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Interaction with GroEL destabilises non-amphiphilic secondary structure in a peptide

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Abstract **The *Escherichia coli* molecular chaperone GroEL can functionally interact with non-native forms of many proteins. An inherent property of non-native proteins is the exposure of hydrophobic residues and the presence of secondary structure elements. Whether GroEL unfolds or stabilises these structural elements in protein substrates as a result of binding has been the subject of extended debate in the literature. Based on our studies of model peptides of pre-formed helical structure, we conclude that the final state of a GroEL-bound substrate is dependent on the conformational flexibility of the substrate protein and the distribution of hydrophobic residues, with optimal association when these are able to present a cluster of hydrophobic residues in the binding interface.**

Key words: GroEL; Molecular recognition; Peptide; Secondary structure

1. Introduction

Molecular chaperones, according to definition, are proteins whose function is to mediate the folding/refolding of other proteins without becoming part of the final folded structure [1]. Of all the known molecular chaperones, the best characterised are the *Escherichia coli* molecular chaperone GroEL (chaperonin 60, Cpn60) and its co-molecular chaperone GroES (chaperonin 10, Cpn10), both structurally very well described proteins (for a recent review see [2]). The chaperonin reaction of protein folding can be considered to comprise two partial reactions, the recognition and binding of a non-native protein, followed by the discharge of the bound polypeptide in a form that may be disposed towards productive folding (for a recent review see [3]). The primary event of chaperonin action has been the focus of many studies and it is now well understood that GroEL discriminates substrates on the basis of electrostatic and hydrophobic interactions [4–17]. The fact that substrate recognition by the chaperone is highly promiscuous, with a large variety of protein folding intermediates capable of interacting with the chaperonin [18,19], suggests that GroEL makes use of general structural features that are common to all unfolded protein substrates, but absent or inaccessible in correctly folded structures. A number of research groups have looked at the conformation of substrate proteins bound to GroEL and have made varying conclusions depending on the protein studied and the conditions under which the studies were conducted [11,16,20–31]. The complex between GroEL and its substrate can be dynamic rather than static, meaning that partial folding may occur in regions of the protein that are not bound to the chaperonin. It is therefore very difficult to establish specific rules for the interaction between large polypeptides and GroEL, and only very few target structures have been identified in an intact protein [17,32–34]. The extent to which GroEL discriminates substrates by mechanisms of structural recognition has long remained controversial [24,25,28,31,33–37].

An alternative strategy to examine the selective recognition of features inherent in non-native proteins may be employed by looking at smaller units than the more complex protein substrates [4,33,38–40]. The rationale behind this approach is the fact that all non-native proteins share common properties such as the exposure of hydrophobic residues, a flexible backbone conformation and the presence of secondary structure elements, where each one of these attributes may be selectively exploited by appropriately chosen peptide mimics. The conclusion from these studies is that the peptide backbone conformation is not an exclusive contributor to binding to GroEL [4,36,38,40], but the presentation of a hydrophobic surface as in amphiphilic secondary structure elements can enhance the binding significantly [4,38]. These results support two classic studies by Landry and Gierasch who suggested that amphiphilic helical structure could be a determinant of recognition by GroEL [41,42]. This was based on the observation by NMR that binding to GroEL, but not to the hsp70 analogue DnaK, induced α-helical conformations in short amphiphilic peptides that were otherwise unstructured in solution. Our own efforts to examine the selective recognition of helical structure in peptides by GroEL centre around the binding of two sets of three model peptides where each set was constructed with a hierarchy of α-helix forming propensity of amphiphilic (the AMPH series) or non-amphiphilic characteristics (the NON-AMPH series) [38]. Having reported on the equilibrium binding to the chaperonin and following Landry and Gierasch's footsteps, we were interested to study the conformation of the peptides in the GroEL-bound state. The question to be answered by the circular dichroism (CD) study presented in this paper is, if binding to the chaperone can induce an α-helical conformation in amphiphilic sequences [4,41,42], whether the interaction with GroEL can affect the pre-formed conformation of a non-amphiphilic helical peptide (NON-AMPH$^+$). We conclude that the absence of a hydrophobic surface in non-amphiphilic helical structure results in the disruption of the conformational stabilisation in the peptide upon recognition and binding by the chaperonin.

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2. Materials and methods

2.1. Protein and peptide preparation

GroEL was purified from a recombinant strain of *E. coli* according to previously published methods [43]. The GroEL concentrations given always refer to the oligomer concentration. The NON-AMPH$^+$ peptide was prepared as described previously [38].

2.2. Circular dichroism spectroscopy

Stock solutions of 198 μM NON-AMPH$^+$ peptide (Ro 47-1615-ALYKIKKIKLLESK-ε-dansyl) and 22 μM GroEL were prepared in 20 mM Tris-HCl (pH 7.6 at 20°C). Two sets of GroEL/NON-AMPH$^+$ binding studies were performed. In a first series of experiments, 150 nM GroEL was titrated with increasing concentrations of NON-AMPH$^+$ (2–71.5 μM) and CD spectra between 185 nm and 320 nm were recorded after each addition. A second series of binding experiments was conducted by titration of NON-AMPH$^+$ (100 μM) with GroEL (40–320 nM). All experiments were performed at 20°C in 20 mM Tris-HCl, pH 7.6. A total of four scans were averaged to obtain each spectrum from which the appropriate buffer and GroEL backgrounds were subtracted. CD signals where the ordinary absorbance of the samples was greater than 1.0 absorbance units were disregarded. All CD spectra were recorded in a 0.02 cm pathlength quartz cell on a Jasco J-720 spectropolarimeter.

3. Results

3.1. Construction of a non-amphiphilic helical peptide

In a recent study we reported on the equilibrium binding of a set of amphiphilic (AMPH) and a set of non-amphiphilic (NON-AMPH) peptides to probe the importance of amphiphilic versus non-amphiphilic secondary structure to GroEL molecular recognition and substrate binding [38]. In the present study we want to focus on the NON-AMPH series peptide, the sequence of which (ALYKIKKIKLLESK) contains a combination of hydrophobic and positively charged residues which would dispose to form a non-amphiphilic α-helix in the event that a conformation were adopted by this peptide (see helical wheel and ribbon structures in Fig. 3 in Preuss et al. [38] for illustration). The ability of the peptide to form the α-helical conformation in solution was modulated by attachment to an α-helix inducing template (Ro 47-1615) which mimics the first turn of an α-helix by placing four hydrogen bond acceptors in parallel arrangement ideal to complement the first four hydrogen bond donors of the attached peptide [44] (see Fig. 2 in Preuss et al. [38]). The formation of these intramolecular hydrogen bonds is thought to lower the conformational entropy of the disordered state in preference for an helical conformation in the peptide. The CD spectrum of the template-conjugated peptide (NON-AMPH$^+$) showed a clear propensity to form α-helical structures in solution (Fig. 1A, thick line).

3.2. Titration of GroEL with NON-AMPH$^+$

In an attempt to study whether the propensity of the NON-AMPH$^+$ peptide to form helical structure would drive its recognition and binding by the chaperonin, as has been implied for amphiphilic sequences [38,41,42], the conformation of the GroEL-bound NON-AMPH$^+$ peptide was described by CD. In a first experiment, a fixed concentration of GroEL (150 nM) was titrated with increasing amounts of NON-AMPH$^+$ (2–71.5 μM) and CD spectra were recorded after each addition. Fig. 1A shows a series of representative far-UV spectra of free (thick line) and GroEL-bound NON-AMPH$^+$ after subtraction of the contribution from GroEL (fine lines). The GroEL-bound peptide showed CD spectra

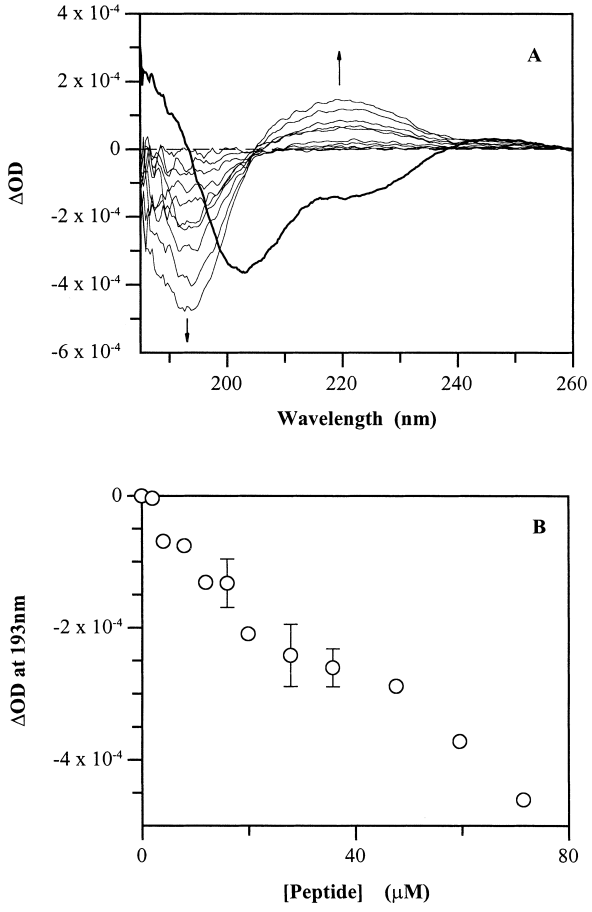

Fig. 1. Titration of GroEL with NON-AMPH$^+$. A: Series of far-UV CD spectra of GroEL-bound NON-AMPH$^+$ (fine lines) after the subtraction of the contribution from GroEL (150 nM). The arrows show the direction of increasing peptide concentration. The CD of free NON-AMPH$^+$ (100 μM, thick line) is shown for comparison. B: CD at 193 nm of the data in A as a function of the NON-AMPH$^+$ concentration with error bars where multiple data points were available. Measurements were made at 20°C in 20 mM Tris-HCl, pH 7.6.

characteristic of a predominantly unstructured conformation, a result that suggested that binding to GroEL did not induce helical structure in the bound peptide. Moreover, any preformed helical structure appeared to be destabilised upon interaction with the chaperonin. Even at a large excess of peptide over GroEL the spectra revealed no significant contribution from helical structures of unbound peptide molecules (Fig. 1A), and when plotted as a function of the peptide concentration the CD in the backbone region showed no tendency to saturate (Fig. 1B). This was consistent with our earlier results from fluorescence equilibrium peptide binding assays and reflected the many peptide binding sites found on the chaperonin (77 ± 9) [38].

3.3. Titration of NON-AMPH$^+$ with GroEL

A second series of reversed CD binding assays was performed in which a high concentration of NON-AMPH$^+$ (100 μM) was titrated with GroEL in eight titration steps (0, 40, 80, 120, 160, 200, 240, 280 and 320 nM). Unfortunately, this was complicated by aggregation of GroEL-peptide complexes (observed as turbidity in the quartz cell) for titra-

tion points beyond 160 nM GroEL. Since the GroEL background could not be accurately subtracted beyond this concentration only spectra for [GroEL]≤160 nM were considered for further analysis; qualitatively, however, all spectra displayed the same trend. Fig. 2 shows the resulting spectra of the GroEL-bound peptide (fine lines) and the spectrum of NON-AMPH$^+$ in the absence of GroEL for comparison (thick line). The presence of an isodichroic point near 203 nm, indicative of a two-state helix-coil transition, and the blue shift of the minimum from 206 to 193 nm demonstrated an increase in the proportion of disordered conformation in the peptide. This was also suggested by the increase of the CD between 205 and 230 nm which may reflect a contribution from aromatic groups (the template and/or the tyrosine residue), known to give rise to positive far-UV CD when the helical content is low [45]. In summary, the data shown in Fig. 2 confirmed that binding of NON-AMPH$^+$ to GroEL resulted in a complete loss of secondary structure-forming propensity in the peptide.

4. Discussion

It is generally accepted that a primary action of molecular chaperones consists of the binding of solvent-exposed hydrophobic amino acid residues which are presented by protein folding intermediates but buried in their native state. As proteins fold, local regions can form secondary structure elements which may also provide binding motifs for recognition by the GroEL binding sites. The determination of relevant structural elements for interaction between GroEL and proteins has been rendered difficult by the observation that early and late protein folding intermediates interact with the chaperone, and that GroEL-bound proteins may display structural fluctuations due to binding and partial folding. The use of peptide substrates as mimetics of non-native proteins circumvents this complication of kinetic competition between substrate folding and binding, and a variety of peptide-based studies have contributed significantly to today's understanding of GroEL molecular recognition [4,38–42]. Gierasch and co-workers were

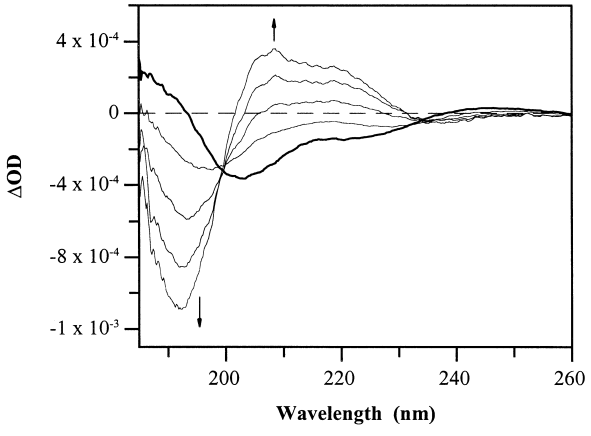

Fig. 2. Titration of NON-AMPH$^+$ with GroEL. NON-AMPH$^+$ (100 μM, thick line) was titrated with GroEL (40 nM, 80 nM, 120 nM and 160 nM). The four spectra of GroEL-bound NON-AMPH$^+$ (fine lines) were corrected for the respective GroEL background. The arrows show the direction of increasing GroEL concentration. Measurements were made at 20°C in 20 mM Tris-HCl, pH 7.6.

the first to present experimental evidence that helical secondary structure may be a determinant of recognition by GroEL [41,42], and have led the way to numerous studies investigating the involvement of secondary structure elements in GroEL molecular recognition [4,36,38–40]. It has now become clear that the binding of substrates to GroEL is largely mediated by the presentation of a hydrophobic array of side chains, regardless of the actual backbone conformation [4,36,40], and is further supplemented by the disposition of charged groups which determine which substrate, or regions within a substrate, may be recognised by the chaperone. The predominance of hydrophobic residues in mediating the association of polypeptides with the chaperonin is strongly supported by the X-ray crystal structure of GroEL which shows a high density of hydrophobic residues on the inside surface of the flexible apical domain where substrate protein is known to bind [5,46–48]. Based on this wealth of experimental evidence we recently suggested that recognition and binding of an amphiphilic helical peptide (AMPH$^+$) was mediated via the cluster of hydrophobic amino acid side chains conveniently presented on one face of the amphiphilic helix for interaction with complementary arrays of binding-competent residues on GroEL [38]. In this event, molecular recognition and initial binding would be followed by little subsequent structural reorganisation. By contrast, it would appear likely that the initial recognition of proteins/peptides with unordered or non-amphiphilic structures are followed by conformational changes in GroEL and its substrate, and that these, and not the initial binding, are driving the interaction. This was suggested by the strong affinity of GroEL for the AMPH$^+$ peptide (apparent K_d 5 ± 1 nM) which presents a denser cluster of hydrophobic residues than its non-amphiphilic helical variant NON-AMPH$^+$ (apparent K_d 62 ± 2 nM). Hence, the weaker affinity of NON-AMPH$^+$ for GroEL may demonstrate that substantial rearrangements of the peptide were necessary in the requirement to optimise the free energy of association. We are now in the position to present experimental evidence to corroborate this proposal. As would be expected, the interaction of GroEL with pre-formed non-amphiphilic α-helical structure was shown to destabilise this conformation (Figs. 1 and 2), possibly forcing NON-AMPH$^+$ to adopt a different, more favourable array of binding-competent residues relative to its solution structure where the hydrophobic side chains are evenly distributed around the helical axis. This result is complementary to experimental evidence by others who showed that association with the chaperone may induce secondary structure conformations in amphiphilic sequences where hydrophobic residues are oriented more readily for interaction with the chaperone [4,41,42].

The hypothesis that secondary structure elements may provide binding motifs for recognition by GroEL has been challenged by the observation that these elements may not necessarily contribute to complex formation [9,26]. Their role in molecular recognition has also been discussed controversially since structural elements within substrate proteins were found to be stabilised [24,29,31,35], or unfolded [25,28,37] upon their interaction with GroEL. Based on evidence presented in this paper, and that of others [4,41,42], we would suggest an explanation for this apparent controversy. It appears that GroEL preferably interacts with sequences in a protein substrate that are able to present a cluster of hydrophobic residues (a hydrophobic surface) in the bound state. Stabilisation

or induction of secondary structural elements may therefore be observed where GroEL interacts with amphiphilic secondary structural elements [38] or sequences with the potential to form amphiphilic secondary structures [4,41,42] respectively. Under these conditions GroEL may even perform a hypothetical role as a partial amphiphilic organiser of folding. The interaction of the GroEL binding sites with pre-formed non-amphiphilic secondary structural elements within a protein substrate on the other hand, appears to result in their destabilisation particularly when the conformational stability is lower than the free energy of binding. In the case of our NON-AMPH$^+$ peptide, we found that the necessary structural rearrangements in NON-AMPH$^+$ did not discourage binding to the chaperonin. A comparative study of NON-AMPH$^+$ and a non-helical variant of the same peptide (NON-AMPH$^-$) demonstrated that both peptides bound to GroEL with almost identical affinity and by the same combination of hydrophobic and electrostatic forces (NON-AMPH$^-$ was constructed by attachment of the peptide to the optical isomer of the template (Ro 47-1614), resulting in a peptide of identical composition but unable to adopt the α-helical conformation) [38].

It seems likely that the 'unfolding' or 'folding' of local structural elements in a protein substrate upon interaction with the chaperonin may result in the rearrangement of kinetically trapped intermediates with low intrinsic stability, allowing them to re-enter the correct folding pathway after release into the cavity or into the bulk solution. Nevertheless, since the destabilisation or stabilisation of such structural elements appears to be essentially a function of the alignment of hydrophobic residues in the substrate protein, these observations are unlikely to point towards a role of GroEL in actively assisting protein folding [25,28,37,49]. We would conclude that there is no universal mechanism for GroEL molecular recognition and binding of substrate proteins; rather, the final state of a GroEL-bound polypeptide is likely to be a function of the distribution of hydrophobic residues in the substrate and the conformational stability of the substrate folding intermediate, and represents a minimum in the free energy of association with GroEL.

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