Basis of Substrate Binding by the Chaperonin GroEL†

Zhulun Wang,‡,§ Hwa-ping Feng,§,⊥ Samuel J. Landry,|| Jennifer Maxwell, and Lila M. Gierasch*

Department of Chemistry, University of Massachusetts, Amherst, Massachusetts 01003

Received May 11, 1999; Revised Manuscript Received July 21, 1999

ABSTRACT: The molecular chaperonins are essential proteins involved in protein folding, complex assembly, and polypeptide translocation. While there is abundant structural information about the machinery and the mechanistic details of its action are well studied, it is yet unresolved how chaperonins recognize a large number of structurally unrelated polypeptides in their unfolded or partially folded forms. To determine the nature of chaperonin-substrate recognition, we have characterized by NMR methods the interactions of GroEL with synthetic peptides that mimic segments of unfolded proteins. In previous work, we found using transferred nuclear Overhauser effect (trNOE) analysis that two 13 amino acid peptides bound GroEL in an amphipathic α-helical conformation. By extending the study to a variety of peptides with differing sequence motifs, we have observed that peptides can adopt conformations other than α-helix when bound to GroEL. Furthermore, peptides of the same composition exhibited significantly different affinities for GroEL as manifested by the magnitude of trNOEs. Binding to GroEL correlates well with the ability of the peptide to cluster hydrophobic residues on one face of the peptide, as determined by the retention time on reversed-phase (RP) HPLC. We conclude that the molecular basis of GroEL-substrate recognition is the presentation of a hydrophobic surface by an incompletely folded polypeptide and that many backbone conformations can be accommodated.

Chaperonins comprise a family of proteins that facilitate the correct folding, assembly, and targeting of other proteins in cells (*1*). They are found ubiquitously in all cell types and in all organisms (*1*); the best studied family member is GroEL, the *Escherichia coli* chaperonin. GroEL is a highly abundant protein that is essential for cell viability under normal growth conditions (*2, 3*). It is a large homooligomeric protein complex composed of 14 identical 60 kDa subunits which are arranged in two stacked heptameric rings (*4, 5*). Functionally, GroEL works in a Mg-ATP[1] and K^+-dependent manner in cooperation with a small co-chaperonin, GroES (or cpn10), which is a heptamer of 10 kDa subunits, constituting a GroE chaperonin machine (*6*). The three-dimensional crystal structures have been determined recently for a GroEL mutant in free (*5*) and nucleotide-bound states (*7*) and for wild-type GroEL in a ternary complex with GroES and ADP (*8*). The important roles of GroE chaperonins in phage assembly, protein folding, and secretion have been identified by both genetic and biochemical studies (*6, 9*−*13*). In vitro, the GroE chaperonin machine has been shown to promote folding and/or assembly of a number of enzymes, including ribulose bisphosphate carboxylase (Rubisco) (*14*), citrate synthase (*15, 16*), ornithine transcarbamylase (OTC) (*17*), and rhodanese (*18*), among others. The promiscuity of GroEL in interactions with a large number of structurally unrelated proteins has been documented, where GroEL is capable of forming stable complexes with at least half of the *E. coli* soluble proteins in their unfolded or partially folded states (*19*), and defective GroE function leads to the accumulation of a number of *E. coli* proteins including citrate synthase (*13*).

The basis of GroEL action is its ability to recognize and stabilize unfolded or partially folded structures and to release them in a cooperative fashion upon binding of the co-chaperonin GroES and ATP, thus preventing inappropriate intra- and intermolecular interactions that would otherwise lead to irreversible, nonspecific aggregation (*20*−*22*). To understand the molecular mechanisms by which chaperonins function, two central questions—how chaperonins recognize and bind their substrates and how they modulate the folding process—must be answered. The latter question is the focus of many studies, the results of which provide important mechanistic details of the function of each part of the chaperonin machinery (for a recent review, see ref *23*). On the other hand, depending on the substrates studied, the mechanisms of the chaperonin-assisted folding vary (for a review, see ref *23*). This observation indicates that certain properties of the substrates, which are not completely characterized, modulate the interactions with chaperonins and are important determinants in the selection of the assisted-folding pathways. Substrate recognition by chaperonins is highly promiscuous, since they can interact with diverse polypeptides sharing little primary sequence homology, provided that these substrate proteins are incompletely folded. In other words, chaperonins can differentiate unfolded or

† This research is supported by NIH Grant GM27616 (L.M.G.).

* To whom correspondence should be addressed. Phone: (413) 545-2318. Fax: (413) 545-0011. Email: gierasch@chem.umass.edu.

‡ Current address: Tularik Inc., 2 Corporate Drive, South San Francisco, CA 94080.

§ These authors contribute equally to this work.

⊥ Current address: Nature Structural Biology, Editorial Office, 345 Park Avenue, New York, NY 10010-1707.

|| Current address: Department of Biochemistry, Tulane University, School of Medicine, 1430 Tulane Avenue, New Orleans, LA 70112.

[1] Abbreviations: ATP, adenine triphosphate; ADP, adenine diphosphate; PMSF, phenylmethylanesulfonyl fluoride; EGTA, ethylenebis-(oxyethylenenitrilo)tetraacetic acid; NMR, nuclear magnetic resonance.

10.1021/bi991070p CCC: $18.00
Published on Web 09/03/1999

partially folded states from the native state, but lack sequence specificity. Hence, chaperonins must recognize features that are only present or accessible in incompletely folded states (*24*). The properties shared by all nonnative states include secondary structural elements, exposed hydrophobic patches or clusters, incompletely packed side chains, and polypeptide chain flexibility. The present study addresses the recognition question by examining interactions of the chaperonin GroEL with a variety of different synthetic peptides which simulate segments of incompletely folded polypeptides.

GroEL recognizes nonnative states in protein folding pathways to prevent aggregation, which principally results from the exposure of hydrophobic surfaces in the early folding stages. It is likely, therefore, that the interaction between GroEL and substrates is primarily hydrophobic in nature. This hypothesis is supported by studies using either calorimetry or mutagenesis which identified a cluster of hydrophobic residues in the apical domain as implicated in substrate binding (*25, 26*). In previous studies, we demonstrated that two 13-residue peptides bound to GroEL in an α-helical conformation, as determined by transferred nuclear Overhauser effect (trNOE) analysis (*27, 28*). Interestingly, these same peptides bind to the *E. coli* hsp70 homologue, DnaK, in an extended conformation (*28*). The question arises as to whether an α-helical backbone conformation is a necessary condition for recognition by GroEL, and if so, what features of the α-helix determine such binding. The peptides studied so far are amphipathic when folded in an α-helical conformation and, therefore, present a hydrophobic surface that could be exploited in the binding interaction. However, for other sequences, nonhelical backbone conformations could also present a hydrophobic aspect.

We have now explored whether the GroEL-binding interaction has any preference for helical arrangements of the polypeptide substrate by studying additional peptides which have different patterns of hydrophobic and hydrophilic residues or are derived from nonhelical secondary structures in native proteins. TrNOE analysis shows that the peptides may adopt conformations other than an α-helix when bound to GroEL, such as an extended β-strand conformation. Furthermore, the relative binding affinities of peptides for GroEL parallel very closely their abilities to cluster hydrophobic residues, as reflected by the retention times of the peptides in reversed-phase high-performance liquid chromatography (RP-HPLC). Thus, we conclude that the binding of substrates to GroEL is based on surface hydrophobicity, regardless of backbone conformation.

MATERIALS AND METHODS

GroEL was purified by overexpression in the *E. coli* strain C1859 bearing plasmid pOF39 using the improved purification procedures described previously (*29*). In brief, cell cultures were grown to late log phase at 37 °C and collected by centrifugation at 4 °C. All subsequent purification steps were carried out at 4 °C except chromatography on Cibacron-blue columns. The cell pellets were resuspended in Tris-saline (50 mM Tris-HCl and 125 mM NaCl, pH 7.5) containing protease inhibitors (10 μM E-64, 10 μM aprotinin, 1 mM PMSF, and 10 mM EGTA) and lysed by sonication. The lysate was then centrifuged at 35000*g* for 20 min, and the supernatant was fractionated by ammonium sulfate precipitation. The 1.8−2.4 M cut was resuspended in Tris-saline and spun at 280000*g* for 120 min. The supernatant was then resuspended in Tris-saline containing 1 mM ATP, 5 mM $MgCl_2$, and 25 mM KCl, and applied to a 5 × 30 cm Sephacryl S400 HR gel-filtration column equilibrated in the same buffer. Fractions containing GroEL/GroES complex were collected, diluted 3-fold with distilled water and applied to a 30 mL Q-Sepharose column equilibrated with 20 mM Bis-Tris-HCl and 50 mM KCl, pH 6.0. GroES and GroEL were eluted with a KCl gradient at ∼0.2 and 0.5 M, respectively. GroEL was further purified by passing through a 2 mL bed of Cibacron-blue agarose 3GA (Sigma, St. Louis, MO). For each preparation, the molar extinction coefficient of GroEL at 276 nm was characterized by UV, with the concentration determined by amino acid analysis. The typical ϵ_{276} of GroEL subunit was about 13 000 M^{-1} cm^{-1}. The protein was stored at 4 °C as the ammonium sulfate precipitate, and washed in a Centricon-30 concentrator (Amicon, Beverly, MA) by three rounds of dilution and concentration with distilled water before an experiment. GroEL used in the T_2 measurements was extensively washed in sodium phosphate buffer (40 mM, pH 6.0) so that the final NH_4^+ concentration is in the nanomolar range. Mg^{2+} was supplemented to the NMR samples containing ATP with a concentration ratio of 1:1. GroES was not included in the experiments.

All peptides were synthesized by solid-phase peptide synthesis using a Milligen 9050 peptide synthesizer (Millipore, Milford, MA) using continuous flow 9-fluorenylmethoxycarbonyl (Fmoc) amino acid protection chemistry. Crude peptides were purified by RP-HPLC on a Vydac C18 column with an acetonitrile/water gradient containing 0.1% trifluoroacetic acid. The purity of the peptide was checked by analytical RP-HPLC. The identity of the peptide was verified by mass spectrometry, amino acid analysis, and sequencing. The peptide concentration was determined by amino acid analysis.

NMR spectra were recorded on either a Varian VXR-500 or a Bruker AMX2 spectrometer operating at a proton frequency of 500 MHz. T_2 measurements were performed with the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence (*30*) with 64 scans per FID. Two-dimensional (2-D) total correlation (TOCSY) and nuclear Overhauser effect (NOESY) spectra were acquired in the phase-sensitive mode, with 6000 Hz spectral widths in both dimensions and a total of 2 × 256 free induction decays (FIDs) with 1024 complex points per FID. The mixing times were 65 ms in the TOCSY and varied from 50 to 300 ms in NOESY. The 2-D data were processed using FTNMR on a Sun4/260 workstation or FELIX (Molecular Simulations, San Diego) on a Silicon Graphics workstation. In general, Gaussian or sine-bell apodization functions were used in both dimensions, and a matrix of 1024 × 1024 real points was formed by zero-filling. Sequential resonance assignment of the peptide was accomplished by TOCSY and NOESY. All data were obtained at 25 °C except the assignment measurements, which were performed at 8 °C. Proton chemical shifts were referenced with respect to the methyl protons of 3-(trimethylsilyl) propionate (TMSP) at 0 ppm included in the samples.

Selection of Peptides. Synthetic peptides were utilized as mimics of segments of incompletely folded polypeptide chains in order to understand whether peptide backbone

Table 1: Experimental Peptides

peptide	sequence	propensity
Rho2(native)	Ac-STKWLAESVRAGK-NH_2	amphipathic α-helix
chirality variants of Rho2[a]		
D-Rho2	Ac-STKWLAESVRAGK-NH_2	amphipathic left-handed α-helix
L,D-Rho2	Ac-STKWLAESVRAGK-NH_2	unable to form α-helix
amphipathic variants of Rho2[b]		
ID	Ac-YKALAESLKASK-NH_2	amphipathic α-helix
KE	Ac-YKLSAEKLSAAK-NH_2	non-amphipathic α-helix
β-strand peptides (native)		
Xhat1(C → S)[c]	**Y**STATL**S**LGHHAVP	β-strand
CRABP3	Ac-SKPHVEIRQDGD	β-strand

[a] Sequence held constant with substitution of D-amino acids (underlined). [b] Sequence altered with amino acid composition held constant. [c] In Xhat1(C → S), the bold-face letters indicate residues that were not in the original hemagglutinin sequence: the first residue Tyr was not in the original β-strand, and the original Cys was changed to Ser.

conformation or hydrophobic surface is involved in GroEL-substrate recognition. The peptides tested here provide examples of amino acid sequences that have different intrinsic abilities to present a hydrophobic surface and to adopt a specific secondary structure in an apolar environment (*31*). Among them are some peptides (see Table 1) designed specifically to examine the following issues: (1) α-helix versus non-α-helix; (2) amphipathic versus nonamphipathic; (3) α-helix versus β-strand.

D-Rho2 and L,D-Rho2 (see Table 1) are "chirality mutants" of the original rhodanese peptide Rho2 (*27*, *28*) in that they bear the same peptide sequence as that of the native Rho2 peptide, but contain amino acid residues in D-form. The D-Rho2 peptide, with all the residues in D-form, can form a *left-handed* α-helix that would be isoenergetic to the right-handed helix of the original Rho2 peptide, whereas the L,D-Rho2 peptide with D-amino acids at alternating positions in the sequence is not likely to form an α-helix at all.

The ID peptide was designed to have characteristics similar to the Rho2 peptide, viz., the ability to adopt an amphiphathic α-helical conformation, but with a simplified amino acid composition. The KE peptide has the same amino acid composition as that of ID but with an altered sequence arrangement which would not yield an amphipathic character if the peptide adopts an α-helical conformation. Thus, the ID and KE peptides share the same overall hydrophobicity but differ in amphipathicity or surface hydrophobicity.

Many of the peptides studied have a high intrinsic tendency to take up an α-helical conformation. To examine whether GroEL is capable of recognizing and binding β-strands, we selected two peptides, Xhat1(C → S) and CRABP3, whose sequences correspond to β-strands in native influenza virus hemagglutinin (*32*) and cellular retinoic acid binding protein (CRABPI) (*33*), respectively.

Rho2 Chirality Variants Bind GroEL. The chirality variants of the Rho2 peptide are not able to form regular *right-handed* α-helix. Their interactions with GroEL thus address the question whether a right-handed α-helix is a necessary structural motif for recognition. Figure 1 shows circular dichroism (CD) spectra of the Rho2 peptide and its chirality variants in aqueous buffer and in 20% (v/v) trifluoroethanol (TFE), a solvent that stabilizes the helical conformation proportional to the helical propensity of the dissolved peptides (*34*, *35*). CD spectra of both Rho2 and D-Rho2 indicate that they adopt largely random conformations in

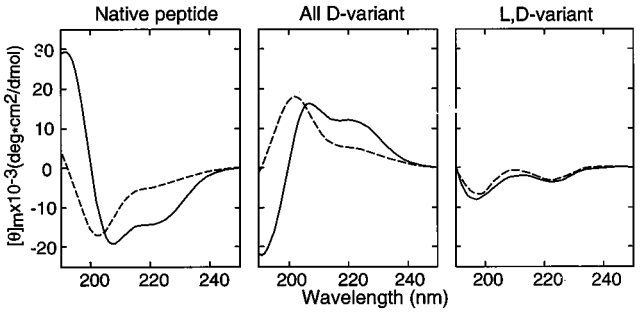

FIGURE 1: CD spectra of the native Rho2 peptide and its chirality variants in pH 6.1 phosphate buffer (dashed line) and in 20% (v/v) TFE (solid line). In 20% TFE, the D-Rho2 peptide forms a left-handed α-helix, while the L,D-Rho2 peptide cannot form an α-helix at all.

aqueous buffer solutions. The CD spectrum of the Rho2 peptide in 20% TFE displays the typical negative double minima characteristic of a right-handed α-helix. By contrast, the CD spectrum of D-Rho2 has positive intensity but the identical double minima shape as its mirror image, demonstrating that it forms a *left-handed* α-helix in 20% TFE. L,D-Rho2, however, does not take up an α-helical conformation even in 20% TFE. Its CD spectrum both in aqueous buffer and in 20% TFE is similar to that of gramicidin A in methanol, which exists in equilibrium between an uncharacterized monomeric form and several double-helical species consisting of π-helices (*36*). The equilibrium is shifted to favor the double-helical species when long-chain alcohol (hydrophobic) is used instead of methanol (*36*). This similarity is expected given the alternating L,D sequences in both of these peptides.

A wealth of information on ligand−macromolecule interactions can be obtained by NMR methods. Specific protein-induced line broadening of ligand resonance peaks indicates differential immobilization, and thus points to the most important ligand−protein interactions. When the free ligand and macromolecule-bound ligand are in the fast exchange regime on the NMR time scale of spin−lattice relaxation, trNOEs provide information that reveals the ligand conformation in the bound state (*37*, *38*). Many factors contribute to the magnitudes of the observed trNOEs and line-broadening effects: affinity, extent of interaction and the internal motion of the bound peptide, magnetization transfer pathways between protein and peptide, and anisotropy of motion (*38*). Thus, the majority of the line broadening and

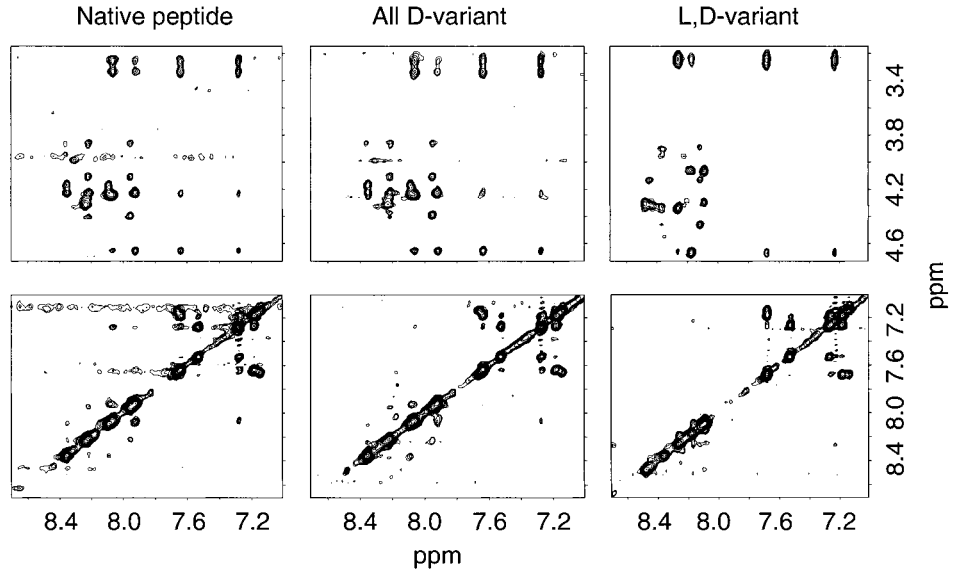

FIGURE 2: 2-D trNOE spectra of rhodanese peptide Rho2 and its chirality variants, D-Rho2 and L,D-Rho2, in the presenc of GroEL. The peptide concentration was 7.9 mM with 200 μM (monomer concentration) GroEL in 40 mM potassium phosphate, pH 6.0, buffer with 10% D_2O and 0.35 mM TMSP. The mixing time was 100 ms.

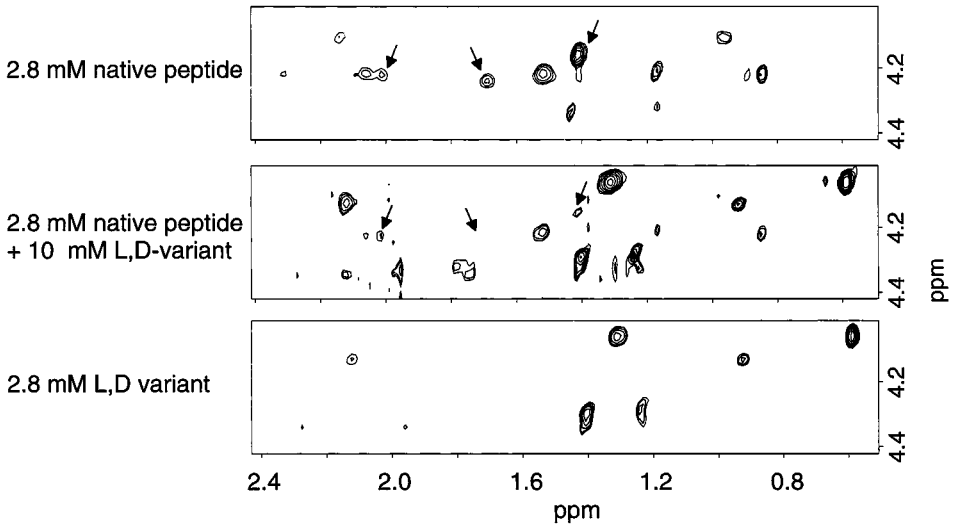

FIGURE 3: Comparison of 2-D trNOE spectra of the rhodanese peptides Rho2, L,D-Rho2 and Rho2 plus L,D-Rho2 in the presence of 50 μM GroEL. TrNOEs of the native Rho2 peptide (2.8 mM) are markedly reduced (some examples are pointed by the arrows) by the addition of an excess amount of the L,D-Rho2 peptide (10 mM), indicating competition for the same GroEL binding sites. The sample buffer condition was the same as described in Figure 2. The mixing time was 100 ms.

trNOE data were interpreted only qualitatively in this study. In the presence of GroEL, one-dimensional (1-D) NMR spectra showed line broadening for all three peptides, the original Rho2, D-Rho2, and L,D-Rho2, indicating that they all bind GroEL. Concomitantly, many trNOE cross-peaks were observed for all three of the peptides in the NOESY spectra. Figure 2 shows a comparison of the trNOEs for the original Rho2 peptide and its D- and L,D-variants. The D-Rho2 peptide, which can form a *left-handed* α-helix, showed trNOEs almost identical to that of the Rho2 peptide, suggesting that the peptide adopts a *left-handed* α-helical conformation in the GroEL-bound state. Note that the *left-handed* α-helical conformation of the D-Rho2 peptide would be equally amphipathic in nature as the right-handed α-helical conformation of the original Rho2 peptide. For L,D-Rho2, the magnitude of the trNOEs are similar to those of the native and all D-peptides, although the pattern of trNOEs is different. In particular, there are none of the NH(i)/NH(i+1) cross-peaks that would be expected in an α-helical conformation (*39*). Both line broadening and trNOEs suggest that the L,D-Rho2 peptide binds to GroEL as well as the original and the all D-variant, even though it adopts neither *right-handed* nor *left-handed* α-helix. The L,D-Rho2 peptide might adopt another type of helix, such as a π-helix, which could also present hydrophobic residues in a cluster on its surface, but there is insufficient data to specify this conformation. The possibility that the original and L,D-Rho2 peptides bind different sites on GroEL was tested by a competition experiment. As shown in Figure 3, the trNOEs of the native peptide were dramatically reduced by the addition of an excess of the L,D-peptide, indicating that the two peptides compete for the same binding sites on GroEL. We conclude that an α-helix is not an essential motif for GroEL-peptide binding.

Disruption of Amphipathic Helical Character Weakens GroEL Binding. To test the importance of amphipathicity

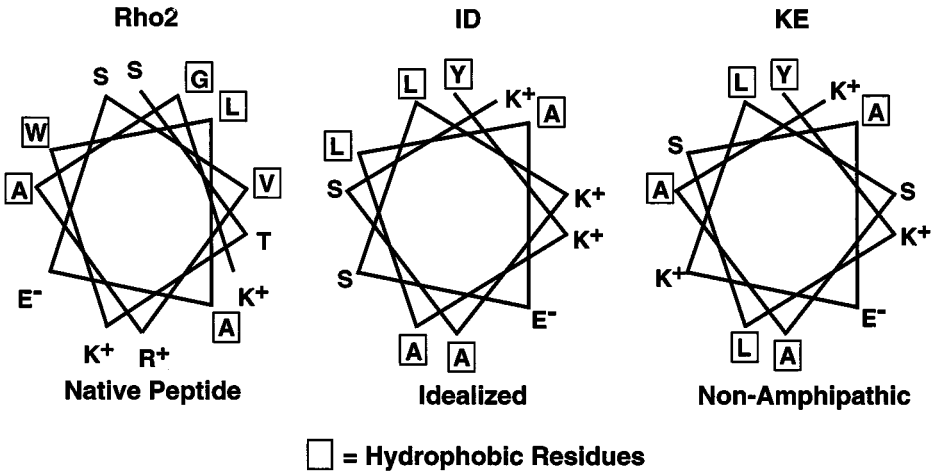

FIGURE 4: Helical wheel representation of three of the peptides used in this study: Rho2, ID, and KE.

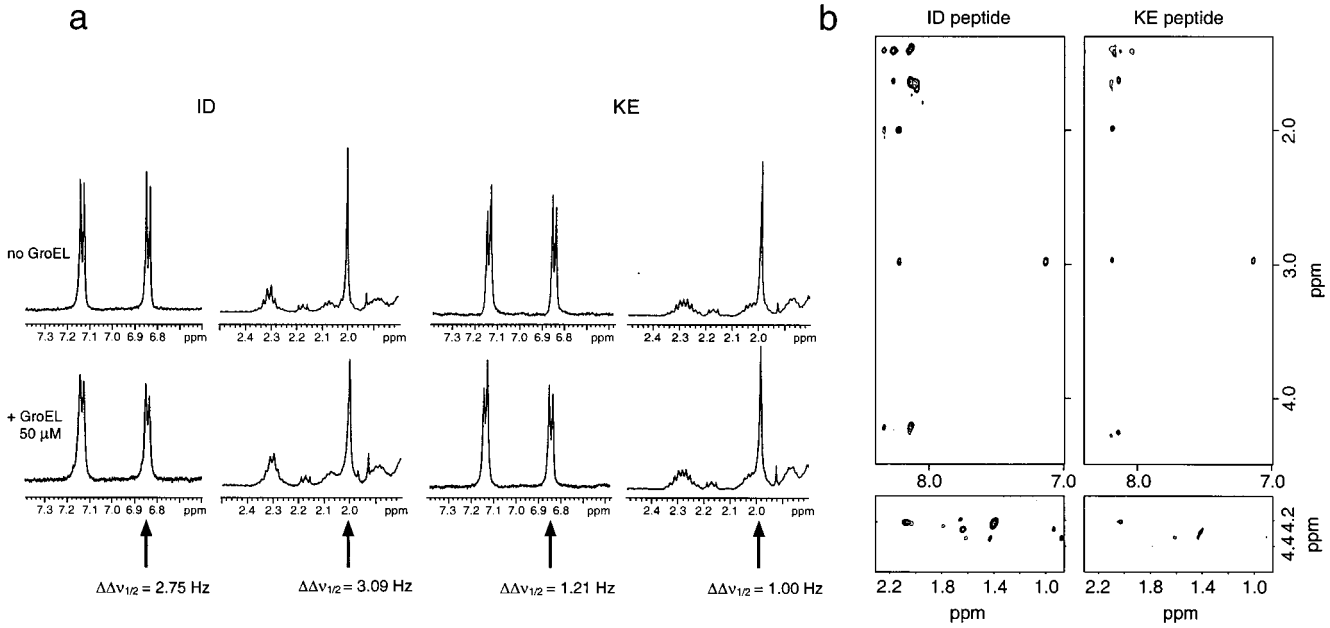

FIGURE 5: Selected regions of 1-D NMR spectra (a) and 2-D trNOE spectra (b) of the ID and KE peptides alone and with GroEL. The KE peptide in the presence of GroEL showed reduced line broadening and fewer trNOEs as compared with those of the ID peptide, suggesting that the amphipathic peptide is capable of binding GroEL with relatively higher affinity than the nonamphipathic one. Samples contained 2.4 mM peptide and 50 μM GroEL in 40 mM K-potassium phosphate, pH 6.0, buffer with 10% D_2O and 0.35 mM TMSP. The mixing time in 2-D NOESY experiments was 150 ms.

in the recognition of peptide substrates by GroEL, we carried out NMR experiments in the presence of GroEL on peptides, which when folded as helices, would present varying amphipathic character. The abilities of the ID and KE peptides to form amphipathic α-helices are compared using helical wheel diagrams in Figure 4; note that the most hydrophobic residues and the charged residues are dispersed around the helix in the KE peptide relative to the ID peptide. CD spectra of both ID and KE peptides in TFE indicate that these peptides have the propensity to form α-helical conformations (data not shown). Figure 5a shows the GroEL-induced line-broadening effect on the peptide resonance peaks for the ID and KE peptides. The "nonamphipathic" KE peptide showed significantly less line broadening in the presence of GroEL than the "amphipathic" ID peptide. Furthermore, smaller trNOEs were observed for the KE peptide as compared with the ID peptide (Figure 5b). These qualitative measures together show that the "nonamphipathic" KE peptide binds GroEL with lower affinity. This result supports the hypothesis that the clustering of hydrophobic residues on one face of the peptide is critical for GroEL-binding.

β-Strand Peptide with Alternating Hydrophobic Residues Binds GroEL in an Extended Conformation. From the behavior of the L,D-Rho2 peptide, we know that it is not necessary for peptides to be α-helical to bind GroEL, although many peptides we have examined do adopt α-helices in the GroEL-bound state. Incompletely folded states of GroEL protein substrates may also contain *β*-strands, and it is of interest to explore whether these features can be bound by GroEL. To assess the ability of GroEL to bind peptides that tend to form a *β*-strand conformation, we have examined two peptides, Xhat1(C → S) and CRABP3. The Xhat1 (C → S) peptide features alternating hydrophobic and hydrophilic amino acid residues in the sequence, and displays a hydrophobic surface in the *β*-strand conformation. Both CD and NMR measurements indicate that the peptide alone in solution lacks a strongly favored conformation (data not

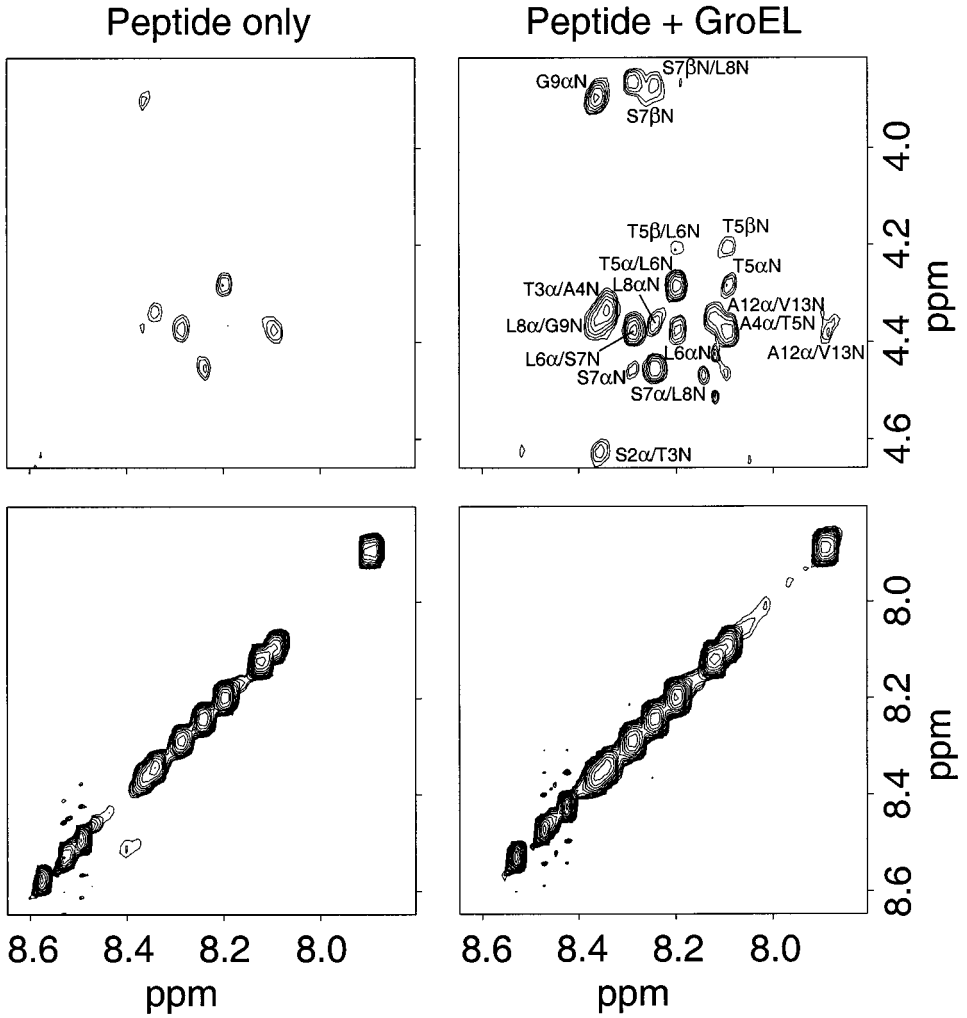

FIGURE 6: 2-D NOESY spectra of the Xhat1 peptide alone and with GroEL. Interresidue $C^{\alpha}H(i)/NH(i+1)$ cross-peaks are labeled with residue numbers. The appearance of many $C^{\alpha}H(i)/NH(i+1)$ and the absence of the $NH(i)/NH(i+1)$ trNOE cross-peaks in the spectrum of the peptide upon addition of GroEL indicate that the peptide adopts a β-strand conformation in the GroEL-bound state. Sample condition: 2.8 mM peptide and 120 μM GroEL in 40 mM sodium acetate-d_3, pH 6.0, with 10% D_2O and 0.35 mM TMSP. The mixing times were 300 ms and 100 ms for the peptide alone and for the peptide with GroEL, respectively.

shown). The addition of GroEL leads to line broadening of the peptide resonance peaks and many strong trNOEs. The $NH(i)/NH(i+1)$ NOE connectivities that would indicate an α-helical conformation (*39*) are lacking as shown in Figure 6. On the other hand, $C^{\alpha}H(i)/NH(i+1)$ trNOEs, as labeled in Figure 6, are much stronger than the intraresidue $NH/C^{\alpha}H$ trNOEs. The presence of strong $C^{\alpha}H(i)/NH(i+1)$ NOEs, combined with the absence of $NH(i)/NH(i+1)$ and characteristic α-helical medium-range NOEs, strongly indicate a predominantly β (extended) conformation for the peptide upon binding to GroEL (*39*). The other β-peptide CRABP3, however, showed almost no binding to GroEL, as very little line broadening and trNOEs were observed for the peptide in the presence of GroEL (data not shown). We found that the CRABP3 peptide differs from the Xhat1(C → S) peptide in the surface hydrophobicity. The results with the above two β-peptides demonstrate (1) the ability of GroEL to recognize an extended or β-conformation, and (2) the importance of a hydrophobic surface.

In summary, among the almost 20 peptides examined in our laboratory by trNOE studies, many but not all show binding to GroEL. The GroEL-binding peptides often, but not always, form α-helices in the GroEL-bound state. Other peptide conformations, such as β-strands, turns (*29*), or perhaps even π-helices, are also found for the GroEL-bound peptides, provided that such a conformational arrangement presents a hydrophobic surface. Evidently, the peptide backbone conformation is not a molecular basis for GroEL recognition; rather the hydrophobic surface of the amphipathic structure is important for GroEL-peptide binding.

GroEL-Peptide Binding Parallels Retention of the Peptides in RP-HPLC. The surface hydrophobicity, a possible GroEL binding motif, has been determined for all the peptides in parallel with the NMR observations. RP-HPLC separates polypeptides primarily by differential surface hydrophobic interactions. The retention times of the peptides on a C18 stationary phase directly reflect the ability of the peptides to present a hydrophobic surface (*40*). The retention times of the peptides on a Vydac analytical C18 RP column were measured using an acetonitrile (ACN)/water gradient containing 0.1% trifluoroacetic acid (TFA). We found that the peptide retention time on RP-HPLC correlates well with the magnitude of trNOE intensity (Table 2). A peptide that binds GroEL with higher affinity also has a longer retention time on the RP-HPLC. The observed correlation between the relative peptide-binding affinity for GroEL and the ability

Table 2: Correlation between Relative TrNOEs and RP-HPLC Retention of Tested Peptides

peptide	sequence	retention (ACN%)	relative trNOEs
P22 tailspike	Ac-DYVKFPGIETLL-NH_2	32.5	+++
VSV-C	Ac-KLIGVLSSLFRPK-NH_2	30.7	+++
Rho2-L6	Ac-STKWLLESVRAGK-NH_2	25.5	+++
AL	Ac-YKALSEALKSAK-NH_2	24.5	+++
ID	Ac-YKALAESLKSAK-NH_2	22.0	+++
Rho2	Ac-STKWLAESVRAGK-NH_2	21.7	+++
D-Rho2	Ac-STKWLAESVRAGK-NH_2	20.8	+++
Xhat1	YSTATLSLGHHAVP	20.5	+++
Rho2-5P6	Ac-STKWLPAESVRAGK-NH_2	20.3	+++
L,D-Rho2	Ac-STKWLAESVRAGK-NH_2	20.2	+++
KE	Ac-YKLSAEKLSAAK-NH_2	18.0	++
ES24D	Ac-KRKEVETKSAGDIVLTGSAA-NH_2	17.3	++
ES24G	Ac-KRKEVETKSAGGIVLTGSAA-NH_2	16.8	++
Rho2-P5	Ac-STKWPAESVRAGK-NH_2	16.8	++
"control"	GPENRGDSCA	15.6	++
Rho2-P5G6	Ac-STKWPGESVRAGK-NH_2	15.5	++
CRAPB3	Ac-SKPHVEIRQDGD	12.0	+
KWK	KWK	6.5	+

of the peptide to present a hydrophobic surface in an apolar environment argues that the GroEL-peptide recognition is hydrophobic in nature.

GroEL-Induced Line-Broadening Effects Can Be Reversed by the Addition of ATP. We have taken advantage of the weak affinity of small synthetic peptides for GroEL to observe trNOE and specific line-broadening effects induced by the formation of complexes. However, GroEL is an 800 kDa macromolecular complex and has many potential surfaces for (weak) peptide binding that may not be functionally significant. To confirm that our results were due to peptide binding at the substrate-binding site of GroEL, we took advantage of the modulation of substrate affinity effected by ATP binding (*41*−*43*), and measured the spin−spin relaxation time (T_2) of several resonances from the peptide Rho2 in three different conditions: (1) peptide only, (2) peptide and GroEL, and (3) peptide with GroEL/ATP, in a buffer containing 40 mM sodium phosphate at pH 6.0. In these experiments, the peptide concentration was 1.5 mM, GroEL monomer concentration was 50 μM, and the final ATP concentration was 4 mM. It has been reported that ATP-binding induces conformational changes in the apical domain of GroEL (*44*), resulting in decreased substrate binding (*41*−*43*), and that in the absence of K^+, the ATP hydrolysis rate is greatly reduced—by a factor as large as 10000-fold (*45*). Line widths of NMR resonances are inversly proportional to T_2, so T_2 measurements represent a different way to quantify the effects of GroEL on peptides. If the peptide binding to GroEL we observed in the NMR experiments is functionally significant, we expect the T_2 relaxation effects to be reversed by the addition of ATP. This is indeed the case (see Table 3): upon addition of GroEL, there was an overall decrease of T_2 values for the peptide resonances; further addition of ATP partially reversed the trend. Significantly, the effects of GroEL or ATP are not uniform throughout the peptide: in both cases, the hydrophobic residues experienced more pronounced changes than the hydrophilic ones. Both observations are consistent with the notion that the peptide is bound in a sided manner at the apical domain of GroEL. It is worth noting that, among many other factors, T_2 is affected by the viscosity of the solution. The effect of GroEL addition on the overall viscosity of the NMR sample is reflected by the change in T_2 of the C-terminal residue (K13), which serves as an internal control in our experiments.

Table 3: T_2 Values of Selected Rho2 Resonances

resonance	peptide only[a]	peptide + GroEL[a]	peptide + GroEL/Mg-ATP[a,b]
L5δ	230 ± 20	73 ± 3	93 ± 4
L5N	130 ± 10	38 ± 1	54 ± 2
W5Nϵ	138 ± 4	58 ± 2	73 ± 6
G12α	202 ± 5	103 ± 3	118 ± 3
K13ϵ	410 ± 10	281 ± 8	272 ± 6

[a] The units for the T_2 values are in msec. Error for each value is estimated from the uncertainty of the fitted parameter in the nonlinear fitting algorithm. [b] The concentration ratio of Mg^{2+}:ATP was 1:1. GroES was not included in the experiments.

DISCUSSION

We have demonstrated that GroEL binds many synthetic peptides, which provides a system to mimic the promiscuity of GroEL-substrate recognition. By systematically varying the seqeunce and the conformational propensity of these peptides, we showed that the most important determinant for GroEL-substrate interaction is the presentation of a hydrophobic surface, and the presentation of such surfaces offers the common motif for promiscuous binding: only those peptides with a high tendency to present a hydrophobic surface demonstrate high affinity for GroEL binding. In an efficient assisted-folding reaction cycle, it is essential that GroEL bind only the unfolded or the partially folded forms but not the native state of proteins. The features that characterize the early folding intermediate such as a molten globule or compact intermediate include a high content of secondary structure, few tertiary interactions, and exposed hydrophobic surfaces. Studies on the interactions between GroEL and a model substrate, α-lactalbumin (αLA), showed that GroEL does not recognize the classical molten globule, the apo-αLA (*46*), but it binds the less organized forms of the same state when one or more of the native disulfide bonds are reduced (*47*, *48*). It is likely that disruption of one or more of the four native disulfide bonds allows flexibility in the apo-αLA chain and enables the protein to expose hydrophobic surfaces for binding. In another study, using tips functionalized with proteins in an atomic force microscope, Vinckier and co-workers showed that hydrophobic

modifications to the tips increase the attractive force to GroEL in an ATP-dependent manner (*49*). These results are consistent with our current study, although our conclusion derives from using synthetic peptides to mimic substrates.

Recent studies using whole proteins as substrates with hydrogen exchange followed by either NMR or mass spectrometry suggest that GroEL recognizes conformations similar to early folding intermediates (*50*−*52*). However, it is difficult to determine from these studies which residues are in contact with GroEL, and in what conformations these residues are presented to GroEL. Using small peptides in conjunction with trNOE analysis, we determined that GroEL is capable of binding substrates in α-helical as well as β-strand conformations. This observation is consistent with the conclusion derived from the crystal structure of GroEL that the central cavity presents a wall of hydrophobic residues for binding but lacks grooves or pockets that recognize defined secondary structures (*5, 53*). This observation is also supported by reports that GroEL binds the folding intermediate of an all β-protein (*54*), and that the binding site of a mutant form of β-lactamase is most likely a β-sheet in the C-terminal region of the protein (*55*). Recent studies using peptides as substrate mimetics have provided an opportunity to examine the sequence requirement for GroEL binding. Alignment of the peptides that are known to interact with GroEL has led to the conclusion that there are no consensus sequence for binding, only an enrichment of hydrophobic residues (*53, 56, 57*). These studies also showed that GroEL exhibits a preference for peptides bearing net positive charges, indicating an electrostatic component in the GroEL-substrate interactions (*53, 58*) that were shown to be important for some proteins as substrates (*48, 59*−*61*). In our current work, we showed that there are no backbone conformation requirements for substrate recognition by GroEL, a conclusion that is corroborated by an independent study using fluorescently labeled peptides (*62*). Since similar conclusions have been drawn from experiments using either whole proteins or peptides as substrates, we believe it is a valid approach to use small synthetic peptides to obtain a detailed structural description of substrates when bound to GroEL.

The most difficult aspect of using peptides as substrates is to demonstrate that binding occurs at the central cavity, the site that has been shown to be functionally relevant (*26, 63*). Our experiment using ATP to reverse the line-broadening induced by binding to GroEL produced small but statistically significant effects and supported the interpretation that we were observing such functional interactions. However, even in the presence of 80-fold excess of ATP over GroEL, the line-broadening effects are not completely reversed (see Table 3). Part of the broadening probably comes from the change in viscosity of the sample upon addition of GroEL, but other explanations may also contribute. (1) ATP binds to the GroEL double-ring complex with positive cooperativity within one ring, but with negative cooperativity between the two rings (*64*). This nested cooperativity sets up GroEL to be intrinsically asymmetric, and this asymmetry is an integral part to the two-stroke folding machinery mechanism (*8, 65*). The ramification of the negative cooperativity for ATP between the rings is that we might not have saturated all the ATP binding sites in GroEL to convert both rings to low affinity simultaneously. (*2*) The crystal structure of GroEL complexed with nucleotides and GroES indicates that the conformational changes resulting from GroES binding lock the nucleotides in their binding pockets and commit the cis cavity (the cavity under GroES) for refolding (*8, 66*). In the absence of GroES, ATP binding to GroEL induces conformational changes reminiscent of those observed in the crystal structure, but perhaps in a dynamic manner (*44, 67*). The crystal structure of GroEL complexed with nucleotide only suggests that ATP may still be able to diffuse out of its binding pocket in this state. Binding to GroES therefore coordinates and locks the conformational changes of GroEL in a cooperative manner. Without GroES, release from the binding sites of GroEL may not be cooperative, and this could result in the partial reversal of the line-broadening effects.

The hsp70 and hsp60 families of proteins constitute a network of chaperones to assist folding inside the cells (*68*). In some cases, hsp70s and hsp60s work in concert, and the substrates are handed off in a vectorial fashion (*69, 70*), while in others, the substrates are shuttled back and forth between the two families of chaperones, and the assisted folding is acheived with the hsp70s and their cohorts, DnaJ and GrpE homologues (*68, 71, 72*). It is becoming increasingly apparent that hsp70s and hsp60s recognize the different structural features of unfolded and partially folded polypeptides. Results on the peptide binding specificity of hsp70s point to a common binding motif for hsp70: short (around 7−8 amino acid residues) sequences enriched in hydrophobic residues (*73*−*75*). The substrate is bound in an extended conformation (*28, 76*) with a long aliphatic side chain buried in the single hydrophobic pocket in the binding site, and the complex is stabilized by hydrophobic interactions as well as many backbone NH/CO hydrogen bonds between the substrate and the chaperone (*76*). We demonstrate that GroEL binds preferentially to peptides which are capable of presenting a hydrophobic surface in a variety of backbone conformations, with the side chains on the peptides mediating the stabilizing interactions for the complex. This result is consistent with the ability of hsp60s to recognize folding intermediates, such as a molten globule or a partially collapsed state. Thus, the peptide-binding properties of hsp70s and GroEL bear a general similarity of hydrophobic interaction, but differ in detailed aspects. Interestingly, our preliminary NMR data on the binding of the Xhat1 (C → S) peptide to the C-terminal peptide-binding domain of BiP, which is an hsp70 family member in the endoplasmic reticulum (ER) of eukaryotic cells (*77*), show that the binding is likely to be localized to only part of the peptide sequence. However, it seems that the entire peptide sequence participates in binding to GroEL. This is consistent with the observation that an increase in length over 7 amino acid residues does not result in a higher affinity between the hsp70s and their substrates, while the minimal length of a substrate that can be co-purified with GroEL through gel filtration is ∼60 amino acid residues (*78*). GroEL has a large binding surface for substrate proteins compared to that of DnaK (*5, 76*), and multiple apical domains from the same ring may engage in substrate binding simultaneously (*79*). A systematic comparison of the binding of hsp70s and hsp60s for the same set of model peptides should improve our current understanding of the general and specific recognition

mechanisms of molecular chaperones in the assisted protein folding pathways.

ACKNOWLEDGMENT

We thank Teddy Scott for his help with several experiments and Muppalla Sukumar for his expert advice on transferred NOE analysis.

REFERENCES

1. Ellis, R. J. (1996) in *Chaperonins* (Ellis, R. J., Ed.) Academic Press Inc, San Diego, pp 2−23.
2. Zeilstra-Ryalls, J., Fayet, O., and Georgopoulos, C. (1991) *Annu. Rev. Microbiol. 53*, 301−325.
3. Hemmingsen, S. M., Woolford, C., van der Vies, S. M., Tilly, K., Dennis, D. T., Georgopoulos, C. P., Hendrix, R. W., and Ellis, R. J. (1988) *Nature 333*, 330−334.
4. Hendrix, R. W. (1979) *J. Mol. Biol. 129*, 375−392.
5. Braig, K., Otwinowski, Z., Hegde, R., Boisvert, D. C., Joachimiak, A., Horwich, A. L., and Sigler, P. B. (1995) *Nature 371*, 578−586.
6. Georgopoulos, C., and Ang, D. (1990) *Semin. Cell Biol. 1*, 19−25.
7. Boisvert, D. C., Wang, J., Otwinowski, Z., Horwich, A. L., and Sigler, P. B. (1996) *Nat. Struct. Biol. 3*, 170−177.
8. Xu, Z., Horwich, A. L., and Sigler, P. B. (1997) *Nature 388*, 74−80.
9. Kusukawa, N., Yura, T., Ueguchi, C., and Akiyama, Y. (1989) *EMBO J. 8*, 3517−3521.
10. van Dyk, T. K., Gatenby, A. A., and LaRossa, R. A. (1989) *Nature 342*, 451−453.
11. Goloubinoff, P., Gatenby, A. A., and Lorimer, G. H. (1989) *Nature 337*, 44−47.
12. Ewalt, K. L., Hendrick, J. P., Houry, W. A., and Hartl, F. U. (1997) *Cell 90*, 491−500.
13. Horwich, A. L., Low, K. B., Fenton, W. A., Hirshfield, I. N., and Furtak, K. (1993) *Cell 74*, 909−917.
14. Goloubinoff, P., Christeller, J. T., Gatenby, A. A., and Lorimer, G. H. (1989) *Nature 342*, 884−889.
15. Büchner, J., Schmidt, M., Fuchs, M., Jaenicke, R., Rudolph, R., Schmid, F. X., and Kiefhaber, T. (1991) *Biochemistry 30*, 1586−1591.
16. Zhi, W., Landry, S. J., Gierasch, L. M., and Srere, P. A. (1992) *Protein Sci. 1*, 522−529.
17. Zheng, X., Rosenberg, L. E., Kalousek, F., and Fenton, W. A. (1993) *J. Biol. Chem. 268*, 7489−7493.
18. Mendoza, J. A., Rogers, E., Lorimer, G. H., and Horowitz, P. M. (1991) *J. Biol. Chem. 266*, 13044−13049.
19. Viitanen, P. V., Gatenby, A. A., and Lorimer, G. H. (1992) *Protein Sci. 1*, 363−369.
20. Ellis, R. J. (1994) *Curr. Opin. Struct. Biol. 4*, 117−122.
21. Gething, M. J., and Sambrook, J. (1992) *Nature 355*, 33−45.
22. Georgopoulos, C., and Welch, W. J. (1993) *Annu. Rev. Cell Biol. 9*, 601−634.
23. Horwich, A. L., and Fenton, W. A. (1997) *Protein Sci. 6*, 743−760.
24. Landry, S. J., and Gierasch, L. M. (1994) *Annu. Rev. Biophys. Biomol. Struct. 23*, 645−669.
25. Lin, Z., Schwartz, F. P., and Eisenstein, E. (1995) *J. Biol. Chem. 270*, 1011−1014.
26. Fenton, W. A., Kashi, Y., Furtak, K., and Horwich, A. L. (1994) *Nature 371*, 614−619.
27. Landry, S. J., and Gierasch, L. M. (1991) *Biochemistry 30*, 7359−7362.
28. Landry, S. J., Jordan, R., McMacken, R., and Gierasch, L. M. (1992) *Nature 355*, 455−457.
29. Landry, S. J., Zeilstra-Ryalls, J., Fayet, O., Georgopoulos, C., and Gierasch, L. M. (1993) *Nature 364*, 255−258.
30. Braun, S., Kalinowski, H.-O., and Berger, S. (1998) *150 and more basic NMR experiments: a practical approach*, Wiley-VCH, New York.
31. DeGrado, W. F., Wasserman, Z. R., and Lear, J. D. (1989) *Science 243*, 622−628.
32. Weis, W. I., Brünger, A. T., Skehel, J. J., and Wiley, D. C. (1991) *J. Mol. Biol. 212*, 737−761.
33. Kleywegt, G. J., Bergfors, T., Senn, H., Le Motte, P., Gsell, B., Shudo, K., and Jones, T. A. (1994) *Structure 2*, 1241−1258.
34. Nelson, J. W., and Kallenbach, N. R. (1986) *Proteins: Struct., Funct., Genet. 1*, 211−217.
35. Lehrman, S. R., Tuls, J. L., and Lund, M. (1990) *Biochemistry 29*, 5590−5596.
36. Chen, Y., and Wallace, B. A. (1997) *Biopolymers 42*, 771−781.
37. Clore, G. M., and Gronenborn, A. M. (1982) *J. Magn. Reson. 48*, 402−417.
38. Campbell, A. P., and Sykes, B. D. (1993) *Annu. Rev. Biophys. Biomol. Struct. 22*, 99−122.
39. Wüthrich, K. (1986) *NMR of proteins and nucleic acids*, J. Wiley & Sons, New York.
40. Buttner, K., Blondelle, S. E., Ostresh, J. M., and Houghten, R. A. (1992) *Biopolymers 32*, 575−583.
41. Laminet, A. A., Ziegelhoffer, T., Georgopoulos, C., and Plückthun, A. (1990) *EMBO J. 9*, 2315−2319.
42. Martin, J., Langer, T., Boteva, R., Schramel, A., Horwich, A. L., and Hartl, F. U. (1991) *Nature 352*, 36−42.
43. Viitanen, P. V., Donaldson, G. K., Lorimer, G. H., Lubben, T. H., and Gatenby, A. A. (1991) *Biochemistry 30*, 9716−9723.
44. Roseman, A. M., Chen, S., White, H., Braig, K., and Saibil, H. R. (1996) *Cell 87*, 251−251.
45. Todd, M. J., Viitanen, P. V., and Lorimer, G. H. (1993) *Biochemistry 32*, 8560−8567.
46. Okazaki, A., Ikura, T., Nikaido, K., and Kuwajima, K. (1994) *Nat. Struct. Biol. 1*, 439−445.
47. Hayer-Hartl, M. K., Ewbank, J. J., Creighton, T. E., and Hartl, F. U. (1994) *EMBO J. 13*, 3192−3202.
48. Aoki, K., Taguchi, H., Shindo, Y., Yoshido, M., Ogasahara, K., Yutani, K., and Tanaka, N. (1997) *J. Biol. Chem. 272*, 32158−32162.
49. Vinckier, A., Gervasoni, P., Zaugg, F., Ziegler, U., Lindner, P., Groscurth, P., Plückthun, A., and Semenza, G. (1998) *Biophys. J. 74*, 3256−3263.
50. Robinson, C. V., Grosse, M., Eyles, S. J., Ewbank, J. J., Mayhew, M., Hartl, F. U., Dobson, C. M., and Radford, S. E. (1994) *Nature 372*, 646−651.
51. Grosse, M., Robinson, C. V., Mayhew, M., Hartl, F. U., and Radford, S. E. (1996) *Protein Sci. 5*, 2506−2513.
52. Goldberg, M. S., Zhang, J., Sondek, S., Matthews, C. R., Fox, R. O., and Horwich, A. L. (1997) *Proc. Natl. Acad. Sci. 94*, 1080−1085.
53. Coyle, J. E., Jaeger, J., Grosse, M., Robinson, C. V., and Radford, S. E. (1998) *Folding Des. R93−R104*.
54. Schmidt, M., and Büchner, J. (1992) *J. Biol. Chem. 267*, 16829−16833.
55. Gervasoni, P., Staudenmann, W., James, P., and Plückthun, A. (1998) *Biochem. 37*, 11660−11669.
56. Richarme, G., and Kohiyama, M. (1994) *J. Biol. Chem. 269*, 7095−7098.
57. Brazil, B. T., Cleland, J. L., McDowell, R. C., Skelton, N. J., Paris, K., and Horowitz, P. M. (1997) *J. Biol. Chem.* 21, 5105−5111.
58. Hutchinson, J. P., Oldham, T. C., ElThaher, T. S. H., and Miller, A. D. (1997) *J. Chem. Soc., Perkin Trans. 2*, 279−288.
59. Katsumata, K., Okazaki, A., and Kuwajima, K. (1996) *J. Mol. Biol. 258*, 827−838.
60. Sparrer, H., Lilie, H., and Büchner, J. (1996) *J. Mol. Biol. 258*, 74−87.
61. Perrett, S., Zahn, R., Stenberg, G., and Fersht, A. R. (1997) *J. Mol. Biol. 269*, 892−901.
62. Preuss, M., Hutchinson, J. P., and Miller, A. D. (1999) *Biochemistry 38*, 10272−10286.
63. Langer, T., Pfeifer, G., Martin, J., Baumeister, W., and Hartl, F. U. (1992) *EMBO J. 11*, 4757−4765.

64. Yifrach, O., and Horovitz, A. (1995) *Biochemistry 34*, 5303−5308.
65. Burston, S. G., Ranson, N. A., and Clarke, A. R. (1995) *J. Mol. Biol. 249*, 138−152.
66. Rye, H. S., Burston, S. G., Fenton, W. A., Beecham, J. M., Xu, Z., Sigler, P. B., and Horwich, A. L. (1997) *Nature 388*, 792−798.
67. Xu, Z., and Sigler, P. B. (1998) *J. Struct. Biol. 124*, 129−141.
68. Buchberger, A., Schröder, H., Hesterkamp, T., Schönfeld, H.-J., and Bukau, B. (1996) *J. Mol. Biol. 261*, 328−333.
69. Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M. K., and Hartl, F. U. (1992) *Nature 356*, 683−689.
70. Gragerov, A., Nudler, E., Komissarova, N., and Gaitanaris, G. A. (1992) *Proc. Natl. Acad. Sci. 89*, 10341−10344.
71. Freeman, B. C., and Morimoto, R. I. (1996) *EMBO J. 15*, 2969−2979.
72. Ayling, A., and Baneyx, F. (1996) *Prot. Sci. 5*, 478−487.
73. Flynn, G. C., Pohl, J., Flocco, M. T., and Rothman, J. E. (1991) *Nature 353*, 726−730.
74. Blond-Elguindi, S., Cwiria, S. E., Dower, W. J., Lipshutz, R. J., Sprang, S. R., Sambrook, J. F., and Gething, M.-J. H. (1993) *Cell 75*, 717−728.
75. Gragerov, A., Zeng, L., Zhao, X., Burkholder, W., and Gottesman, M. E. (1994) *J. Mol. Biol. 235*, 848−854.
76. Zhu, X., Zhao, X., Burkholder, W. F., Gragerov, A., Ogata, C. M., Gottesman, M. E., and Hendrickson, W. A. (1996) *Science 272*, 1606−1614.
77. Munro, S., and Pelham, H. R. B. (1986) *Cell 46*, 290−300.
78. Hlodan, R., Tempst, P., and Hartl, F. U. (1995) *Nat. Struct. Biol. 2*, 587−595.
79. Thiyagarajan, P., Henderson, S. J., and Joachimiak, A. (1996) *Structure 4*, 79−88.

BI991070P