformations biased toward extended states (Wright et al., 1988). Addition of GroEL (peptide:GroEL monomer ratio 28:1) leads to line broadening of the peptide resonances (see below); no new resonances arise from bound peptide or from protein, indicating that the peptide is in intermediate to fast exchange and that the long correlation time of GroEL broadens the protein signals such that they are not observable. As shown in Figure 1b, many strong cross peaks appear in the NOESY spectrum of the sample containing 100 μM GroEL (monomer). The pattern of trNOEs observed (summarized in Figure 2) reveals that the peptide takes up an α-helical conformation in its bound state (Wüthrich, 1986). A continuous series of strong NH-(i)/NH$(i+1)$ trNOEs extends from Trp4 to Arg10. Several "medium-range" trNOEs are observed at longer mixing times (summarized in Figure 2, data not shown).

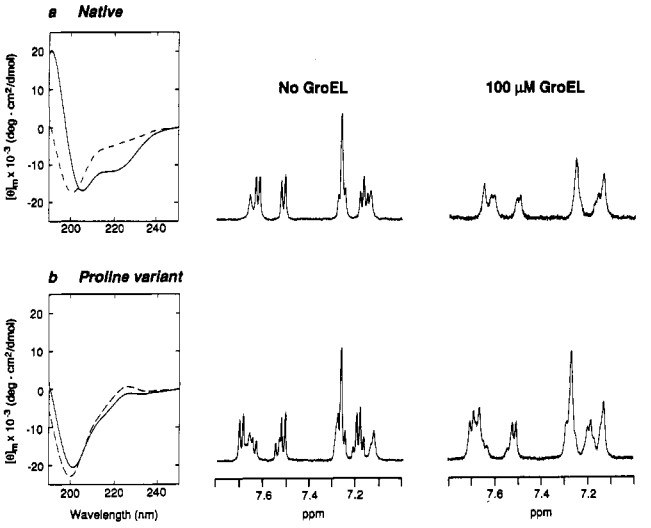

FIGURE 3: Circular dichroism spectra in 20% TFE (solid line) and in buffer (dashed line) (left panel) and 1D NMR spectra of the aromatic region showing line broadening effects (right panel) in the native peptide (a) and the proline variant (b). Note, for example, that the splitting of the 7.52 ppm resonance line (assigned to the ζ2 proton of Trp3) is less well resolved in the presence of GroEL as a result of chemical exchange with the bound form. This broadening effect is more pronounced for the native peptide.

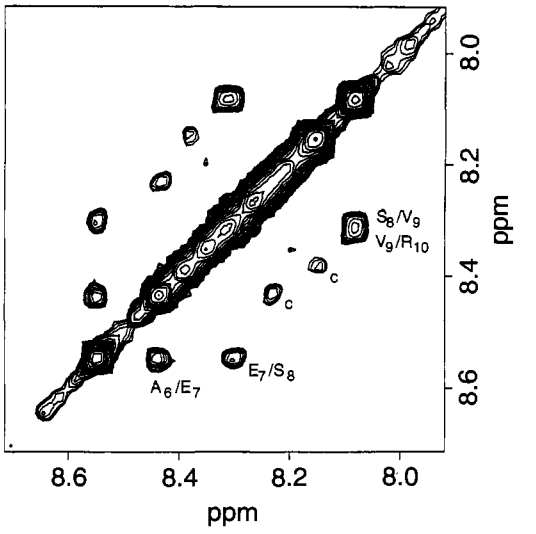

FIGURE 4: NH–NH region of the NOESY spectrum of the proline variant (Ac-STKWPAESVRAGK-NH_2) in the presence of GroEL showing trNOEs indicative of α-helix formation; pH 6.1, [proline variant] = 3.2 mM, [GroEL as monomer] = 100 μM, mixing time 300 ms. The off-diagonal peaks labeled c arise from a small population of a cis Trp–Pro bond containing isomer.

Propensity to assume an α-helical conformation could be an important characteristic of peptides that bind to GroEL. To test this possibility, a variant of the rhodanese peptide was synthesized with Leu5 replaced by Pro; this peptide is expected to have substantially lower helix-forming tendency. Circular dichroism (CD) spectra of the two peptides in buffer and in 20% (v/v) trifluoroethanol (TFE)/buffer as a helix-promoting solvent confirm this expectation (see left panel of Figure 3). In buffer, both peptides are largely unstructured. In 20% TFE, the peptide with native sequence has high helix content (ca. 50%), but that of the proline variant is substantially lower (<20%) (Greenfield & Fasman, 1969; Chen et al., 1974).

The 1D NMR spectrum of the proline-containing peptide shows considerably less line broadening upon addition of GroEL than does that of the native peptide, indicating that binding of the proline variant is significantly weaker (Figure 3, right panel). Consistent with weak binding, the 2D NOE spectrum of the variant in the presence of GroEL exhibits only small trNOEs. [Note: The weakness of trNOEs observed for this peptide rules out the possibility that trNOEs observed with the native peptide are merely NOEs that arose from increased viscosity of the sample containing GroEL.] However, those trNOEs for the proline variant that are observable (Figure 4) indicate that the bound peptide forms a helix extending from Trp4 to Val9. The only conformationally diagnostic trNOEs observed were NH(i)/NH(i + 1) or analogous NH/δ, δ/NH (for proline) trNOEs. The steady-state level of this peptide/GroEL complex is probably too low, as a result of low binding affinity, to produce medium-range trNOEs.

Discussion

The transferred NOE method is ideal for the questions posed in this study. Only if there is binding of the peptide to the chaperone will the efficiency of magnetization transfer between protons be great enough to give rise to sizable NOEs. Hence, observation of trNOEs immediately demonstrates that the rhodanese peptide binds to GroEL. Because the peptide resonances are broadened and large trNOEs are seen, we can estimate the equilibrium constant for peptide dissociation from GroEL to be in the range 10–1000 μM, assuming a diffusion-controlled on rate (Clore & Gronenborn, 1982).

During the initial, linear buildup of the NOE, its magnitude is proportional to the rate of magnetization transfer (cross-relaxation) between two protons and the concentration of the peptide/GroEL complex (Clore & Gronenborn, 1983). The cross-relaxation rate is, in turn, proportional to the inverse sixth power of the distance between the protons in the bound state, thus providing the sensitive distance measure useful for structure determination. Thus, the conformation of the bound peptide is revealed by the pattern of trNOEs observed, which is consistent with an α-helical conformation (Wüthrich, 1986). The most persistently helical part of the peptide extends from Leu5 through Val9.

The comparison of the impact of GroEL addition on the spectrum of the native rhodanese peptide and on that of a proline-containing variant with a reduced tendency to fold as a helix argues persuasively that the affinity of a sequence for GroEL will be greater if the intrinsic α-helix forming tendency is greater. Nonetheless, the proline-containing peptide binds to GroEL more weakly than the native peptide but also in an α-helical conformation. This result is striking in light of the low content of helix present in solutions of the proline variant in TFE/water. Clearly, the environment presented by the GroEL binding site can induce a helical conformation, even in a reluctant helix former.

Taken together, these results support the hypothesis that tendency to form helix is one attribute of protein sequences bound by GroEL. This conclusion is reasonable since α-helices are common structures that form early in the folding path of several proteins (Roder et al., 1988; Bycroft et al., 1990; Miranker et al., 1991) and are likely to be populated to some extent even in short segments of polypeptide chain (Marqusee et al., 1989). It is additionally reasonable that the helices bound by a chaperone like GroEL would be amphipathic, since the binding motif should be characteristic of incompletely folded chains that have not optimally sequestered their hydrophobic surface area. In vivo, GroEL may assist folding by binding polypeptides during translation, thus protecting nascent chains from interactions that lead to aggregation. The present results suggest that amino-terminal sequences could associate with GroEL as α-helices. More favorable intra-

molecular, tertiary interactions would develop as the rest of the protein is made, and so the polypeptide would partition from GroEL into the native structure. Whether the formation of helix upon GroEL binding directly aids the adoption of native structure is an open question. It is possible from our results since the rhodanese peptide is helical in the final folded protein.

Several avenues of investigation follow logically from these results. First, variation of the sequence of the rhodanese peptide should define further the patterns of charge and hydrophobicity that favor GroEL binding. The present findings can be correlated to the GroEL-assisted in vitro refolding of rhodanese by making corresponding alterations in rhodanese, by testing for competition by addition of peptides, and by seeking other potential sites in the rhodanese sequence that may interact with GroEL. We are exploring interactions of chaperones from other classes to see whether the present binding motif is generally exploited in chaperone recognition.

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