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Multiple Cycles of Global Unfolding of GroEL-bound Cyclophilin A Evidenced by NMR

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GroE, the chaperonin system of *Escherichia coli*, prevents the aggregation of partially folded or misfolded proteins by complexing them in a form competent for subsequent folding to the native state. We examined the exchange of amide protons of cyclophilin A (CypA) interacting with GroEL, using NMR spectroscopy. We have applied labeling pulses in H_2O to the deuterated GroEL-CypA-complex. When ATP and GroES were added after the labeling pulse, refolding of CypA could be accelerated to rates comparable to the amide proton exchange. This allowed the calculation of protection factors (*PF*) for the backbone amide protons in the GroEL-bound substrate protein. A set of highly protected protons in the native state (*PF* 10^5 to 10^7) was observed to be much less protected (*PF* 10^2 to 10^4) in complex with GroEL and, in contrast to the native structure, the protection factors were found to be quite uniform along the sequence suggesting that CypA with native-like structure undergoes multiple cycles of unfolding while bound to GroEL, which are faster than unfolding in free solution. Because of the small sequence dependence of the protection factors, unfolding must be global, and in this way the chaperone appears to resolve off-pathway intermediates and to support protein folding by annealing. Although in the complex with GroEL native-like states still predominate over globally unfolded states, this equilibrium is shifted 10^2 to 10^4-fold toward the unfolded state when compared to CypA in free solution. Repeated global unfolding may be a key step in achieving a high yield of correctly folded proteins.

Keywords: protein folding; amide proton exchange and NMR; molecular chaperones; GroEL; cyclophilin A

Introduction

Protein folding *in vivo* is mediated by helper proteins, the ''molecular chaperones'' (Gething & Sambrook, 1992). Among the best characterized in terms of structure and function are the *Escherichia coli* chaperonins GroEL and GroES. GroEL is a large complex of 14 identical 57 kD subunits that form a double toroid with a central cavity (Saibil *et al.*, 1993; Braig *et al.*, 1994; Roseman *et al.*, 1996). Each subunit is made up of three domains, a large equatorial domain forming the central core of the ring structure and containing the nucleotide binding site, a small intermediate domain and a large, flexible apical domain. The apical domain contains the binding sites for non-native proteins and GroES (Chen *et al.*, 1994; Fenton *et al.*, 1994). By binding to unfolded proteins, mainly by hydrophobic interactions (Fenton *et al.*, 1994; Zahn & Plückthun, 1994; Zahn *et al.*, 1994a; Braig *et al.*, 1995; Lin *et al.*, 1995), GroEL creates an environment with high affinity for non-native proteins. By reversible binding of aggregation-prone intermediates the aggregation of the polypeptide is prevented (Goloubinoff *et al.*, 1989; Buchner *et al.*, 1991; Zahn & Plückthun, 1994).

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Abbreviations used: 2D, two-dimensional; CypA, cyclophilin A; D, ^{2}H; *DF*, deprotection factor; DTT, dithiothreitol; H, ^{1}H; HSQC, heteronuclear single quantum coherence; MES, 2-[*N*-morpholino]-ethane sulphonate; nat, native; NMR, nuclear magnetic resonance; *PF*, protection factor; PPIase, peptidyl-prolyl *cit/trans*-isomerase; rc, random coil.

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GroES is a dome-like heptamer with 10 kDa subunits (Hunt *et al.*, 1996) that binds to one end of the GroEL-cylinder in the presence of adenine nucleotides, thus forming an asymmetric complex which contains two distinct substrate binding sites on the two toroids (Chen *et al.*, 1994). Initially, non-native proteins bind to the side opposite from GroES, but they can be enclosed by subsequent binding of GroES to the same side, and folding of substrate proteins may occur at least partially in the complex (Zahn & Plückthun, 1992; Gray & Fersht, 1993; Weissman *et al.*, 1995, 1996; Mayhew *et al.*, 1996). GroEL may unfold proteins because it binds with high affinity to the unfolded form and thereby simply shifts the equilibrium towards the unfolded state (Laminet *et al.*, 1990; Walter *et al.*, 1996).

Binding and release of substrate proteins and GroES are controlled by the ATPase activity of GroEL (Martin *et al.*, 1993; Todd *et al.*, 1994; Weissman *et al.*, 1994; Burston *et al.*, 1995). Negative cooperativity of substrate and GroES binding has been demonstrated: when one ring is occupied, the second ring has lower affinity for substrate and GroES (Chen *et al.*, 1994; Todd *et al.*, 1994; Yifrach & Horovitz, 1996). GroEL has high affinity for non-native proteins in the absence of nucleotides or in the presence of ADP, but low affinity in the presence of ATP (Yifrach & Horovitz, 1996). ATP and GroES cause dissociation of stable GroEL-protein-complexes, and ATP turnover is observed in concert with cycles of substrate binding and release (Todd *et al.*, 1994).

Although many detailed biochemical studies have been performed in order to understand the molecular mechanism of GroEL action, very little is known as yet about the conformational properties of non-native proteins bound to the chaperonin. Because of the large size of the complex, these studies are inherently difficult. However, amide proton exchange studies in combination with two-dimensional (2D) nuclear magnetic resonance (NMR) spectroscopy have been shown to be a powerful tool to investigate the structure of folding intermediates (Roder & Wüthrich, 1986; Roder *et al.*, 1988; Udgaonkar & Baldwin, 1988). Proton/deuterium-exchange experiments monitored by 2D NMR have been used to study the conformation of GroEL-bound cyclophilin A (CypA) (Zahn *et al.*, 1994b) and barnase (Zahn *et al.*, 1996a,b). For α-lactalbumin (Robinson *et al.*, 1994), β-lactamase (Gervasoni *et al.*, 1996) and dihydrofolate reductase (DHFR) (Groß *et al.*, 1996) similar experiments were monitored by mass spectrometry. Amide proton exchange may be slowed down by hydrogen bonding, steric blockage and electrostatic interactions in folded proteins, as expressed by a protection factor (*PF*) that can be related to the local stability of secondary or tertiary structure (Wüthrich, 1986; Bai *et al.*, 1994). From these studies, a wide range of conformational states has been proposed for different substrate proteins. In the case of α-lactalbumin with one of its disulfide bonds reduced and the other three reshuffled, the bound protein was weakly protected from exchange to an extent similar to that of an uncomplexed molten globule state (Robinson *et al.*, 1994). For β-lactamase very slow exchange rates were observed, suggesting that a native-like state, yet devoid of enzymatic activity, is bound at high temperature (Gervasoni *et al.*, 1996). In GroEL-bound DHFR some 20 deuterons remained, demonstrating that there are sites highly protected from hydrogen exchange (Groß *et al.*, 1996). For barnase, the exchange of deeply buried amide protons was shown to be accelerated by catalytic amounts of GroEL (Zahn *et al.*, 1996a).

In the present studies, CypA was used as substrate protein, because it had been extensively characterized by NMR and crystallography (Wüthrich *et al.*, 1991; Kallen *et al.*, 1991; Braun *et al.*, 1995; Ottiger *et al.*, 1997) and its peptidyl-prolyl *cis/trans*-isomerase (PPIase) activity (Fischer *et al.*, 1984) can readily be measured. Native CypA possesses a number of very slowly exchanging amide protons, which can be used to test the substrate protein for exchange enhancement when bound to GroEL. It was previously shown that all amide protons of CypA exchange with deuterium during three association-dissociation cycles with GroEL, indicating that the complete secondary structure of CypA is considerably destabilized in the complex with GroEL (Zahn *et al.*, 1994b). In order to get more detailed information about amide proton exchange in the GroEL-bound polypeptide, we extended the previous NMR experiments by inclusion of a labeling pulse of variable length (two minutes and 15 minutes) in H_2O after CypA was deuterated during three association-dissociation cycles. From the changes in signal intensities during the labeling pulse, amide proton exchange rates in GroEL-bound CypA were calculated and compared with exchange rates in the native CypA and in random coil polypeptides to obtain refined information on the conformational state of the bound protein.

Results

As a reference for structural interpretations of the amide proton exchange data presented in this paper, Figure 1 shows the locations of the regular secondary structural elements in the three-dimensional structure of native CypA (Figure 1a). All amide protons with exchange rates smaller than 1×10^{-3} min^{-1} in the native conformation (Figure 1b) are involved in backbone-backbone hydrogen bonds in regular secondary structural elements or turns (Ottiger *et al.*, 1997).

Influence of pH on binding of CypA to GroEL

In order to establish conditions of maximal CypA-GroEL-complex formation without irreversible denaturation of the substrate protein upon release, we examined the complex formation and

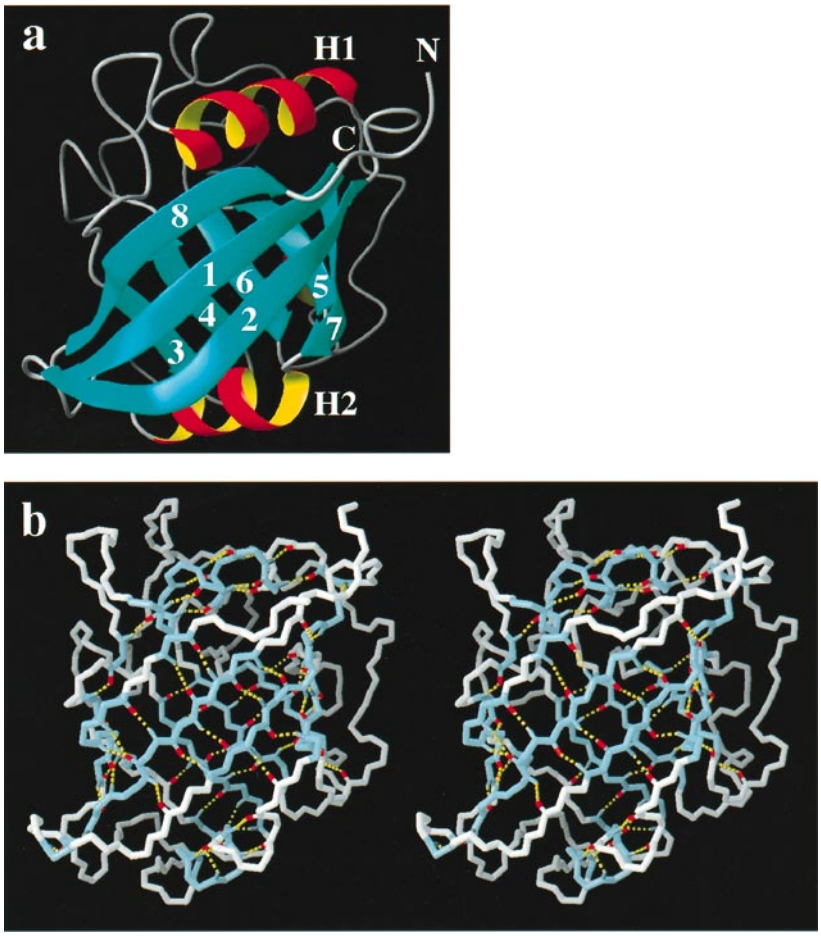

Figure 1. Views of the polypeptide backbone of human CypA. (a) Ribbon drawing of the structure of CypA including eight β-strands, labeled sequentially (residues 5 to 12, 15 to 24, 53 to 57, 61 to 64, 97 to 100, 112 to 115, 127 to 134 and 156 to 163), two α-helices, labeled H1 and H2 (residues 30 to 41, 136 to 145) and a short 3_{10}-helix (residues 120 to 122). (b) Stereo view of the polypeptide backbone in one of the 20 conformers used to represent the 3D NMR solution structure of CypA (Ottiger *et al.*, 1997). The backbone atoms N, C^{α} and C′ are shown for all residues. The 56 backbone amide protons with slow exchange on the time scale of the experiments used are also shown and the corresponding residues (6 to 12, 15, 20 to 25, 29, 33 to 41, 48, 51, 53, 57, 60 to 64, 97 to 100, 112 to 115, 129 to 133, 139 to 143, 157 to 160, 162) are colored in blue. The hydrogen bonds which show slow amide proton exchange are represented by yellow broken lines, and the acceptor CO group is shown in red. The orientation of the molecule is the same as in (a). These drawings were prepared with the program MOLMOL (Koradi *et al.*, 1996).

inactivation of CypA in the presence of equimolar amounts of GroEL by measuring the enzymatic activity during complex formation. The percentage of complexed, inactive CypA is strongly dependent on pH (Figure 2a). The lower the pH, the more complex is formed. These results are consistent with the observed decrease of the melting temperature of CypA with pH (data not shown). Below pH 5.0, however, CypA aggregates irreversibly and GroEL loses function. An important additional observation with regard to proton exchange studies is that complex formation between GroEL and CypA in H_2O and ^2H_2O leads to the same results, when identical pH and pD_{corr} values are used (Figure 2b). pD_{corr} is the value which has been corrected for the isotope effect at the glass electrode: $pD_{corr} = pD_{read} + 0.4$ (Glasoe & Long, 1960). Unless stated otherwise, we always mean pD_{corr} when referring to pD.

Kinetic analysis of the refolding of CypA from the complex with GroEL under various conditions

Dissociation of the GroEL-CypA-complex can in principle be achieved either by lowering the temperature, or by the addition of GroES and either ATP or ADP. In the absence of nucleotides, refolding of CypA from the complex with GroEL was found to be very slow, with a rate of 1.4×10^{-3} min^{-1} at 6°C (Table 1), which is in good agreement with earlier measurements (Zahn *et al.*, 1994b). The yield of active protein was only 74% under these conditions. However, to apply a labeling pulse to GroEL-bound CypA with a sharp end point, refolding has to be fast compared to the amide proton exchange. In search for suitable conditions we measured refolding kinetics in the presence of nucleotides and the co-chaperonin GroES at different temperatures (Table 1). Under all conditions tested, the recovery of CypA activity follows first order kinetics. The addition of ADP to the refolding buffer increases the rate to 3.5×10^{-3} min^{-1} and the yield to 78%, while ATP gives 4.8×10^{-3} min^{-1} and 88% yield. If, additionally, GroES is present during refolding, the yield reaches about 100%, and the rates increase by another factor of 4 to 6. At higher temperatures refolding becomes even faster: at 30°C in the presence of GroES in addition to either ADP or ATP reactivation reaches rates as high as 5.6×10^{-1} min^{-1} and 7.2×10^{-1} min^{-1}, respectively. The results do not depend significantly on whether ADP or ATP is used, leading to the conclusion that no cycling of GroES is necessary for the release of active CypA from a kinetically stable CypA-GroEL-complex. The fastest reactivation rate in Table 1 is now only 140-fold slower than the amide proton exchange in a com-

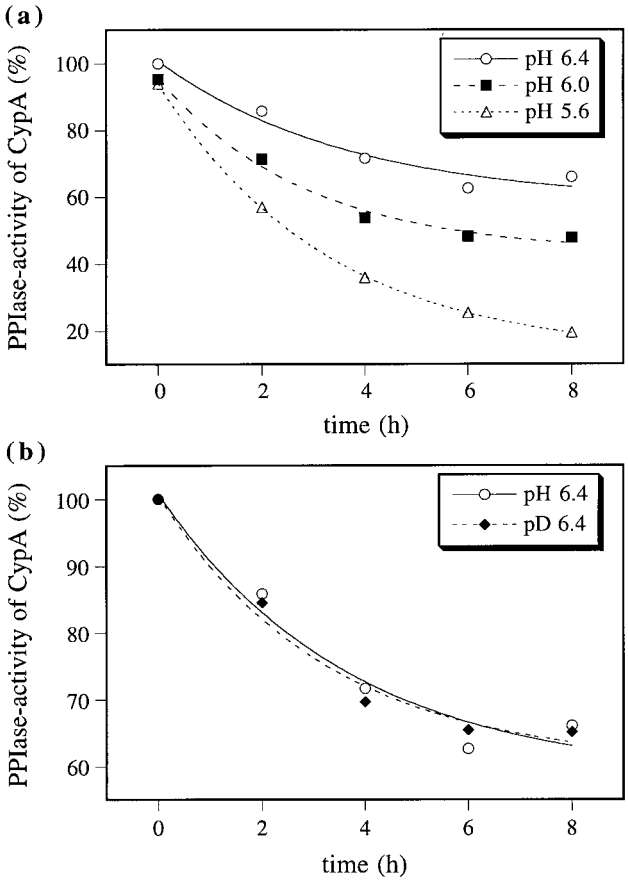

Figure 2. GroEL-dependent inactivation of CypA. The enzymatic activity of an equimolar CypA/GroEL-solution was recorded as a function of time at 30°C. (a) Unfolding in H_2O at pH 6.4, 6.0 and 5.6. (b) Unfolding in H_2O and ^2H_2O at the same values of pH and pD_{corr}, respectively. Under the same conditions, but in the absence of GroEL, the enzymatic activity of CypA at 30°C does not change during the observed time period.

pletely unstructured random coil peptide, thus making the kinetic evaluation of amide proton exchange in a stable GroEL-bound state a realistic proposal.

Experimental design of amide proton exchange studies with GroEL-bound CypA

To characterize the conformation of the GroEL-bound substrate protein, we carried out measurements of amide proton exchange rates by quantification of the $H/^2H$ ratio by NMR (Figure 3). The intended goal of the experiment was to apply short labeling pulses to the GroEL-bound protein, and to determine the exchange rate for each residue in the bound state. From these data we should obtain information on local *versus* global unfolding, and on the conformational stability of CypA while bound to GroEL. In the absence of nucleotides and GroES, binding of CypA to GroEL occurs at 30°C, and dissociation at 6°C (Zahn *et al.*, 1994b). The extent of complex formation is also dependent on pH (Figure 2). Based on these data, we pulse-labeled the deuterated GroEL-bound CypA with protons as outlined in Figure 3. Since CypA can be renatured to recover PPIase activity in good yield only in the presence of GroEL (unpublished results), a mixture of GroEL and CypA was subjected to three association-dissociation cycles in ^2H_2O to ensure that most substrate molecules had been bound at least once (Figure 3, top). Repeated cycles were necessary, because only about 50% of the total CypA were bound to GroEL in each individual cycle under the experimental conditions used: after a solution of 20 μM CypA and 25 μM GroEL in ^2H_2O-buffer was kept at 30°C, pD 6.1 for eight hours and incubated at 6°C for 16 hours (three cycles), about 44% of the initial PPIase activity was retained and 56% of CypA was in complex with GroEL (Figures 4 and 5b). To measure amide proton exchange that had occurred during these cycles, an aliquot of the CypA-GroEL-complex was dissociated at 6°C (Figure 3, A) without any further dilution.

In Figure 3, B and C, an H_2O labeling pulse of variable duration was applied to aliquots 2 and 3 of the complex. The duration is defined by a first dilution of the complex into H_2O, which starts the labeling exchange, and a second dilution into dissociating buffer, which stops the exchange. A mutual control with refolding in either ^2H_2O (Figure 3, B) or H_2O (Figure 3, C) was carried out to check for exchange subsequent to quenching, i.e. after the labeling pulse. The yield of active protein after refolding in the presence of GroES and ATP was 100%. After completed folding, CypA, which was now dissociated from GroEL and exchange-inert at the positions of prime interest here (Wüthrich *et al.*, 1991; Ottiger *et al.*, 1997), was isolated, concentrated and 2D [^{15}N,^1H] single quantum coherence (HSQC) NMR spectra were recorded.

To estimate the optimal pulse length in the experiments of Figure 3, B and C, we calculated the intrinsic random coil exchange rate of each individual amide proton (k_{rc}) from the amino acid sequence of CypA according to Bai *et al.* (1993) (Figure 6b). Most of the very slowly exchanging backbone amide protons ($k_{nat} < 10^{-3}$ min^{-1}) are involved in backbone-backbone hydrogen bonds belonging to regular secondary structure elements (Figures 1 and 6a). The protection factors ($PF = k_{rc}/k_{nat}$) (Figure 6c) are therefore a measure for the stability of the various secondary structures. The average exchange rate in a random coil peptide under the experimental conditions used here (pH 6.0, 30°C) is about 10^2 min^{-1}, which corresponds to $\tau_{1/2} = 6.9 \times 10^{-3}$ min. To obtain detectable exchange, we used time intervals of two minutes and 15 minutes for the labeling in H_2O. In the random coil polypeptide under these conditions, this corresponds to about 285 and 2140 amide proton exchange half lives, respectively. We did not use higher pH and shorter labeling times, since the intrinsic stability of CypA is strongly pH

Table 1. Apparent refolding kinetics of CypA from a GroEL-CypA-complex

Temperature (°C)	6	6	6	6	6	16	30	30
Nucleotides	–	ADP	ATP	ADP	ATP	ADP	ADP	ATP
GroES (μM)	–	–	–	2	2	2	2	2
Rate (min^{-1})	$(1.4 \pm 0.3) \times 10^{-3}$	$(3.5 \pm 0.7) \times 10^{-3}$	$(4.8 \pm 0.7) \times 10^{-3}$	$(2.1 \pm 0.1) \times 10^{-2}$	$(2.0 \pm 0.1) \times 10^{-2}$	$(5.7 \pm 0.3) \times 10^{-2}$	$(5.6 \pm 0.5) \times 10^{-1}$	$(7.2 \pm 0.5) \times 10^{-1}$
Final activity (%)	74 ± 6	78 ± 6	88 ± 6	99 ± 5	103 ± 5	98 ± 5	102 ± 5	104 ± 5

The rate constants for reactivation of CypA from a complex with GroEL were examined in the absence or presence of ADP (1 mM) or ATP (1 mM) and GroES at different temperatures. Refolding was initiated by a 1:20-dilution of the complex into refolding buffer. The starting activity is the enzymatic activity of the equimolar mixture (20 μM) before the dilution. The activity of a 20 μM CypA-solution in the same buffer without addition of GroEL is set to 100%. Standard deviations from duplicate experiments are shown.

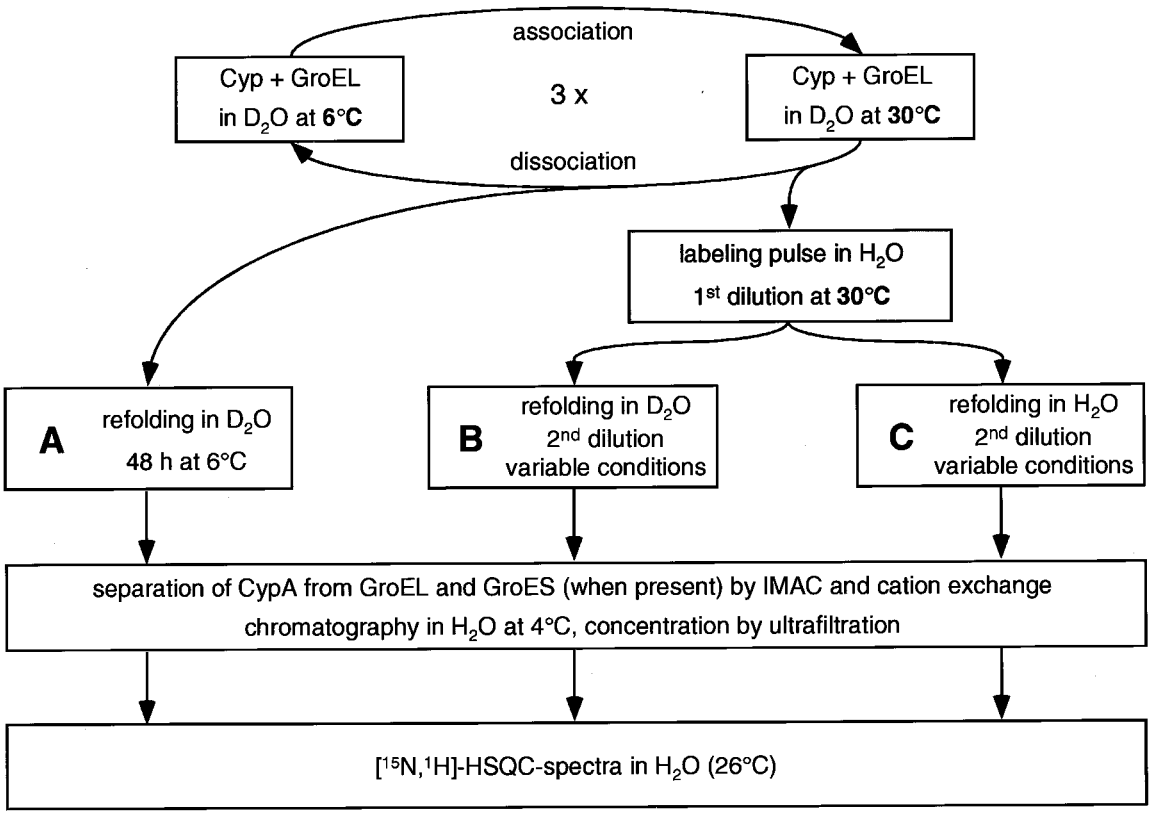

Figure 3. Flow diagram of the NMR experiments used to monitor amide proton exchange of CypA in the presence of GroEL. Depending on the external conditions, the polypeptide associates with or dissociates from GroEL. A mixture of the two proteins is subjected to three association-dissociation cycles in ^2H_2O to ensure maximal ^2H-labeling of all exchangeable amide proton positions. (A) Control: after the third association phase, the complex is dissociated for 48 hours in ^2H_2O to measure the extent of deuteration of CypA during the three temperature cycles. (B) Refolding in ^2H_2O: after the third temperature cycle, the complex is subjected to a labeling pulse (shaded) of variable duration in H_2O under conditions of stable complex maintenance; this is followed by quenching of the exchange by refolding in ^2H_2O. (C) Refolding in H_2O: same as B, except that the quench by refolding is performed in H_2O.

dependent, which could interfere with the interpretation of such data.

Analysis of the NMR data obtained with the pulse labeling experiments

The same general considerations apply for the NMR analysis of the 15 minutes labeling pulse experiment and the two minutes experiment (Figure 5). Before the labeling pulse the signal intensity is taken to be zero, since the protons of GroEL-bound CypA have been exchanged to deuterons (Figure 5). During the labeling pulse in H_2O, deuterons in GroEL-bound CypA are exchanged with protons. CypA refolds when it is diluted into refolding buffer containing ATP and GroES at 30°C, but slowly exchanging protons are still to some extent exchange-competent during the folding step. Because refolding is carried out for one aliquot in ^2H_2O (sample B) and for the other aliquot in H_2O (sample C), exchange should occur in both samples to the same extent, albeit in different directions ($H \rightarrow {}^2H$ *versus* $^2H \rightarrow H$). Taking the isotope effect into account, values for the intensities directly after the labeling pulse, I_X, have been back-calculated from the measured spectra. These manifest the levels of the plateaus B and C on the far right of Figure 5a. Since further exchange during sample preparation can be neglected (see below), proton exchange occurring subsequently to the pulse is limited to the folding reaction (15 to

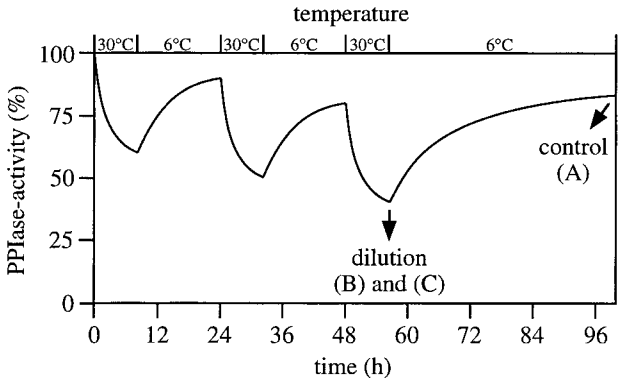

Figure 4. PPIase activity of CypA during the association-dissociation cycling. The activity before the first heating step is set to 100%. The final activity after refolding of sample A (control) at 6°C was 80%.

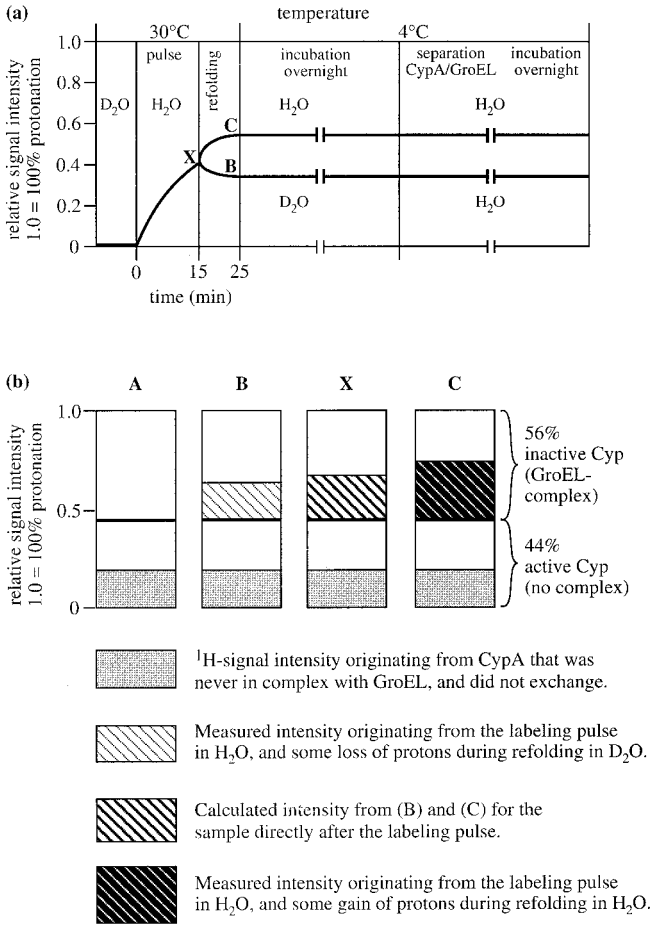

Figure 5. (a) Schematic representation of the protonation state of one slowly exchanging proton position during the different experimental steps of the experiments in Figure 3 B and C. (b) Scheme illustrating the calculation of the protonation in the GroEL-bound protein at the end of the labeling pulse (point X in (a)), as described in Experimental Procedures.

25 minutes in Figure 5a). Assuming monoexponential behavior the exchange rate during the pulse (0 to 15 minutes), where the intensity changes from $I_0 = 0$ to I_X, was calculated with equation (13) (see Experimental Procedures).

Figure 7 shows the relative intensities of the peaks from the [^{15}N,^{1}H]-HSQC spectra obtained from samples A (control, no pulse), B and C (15 minutes labeling pulse). During three association-dissociation cycles, about 80% of all slowly exchanging proton positions became deuterated. This leads to an average signal intensity for the slowly exchanging protons of about 0.2 (Figure 7a). The residual intensities originate from molecules that have never been bound to GroEL (Figure 5b). Compared to the control spectrum, the intensities of the slowly exchanging protons had increased after the 15 minutes labeling pulse (Figure 7b and c), indicating that these positions were solvent-accessible in the complex and could thus exchange during this time. Since folding is not infinitely fast, further exchange during refolding could not be completely avoided (Figure 5). In Figure 7d the differences between the signals from samples B and C are plotted *versus* the amino acid sequence. Most of the negative differences are associated with overlapping peaks, or with broad peaks with small intensities, which are both difficult to integrate. The rapidly exchanging amide protons are all 90% protonated, since the NMR spectra were measured in 90% H_2O/10% ^2H_2O.

After refolding, both samples were stored overnight at 4°C until CypA was separated from GroEL and GroES by chromatography. Under these conditions, no CypA-GroEL-complex is formed. The theoretically expected further change of signal intensities during the preparation of the samples for the NMR measurements was calculated using the exchange rates for native CypA (Ottiger *et al.*, 1997), converted to the actual experimental conditions of pH and temperature during incubation and separation. For the 54 slowly exchanging protons which were used in the present analysis, this anticipated change in signal intensity is less than 2% for 47 positions, and between 2% and 5% for seven positions, and was therefore neglected as indicated by the horizontal lines in Figure 5a.

The analysis of the NMR data had to account for the presence of CypA that had never been in complex with GroEL after three cycles (Figure 3), so that the slowly exchanging protons had not been exchanged (Figure 5b). This residual intensity (15 to 20%, depending on the pH used) is the same for all three samples A, B and C (Figure 3). Furthermore, one has to take into account that during the labeling pulse only 56% of CypA are in complex

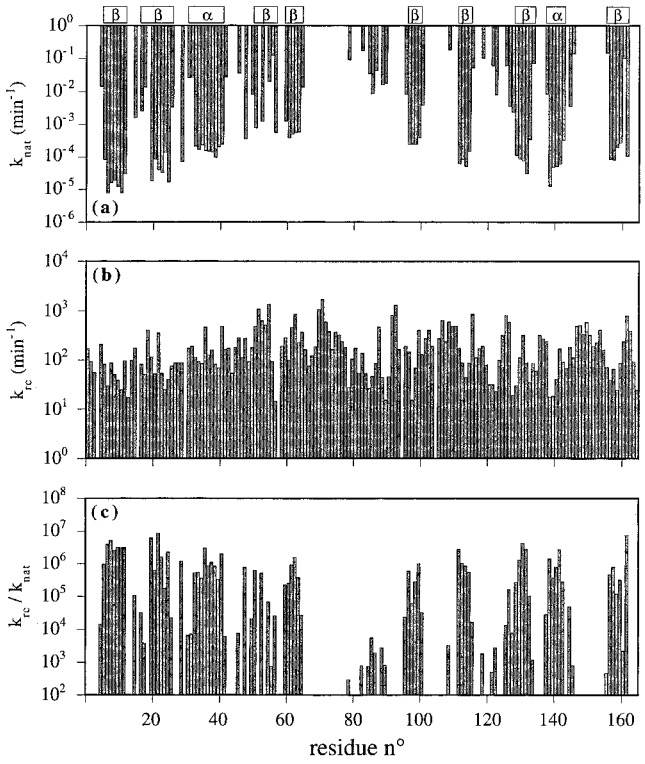

Figure 6. Exchange rates of backbone amide protons in $CypA_{nat}$ (native protein) and $CypA_{rc}$ (random coil) and resulting protection factors plotted against the amino acid sequence. (a) H/^{2}H-exchange rates in native CypA (k_{nat}) at pH 6.0 and 30°C as determined by Ottiger *et al.* (1997). (b) Calculated intrinsic H/^{2}H-exchange rates, k_{rc}, in a random coil polypeptide with the amino acid sequence of CypA (see the text). (c) Protection factors ($PF = k_{rc}/k_{nat}$) for the amide protons in native CypA.

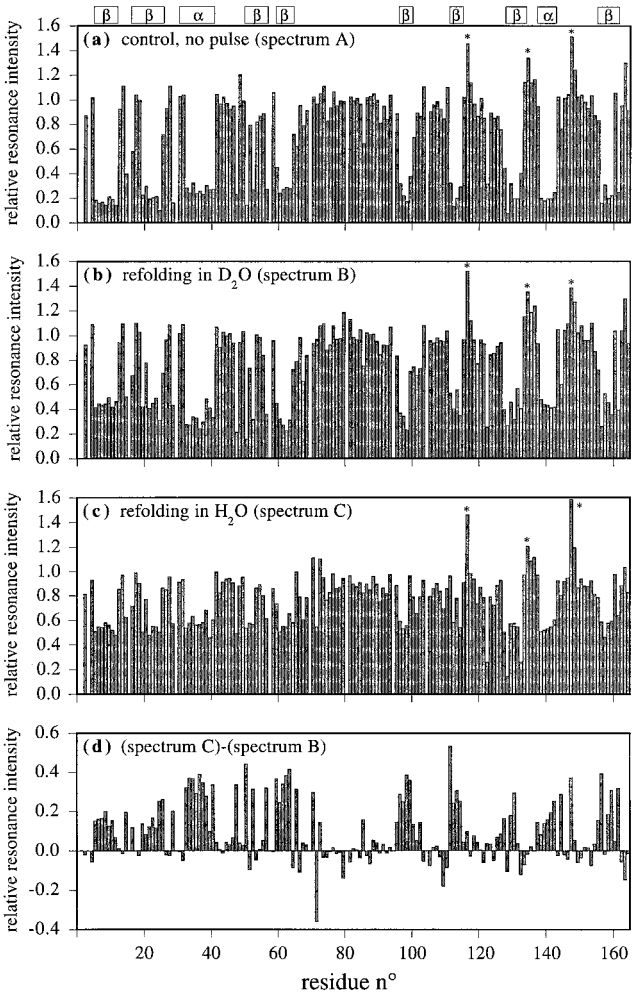

Figure 7. Relative resonance intensities of the peaks from the [^{15}N,^{1}H]-HSQC spectra obtained from samples A to C plotted *versus* the amino acid sequence of CypA. All spectra were normalized by dividing by the corresponding peak volumes from a reference spectrum of fully protonated CypA. To have a direct measure for the protonation at individual positions, the average resonance intensity of the rapidly exchanging protons was set to 1.0. (a) Sample A (control). (b) Sample B (refolding in ^2H_2O). (c) Sample C (refolding in H_2O). (d) Difference between the signal intensities from spectra C and B. The outlying data for the residues 117, 135 and 148 (marked with asterisks) are probably caused by the different pH used for the reference spectrum (pD_{corr} 6.5).

with GroEL (Figure 4) and thus exchange-competent. On this basis, the measured NMR intensities can be related to the intensities for the GroEL-bound protein (I_X) at the end of the pulse (Figure 5a and b). Model calculations showed that the order of magnitude of the calculated exchange rates for GroEL-bound CypA is very robust with respect to the corrections for unbound CypA, or for exchange during the NMR sample preparation.

Protection factors in GroEL-bound CypA

If the degree of protonation at an individual sequence position is known before and after a labeling pulse of known duration, the first order rate constant for the amide proton exchange can be evaluated (for details see Experimental Procedures). In Figure 8a these rates (k_{EL}), as calculated for 54 slowly exchanging protons from the 15 minutes labeling pulse, are plotted against the amino acid sequence. There is no significant variation along the sequence, with values varying between 10^{-2} and 10^{-1} min^{-1}. Protection factors for these amide protons in GroEL-bound CypA are thus much lower than for the native protein and vary between 10^2 and 10^4 (Figure 8b). Correspondingly, compared to the native protein, the rate constants in the GroEL-bound state are increased by a deprotection factor of 10^2 to 10^4 (Figure 8c).

Figure 9 summarizes the results from the two minutes labeling pulse experiment. Rates and protection factors were calculated for 45 backbone amide protons (Figure 9a and b). Nine rate constants given in Figure 8 could not be determined, because the differences between the signal intensi-

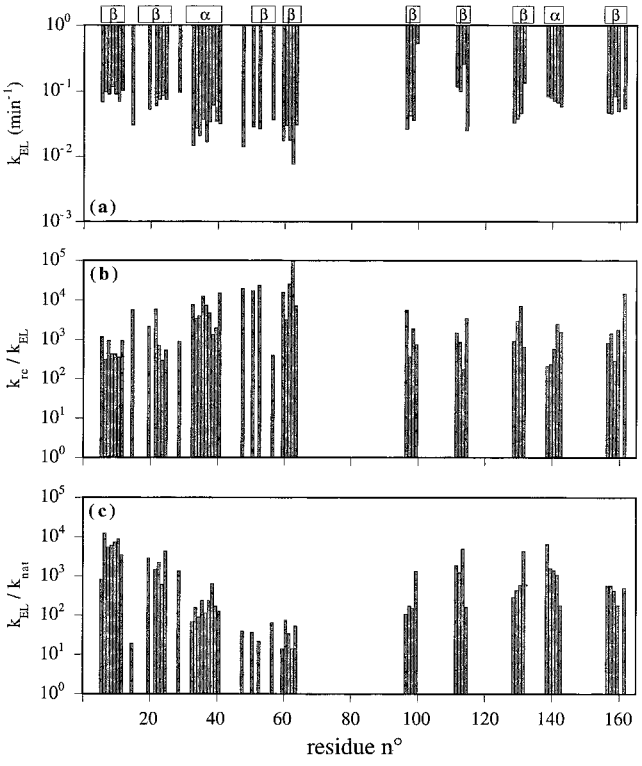

Figure 8. Results from the 15 minutes pulse labeling experiment. (a) Exchange rates in GroEL-bound CypA for 54 sequence positions with ''slow exchange'' in native CypA (Wüthrich *et al.*, 1991; Ottiger *et al.*, 1997) plotted *versus* the residue number. (b) Protection factors relative to the random coil CypA sequence. (c) Deprotection factors relative to native CypA calculated for GroEL-bound CypA.

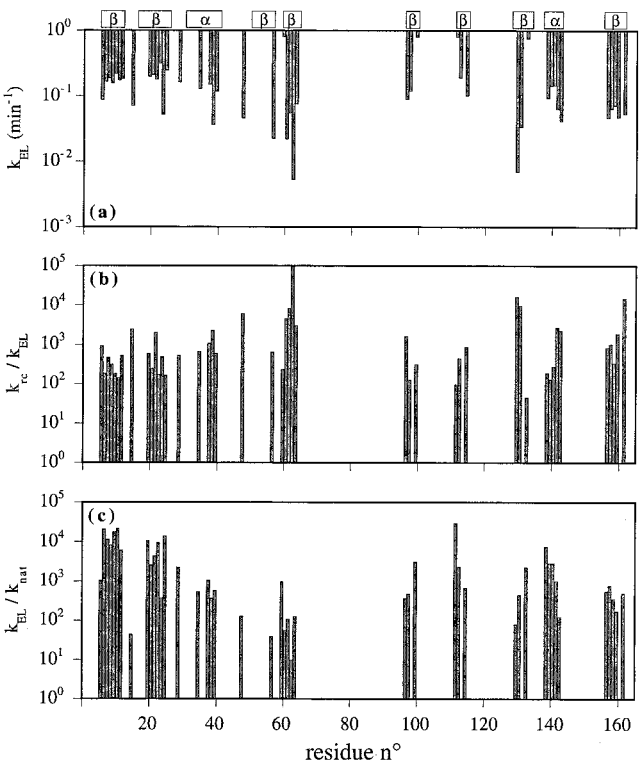

Figure 9. Results from the two minutes pulse labeling experiment. (a) Exchange rates in GroEL-bound CypA for 45 sequence positions with ''slow exchange'' in native CypA plotted *versus* the residue number. (b) and (c) Protection factors and deprotection factors calculated for GroEL-bound CypA as in Figure 8.

ties before and after the pulse were too small. The protection factors and deprotection factors (Figure 9b and c) are in good agreement with the corresponding data from the 15 minutes labeling pulse experiment (Figure 8).

Exchange during refolding at 6°C in the absence of GroES and ATP

We also investigated the exchange after a ten seconds labeling pulse which was initiated by a 1:10-dilution of the deuterated CypA-GroEL-complex into H_2O at 30°C and stopped by a second 1:10-dilution into ice-cold buffer in ^2H_2O or H_2O devoid of ATP or GroES. Under these conditions the protein folds much more slowly (Table 1). The resulting NMR spectra (data not shown) showed that proton exchange of CypA was not limited to the ten seconds-pulse, but continued measurably after cooling on ice during the slow complex dissociation and refolding of CypA.

Proton exchange at 30°C, pD_{corr} 6.4

The following observations confirmed that the presently studied system depends critically on pH (Figure 2). Equimolar amounts of CypA and GroEL were incubated in ^2H_2O-buffer at pD 6.4, instead of pD 6.1 as used in the experiments of Figures 7 to 9. After one hour less than 10% of the slowly exchanging protons were deuterated, and after 7.5 hours the exchange was only approximately 20%. If three association-dissociation cycles were applied as usual, with binding at 30°C (eight hours) and dissociation at 6°C (16 hours), only about 50% of the protons with $k_{nat} < 10^{-3}$ min^{-1} were exchanged, as compared to >80% at pD 6.1 (Figure 7a).

Amide proton exchange of native CypA in the presence of ''catalytic'' amounts of GroEL

After addition of 0.004 stoichiometric equivalents of GroEL, or 0.004 equivalents of GroEL and 0.008 equivalents of GroES to CypA in ^2H_2O-buffer (pD 6.4), the solution was tested for acceleration of the proton exchange in the presence of ADP at 26°C by NMR-measurements. No acceleration of the backbone amide proton exchange from positions with slow exchange in native CypA was detected. These ''catalytic'' amounts of chaperonin thus apparently do not cause unfolding or destabilization

of native CypA that would give rise to an increase in the proton exchange rates, indicating that the CypA-GroEL system behaves differently from the recently studied barnase-GroEL system (Zahn *et al.*, 1996a).

Discussion

The present solvent pulse labeling NMR experiments with human cyclophilin A indicate that most of the slowly exchanging amide proton positions in native CypA have protection factors around 10^3 in the GroEL-bound protein (Figure 8b), and do not reflect the same patterns along the sequence as the protection factors in the native protein (Figure 6c). In the native protein, the amide proton exchange rates in different β-strands differ by as much as two orders of magnitude indicating variable local unfolding events in the native protein. In the GroEL-bound protein this exchange pattern is not maintained, and a rather uniform exchange behavior is observed. The ratios of some exchange rates are even inverted in the GroEL-bound state when compared to free CypA. We can account for this small apparent inversion as being caused by the exchange that takes place during refolding subsequent to the pulse, leading to larger differences between the refolding experiments in ^2H_2O or H_2O at these positions (Figure 7d). Thus the exchange rates k_{EL} are very homogeneous along the polypeptide chain. We conclude that GroEL cannot simply enhance the local unfolding modes of the native CypA, since otherwise the same relative rates would be observed in the bound state and in the native structure. Rather, we must conclude that the observed homogeneity of the exchange rates k_{EL} (Figure 8a) indicates global unfolding in the GroEL-bound state.

The implicated global unfolding mechanism in the GroEL-bound state causes a given molecule of bound CypA to lose all protons at once, with nearly identical rates at all positions along the polypeptide chain. Since the experiments have been carried out with a stable GroEL-CypA-complex, which was pulsed isothermally and at the same pH as used during complex formation, we have taken a snapshot of a form of the complex that is in existence for extended times. This stable GroEL-CypA-complex may correspond to an arrested intermediate of the chaperonin reaction cycle, which in the cell would not exist for such a long time because of the presence of nucleotides and GroES.

Since the amide proton exchange rates in GroEL-bound CypA are 10^3 to 10^4 times slower than the random coil values, we postulate a dynamic model for the GroEL-CypA-complex, where CypA would equilibrate between unstructured and native-like states while remaining bound to the chaperonin. Exchange from the random coil state would be dominant, since the global unfolding is sufficiently frequent. The experimental results indicate that variability of exchange rates along the sequence in the native protein has vanished, and thus local unfolding plays a subordinate role in the exchange. Compared to free CypA the equilibrium between native-like and random coil states would be partially shifted, about 10^3-fold, towards the unfolded state of CypA, presumably by interactions of hydrophobic amino acids with the hydrophobic lining of the cavity of GroEL. This is directly reflected by the deprotection factor of about 10^3 between native and GroEL-bound CypA. However, since native CypA is very stable against global unfolding in the absence of GroEL, giving rise to *PF* values of 10^7, a 10^3-fold destabilization in the complex would still leave ample native-like state in equilibrium, as reflected in the *PF* of GroEL-bound protein of 10^2 to 10^4. Overall, the main effect of GroEL thus seems to be the promotion of dynamic global unfolding of CypA in the complex. Formally, the close similarity of the protection factors for the different individual amide protons could also be explained by exchange occurring from an ensemble of folding intermediates, in which on average all amide protons would show a similar equilibrium constant for deprotection. With a large number of different conformers, all differences could be averaged. However, we consider it rather unlikely that these intermediates would show no differences in stability along the sequence.

The idea of a dynamic equilibrium of the GroEL-bound protein is consistent with the global destabilization deduced from earlier measurements with longer labeling periods (Zahn *et al.*, 1994b), which lead to complete exchange (Figure 10). It suggests that GroEL may support the folding of proteins by annealing through repeated dynamic opening and closing of the GroEL-bound form of CypA. Unproductive, off-pathway intermediates may be resolved because of the global nature of this unfolding. This is achieved by a stabilization of the globally unfolded state when the substrate protein is complexed to GroEL. The unfolded form may be bound by GroEL with high affinity through the contacts with hydrophobic side-chains that are buried in the native state, and therefore CypA reversibly unfolds in the complex. The ''structured'' bound state ($EL \cdot N'$ in Figure 10) still has to have sufficient binding energy to prevent dissociation at pH 6.0, 30°C in the absence of ATP and GroES. Whether a protein forms kinetically stable complexes (such as observed here) or short-lived complexes (barnase, Zahn *et al.*, 1996a,b) will depend on the protein and its folding properties, and possibly on whether the native-like state maintains exposed hydrophobic patches that allow some residual interaction (such as in β-lactamase at high temperature; Gervasoni *et al.*, 1996).

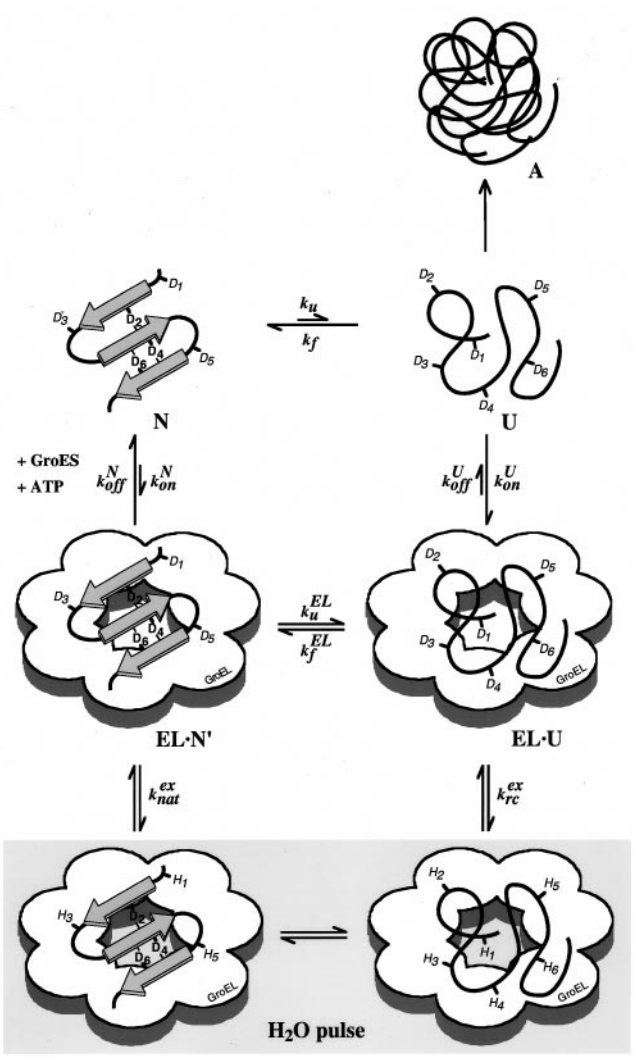

Figure 10. Scheme of GroEL-enhanced H/^{2}H-exchange in a fragment of the substrate protein CypA. Six amide protons are individually numbered. Bold and italic letters represent slowly and rapidly exchanging amide protons, and thick and thin lines represent covalent bonds and hydrogen bonds, respectively. In the folded substrate protein amide proton exchange occurs with the exchange rates in the native protein, k_{nat}. When bound to GroEL, the substrate is in a dynamic equilibrium between a native-like conformation N′ and a globally unfolded state U, in which amide protons exchange with the random coil rates k_{rc}. The net exchange rate from EL·N′ is therefore no longer governed by local unfolding, but by global unfolding *via* EL·U. In the absence of GroEL the unfolded state aggregates irreversibly, whereas GroEL supports folding by allowing the substrate protein to reach the native conformation by several dynamic opening and closing cycles in the bound state. The unfolding rate in the complex is accelerated compared to the free state in solution. While the native-like state N′ is still favored over U in the complex, the equilibrium is shifted 10^2 to 10^4-fold toward a globally unfolded state U when compared to the folded protein in solution.

H/^{2}H-exchange of individual buried amide protons (N–H) in a protein takes place when there are structural fluctuations that expose these protons. Therefore, the exchange rates are a measure of the local flexibility within the protein during a labeling pulse. In ^2H_2O solution the proton exchange follows the basic kinetic mechanism (equation (1)):

$$N_c{-}H \underset{k_f}{\overset{k_u}{\rightleftharpoons}} N_o{-}H \xrightarrow{k_{rc}} N_o{-}{}^2H \underset{k_u}{\overset{k_f}{\rightleftharpoons}} N_c{-}{}^2H \qquad (1)$$

where the indices c and o refer to the closed and open forms, respectively. k_u and k_f are the relevant rates of local or global unfolding and folding, and k_{rc} is the intrinsic random coil exchange rate under the experimental conditions. Under steady state conditions favoring the folded state ($k_f \gg k_u$) the observed exchange rate, k_{ex}, is

$$k_{ex} = \frac{k_u \times k_{rc}}{k_f + k_{rc}} \qquad (2)$$

Depending on the ratio of k_f to k_{rc}, one may distinguish two limiting situations (Hvidt & Nielsen, 1966; Englander & Kallenbach, 1984; Englander *et al.*, 1996). If $k_{rc} \ll k_f$ (EX2 conditions), equation 2 can be simplified to

$$k_{ex} = \frac{k_u}{k_f} k_{rc} \qquad (3)$$

In this case the observed exchange rate k_{ex} is the intrinsic, pH-dependent exchange rate k_{rc} weighted by the thermodynamic stability constant k_u/k_f, which is equal to the ratio of the equilibrium concentrations, $[o]_{eq}/[c]_{eq}$, of the open and closed forms. If in contrast $k_{rc} \gg k_f$, the limit of equation (2) goes to

$$k_{ex} = k_u \qquad (4)$$

This would describe immediate, pH-independent exchange of all protons as soon as complete unfolding has taken place. Under these conditions, the so-called EX1 mechanism takes place, where exchange is limited by the unfolding rate k_u. Proton exchange for most proteins achieves the EX2 limit (Perrett *et al.*, 1995; Englander *et al.*, 1996). Even global unfolding reactions often reach the EX2 limit (Bai *et al.*, 1994; Perrett *et al.*, 1995; Zahn *et al.*, 1996b). Exchange under EX1 conditions has been observed, however, when transient global unfolding is favored (Roder *et al.*, 1985; Perrett *et al.*, 1995), or at high denaturant concentrations, i.e. under unfolding conditions (Kiefhaber & Baldwin, 1995). Here we cannot distinguish between EX1 and EX2 exchange from the pH-dependence of the observed exchange rates, since the pH has a pronounced effect on the thermodynamic stability of CypA and on the GroEL-CypA complexation equilibria (Figure 2). The measured exchange rates, k_{EL}, in the complex are not accurate enough to decide whether they follow the intrinsic rates, and thus this does not give an indication for or against an EX2 mechanism. The observed exchange rates k_{EL} for GroEL-bound CypA are about 20-fold faster

than the net complex formation rates, and complex formation is probably limited by the rate of unfolding of native CypA k_u. Since the exchange rates k_{EL} cannot be faster than the unfolding rate, k_u has to be increased in the complex when compared to the free state in solution. This is consistent with the observed shift in overall stability, leading to an increase in k_u/k_f.

In the presently proposed dynamic model the protein may undergo many cycles of unfolding and refolding in the complex. The stable CypA-GroEL-complex has been maintained for eight hours (or 70 half-lives of proton exchange in GroEL-bound CypA) which, if equation (4) applies, would be identical to the number of half-lives of unfolding in the complex. If equation (3) holds, on the other hand, unfolding would be even faster and even more cycles of folding and unfolding could occur during the lifetime of the complex. Thus, independent of the type of exchange (EX1 or EX2), we can conclude that there are multiple cycles of folding and unfolding in the complex, which supports the ''annealing'' model.

To observe proton exchange, the GroEL-CypA-complexes must be preformed from stoichiometric quantities. The unfolding rate of CypA in solution, observed by loss of activity, under these conditions (where folding is reversible) is very slow. This leads to very slow complex formation rates. Furthermore, once the complex is formed, it is very stable and shows no appreciable off-rate at 30°C. Thus, there is no evidence for dynamically dissociating and reassociating GroEL-CypA-complexes in the absence of nucleotides and GroES. Neither does substoichiometric GroEL by itself cause proton exchange of CypA (Zahn *et al*., 1994b) nor does a substoichiometric amount of a complex of GroEL, GroES and ATP or ADP catalyze this reaction. In the presence of GroES in addition to either ATP or ADP the substrate dissociation rate is increased so that the residence time may be too short to promote significant proton exchange. Overall, the energy-barrier to global unfolding seems to be higher for CypA than, e.g., for barnase (Zahn *et al*., 1996a,b). ATP hydrolysis allows multiple rounds of binding and release during the chaperonin cycle. Thus, under conditions prevailing in the cell, where nucleotides and GroES are present, the annealing may involve both folding and unfolding in the complex and multiple rounds of binding and release of the substrate protein (Todd *et al*., 1994,1996; Corrales & Fersht, 1996).

In conclusion, GroEL appears to catalyze global unfolding once the substrate protein is bound, and to stabilize globally unfolded states, presumably by interactions between hydrophobic amino acids of the substrate that are buried in the folded state, and the central cavity of GroEL. By multiple cycles of folding and global unfolding, kinetically trapped, folding-incompetent conformers would be allowed to find pathways leading to the correctly folded state. The equilibrium of different bound states still favors native-like conformations, thereby allowing native-like states to dissociate with a certain frequency. Proteins with a high interaction energy, such as CypA, require GroES and nucleotide for fast release, presumably because these effector compounds are needed for lowering the interaction energy of the central cavity.

Experimental Procedures

Protein expression and purification

Recombinant ^{15}N-labeled or unlabeled CypA was expressed in *E. coli* and purified as described previously (Weber *et al*., 1991). GroEL was purified from *E. coli* W3110 harboring the plasmid pOF39 as described by Zahn *et al*. (1994a), except for an additional Q-Sepharose chromatography step (Pharmacia Biotech) in 50 mM Tris-(hydroxymethyl)-aminomethane (pH 7.2), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), and gradient elution with 0 to 500 mM NaCl. GroES was expressed with a C-terminal hexahistidine tail in *E. coli* JM83, and isolated as will be described elsewhere. Protein concentrations were determined using the bicinchoninic acid assay (Pierce) and are always given for the oligomeric form.

Folding assays

To follow the unfolding and refolding reactions during CypA-GroEL-complex formation and dissociation, the PPIase activity of CypA was measured at 8°C as described by Fischer *et al*. (1984). 2 to 10 µl of the reaction mixture containing 1 to 20 µM CypA were diluted together with 10 µl of 1 mM chymotrypsin (Sigma) into 1 ml assay solution containing 10 mM *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sigma) at pH 8.0.

To determine unfolding kinetics, 20 µM CypA were incubated with 20 µM GroEL in 20 mM 2-[*N*-morpholino]-ethane sulphonate (Mes), 1 mM DTT in H_2O or ^2H_2O at different pH or pD, and the enzymatic activity was measured as a function of time. The pD_{corr} of ^2H_2O-buffers was measured at 25°C, taking into account the isotope effect at the glass electrode ($pD_{corr} = pD_{read} + 0.4$; Glasoe & Long, 1960). For the determination of refolding kinetics, 20 µM CypA and 20 µM GroEL were incubated in 20 mM Mes (pH 6.0), 1 mM DTT at 30°C for 16 hours and diluted 1:20 into the same buffer, which was pre-equilibrated at various temperatures and contained either no additives, or 2 mM KCl and 2 mM $MgCl_2$ combined with either ADP (1 mM), ATP (1 mM), GroES (2 µM) and ADP, or GroES and ATP to initiate refolding.

Amide proton exchange experiments

For the pulse labeling experiments, 20 µM CypA and 25 µM GroEL in 40 ml ^2H_2O-buffer (99.8% ^2H_2O, 20 mM Mes pD_{corr} 6.1, 1 mM DTT) were incubated at 30°C for eight hours to form a CypA-GroEL-complex. To dissociate the complex, the solution was incubated at 6°C for 16 hours. This association-dissociation cycle was repeated three times to ensure that most of the CypA molecules were bound to GroEL at least once. During the cycle, the PPIase activity of CypA was measured (Figure 4). The expected exchange during the association-dissociation cycles was calculated under the assumption that all amide protons in a CypA molecule exchange when this CypA molecule is bound to GroEL

and has lost its enzymatic activity. This calculated value was in good agreement with the measured relative intensities for the slowly exchanging protons in sample A (see Figure 3).

After the third incubation at 30°C, the sample was divided into three portions (Figures 3 and 4): 10 ml were incubated at 6°C in the same buffer for 48 hours without any further dilution (sample A) and applied onto an S-Sepharose column (Pharmacia Biotech) equilibrated with H_2O-buffer (20 mM Mes pH 6.0, 1 mM DTT) at 4°C to separate CypA from GroEL. CypA-containing fractions eluted with 150 mM NaCl were pooled and concentrated in a Centriprep-10 filtration unit (Amicon). Two 15 ml portions (samples B and C) were diluted into 60 ml of H_2O-buffer (20 mM Mes pH 6.0, 1 mM DTT, final ^2H_2O-content 20%) at 30°C to label exchange-competent proton positions in GroEL-bound CypA. To quench the labeling, samples B and C (75 ml) were each diluted into 300 ml of refolding buffer (20 mM Mes in ^2H_2O pD_{corr} 6.4 (sample B), or 20 mM Mes in H_2O pH 6.0 (sample C)) containing 1 mM ATP, 2 mM $MgCl_2$, 2 mM KCl and 2 μM GroES (final concentrations) at 30°C. After ten minutes the samples were cooled on ice and stored overnight at 4°C until CypA was separated from GroEL and GroES at 4°C. Before the solution was applied onto a Ni-NTA-Superflow column (Qiagen) coupled directly to an S-Sepharose column, it was centrifuged for 20 minutes at 23,000 *g* to remove precipitated protein (mainly GroEL and GroES as shown by SDS-PAGE). After the samples were loaded, the Ni-NTA-Superflow column to which GroES-His_6 had bound was removed from the system and CypA was eluted from the S-Sepharose with 150 mM NaCl. CypA-containing fractions were concentrated and stored at 4°C until the NMR measurements were performed. Directly before the NMR measurements, 10% ^2H_2O-buffer was added to each sample.

NMR measurements were performed on a Bruker AMX600 spectrometer equipped with four channels, using samples of ^{15}N-labeled CypA (150 to 300 μM) in 90% H_2O/10% ^2H_2O or in 99.5% ^2H_2O. 2D [^{15}N,^1H]-HSQC spectra (Bodenhausen & Ruben, 1980) were recorded at 26°C (pH or pD_{corr} 6.0) as data matrices of 180×1024 complex points, with $t_{1,max}(^{15}N) = 84.6$ ms and $t_{2,max}(^1H) = 112.6$ ms. Quadrature detection in the indirect dimension was achieved using the States-TPPI method (Marion *et al*., 1989). The water signal was suppressed by spin-lock pulses (Messerle *et al*., 1989). For data processing and spectral analysis we used the programs PROSA (Güntert *et al*., 1992; Bartels *et al*., 1995a) and XEASY (Bartels *et al*., 1995b), respectively.

To allow direct comparison of the [^{15}N,^1H]-HSQC spectra obtained from different samples, the relative signal intensities were normalized to eliminate effects from different protein concentrations in different samples and from different relaxation characteristics of the individual amide protons. First, the individual peak intensities were divided by the corresponding values obtained from a reference spectrum of a fully protonated sample. Then the spectra were normalized by setting the average of the intensities corresponding to 63 rapidly exchanging protons with exchange rates higher than 10^{-2} min^{-1} in the native protein to 1.0 (corresponding to 100% protonation). In all spectra, the positions of rapidly exchanging protons had a signal intensity around 1.0, because measurements were performed in H_2O.

Determination of hydrogen exchange rates and protection factors from the pulse labeling experiments

Because peptide group hydrogen exchange reactions are influenced by a large solvent isotope effect (Connelly *et al*., 1993), exchange rates were calculated for the conditions of each individual experimental step (Figure 3), using the numbers given by Connelly *et al*. (1993). The H_2O- and ^2H_2O-contents of the buffer were considered using the following equation:

$$k_{\exp} = f_{H_2O} k_{H_2O} + f_{^2H_2O} k_{^2H_2O} \quad (5)$$

k_{exp} is the observed exchange rate in a mixed solvent, f_{H_2O} and $f_{^2H_2O}$ are the fractions of H_2O and ^2H_2O in the buffer, and k_{H_2O} and $k_{^2H_2O}$ are the intrinsic exchange rates at the appropriate pH, or pD_{corr}, respectively. To calculate exchange rates at different temperatures, T, relative to a reference temperature, T_{ref}, each rate constant was corrected using equation (6) (Bai *et al*., 1993) with an activation energy $E_a = 17$ kcal/mol, where R is the general gas constant:

$$k(T) = k(T_{ref}) e^{-\frac{E_a}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)} \quad (6)$$

To calculate exchange rates at different pH values, the following conversion formula was used:

$$k(pH) = k(pH_{ref}) \frac{k_A[H^+] + k_B[OH^-] + k_W}{k_{A,ref}[H^+]_{ref} + k_{B,ref}[OH^-]_{ref} + k_{W,ref}} \quad (7)$$

k_A, k_B, k_W and $k_{A,ref}$, $k_{B,ref}$, $k_{W,ref}$ are the rate constants for a ''standard'' peptide, which depend on the solvent (H_2O or ^2H_2O) and the direction of the exchange (N-H → N-^{2}H or N-^{2}H → N-H) according to Bai *et al*. (1993) and Connelly *et al*. (1993). The molar ionization constants for H_2O and ^2H_2O at 20°C are $10^{-14,17}$ and $10^{-15.05}$, respectively (Connelly *et al*., 1993).

Protection factors (*PF*) for backbone amide protons in native CypA and in the GroEL-bound substrate were calculated as

$$PF = k_{rc}/k_{obs} \quad (8)$$

k_{rc} is the calculated intrinsic exchange rate under the reaction conditions used (here: pH 6.0, 30°C), and k_{obs} is either the measured exchange rate in the native protein, k_{nat}, (converted to pH 6.0, 30°C using equation (5) to (7)) or the exchange rate in GroEL-bound CypA, k_{EL}, calculated from the pulse labeling experiments (see below). The exchange rates in the native protein have been determined at pD_{corr} 6.5, 26°C by Ottiger *et al*. (1997). The conversion of these values to pH 6.0, 30°C is possible since these small differences between the experimental conditions do not have a significant influence on the thermodynamic stability of CypA (Weber, 1991).

To estimate the changes in signal intensities in the NMR-spectra for each individual amide proton in native CypA during the final preparation of the NMR sample (from B and C in Figure 5a until the NMR measurement), equation (9) was applied

$$I(t) = I_{eq} + (I_{initial} - I_{eq}) e^{-k_{exp} t} \quad (9)$$

where k_{exp} is given by equation (5). $I(t)$ is the signal intensity after the experimental step considered, where $I = 1$ corresponds to 100% protonation. The exchange rates k_{H_2O} and $k_{^2H_2O}$ refer to the native protein, taking into account the actual experimental conditions of pH or pD, and temperature. I_{eq} is the maximal relative intensity

which can be reached under equilibrium conditions and is equal to the H_2O-content in the respective buffer f_{H_2O}. $I_{initial}$ is the signal intensity at the beginning of the experimental step.

For the calculation of the proton exchange rates in GroEL-bound CypA, the relative signal intensities at the beginning (I_0) and at the end of the labeling pulse (I_X) had to be known. A scheme to explain the principle of the calculations is shown in Figure 5a and b. We assume that in every CypA molecule that was bound to GroEL at least once, all backbone amide protons exchanged quantitatively, i.e. $I_0 = 0$. This assumption was checked and confirmed to be correct by comparisons between the decrease in enzymatic activity during complex formation and the extent of exchange in the same sample (control A). To calculate the relative signal intensity at the end of the pulse (I_X), data from samples B and C were used. Since after three association-dissociation cycles, there still remained some CypA molecules that had never been bound to GroEL, the signal intensity caused by these molecules had to be considered in the calculation and was available from the data from control A. Thus, during the labeling pulse only a certain percentage of CypA molecules in the solution were exchange-competent, as determined by measurements of the enzymatic activity. Therefore, values for the intensities corresponding to samples B and C were divided by the fraction f_{ex} of molecules bound to GroEL, leading to the following equations:

$$I_B = \frac{I_{B,exp} - I_{A,exp}}{f_{ex}} \tag{10}$$

$$I_C = \frac{I_{C,exp} - I_{A,exp}}{f_{ex}} \tag{11}$$

I_B and I_C are the corrected relative intensities for an individual amide proton considering only bound CypA in the samples B and C. $I_{A,exp}$, $I_{B,exp}$ and $I_{C,exp}$ are the experimentally measured corresponding intensities from samples A, B and C. In the 15 min and the 2 min pulse experiment, f_{ex} was 0.56 and 0.35, respectively. This is summarized graphically in Figure 5b. The exchange during the folding reaction can be estimated by the following equation for the calculation of the intensities at point X (I_X):

$$\frac{I_C - I_X}{I_X - I_B} = \frac{k_C}{k_B} \tag{12}$$

k_B and k_C are the exchange rates during folding in H_2O and ^2H_2O, which are unknown and are presumably complicated functions of time. However, the ratio of k_C and k_B depends only on the H_2O- and ^2H_2O-contents in the refolding buffer, on pH or pD, and on the isotope effect, and can therefore be calculated with equations (5) to (7) according to Bai *et al*. (1993) and Connelly *et al*. (1993) for the experimental conditions used. Equation (12) can be solved for the desired intensity I_X, using the calculated ratio k_C/k_B.

$$I_X = \frac{I_C + (k_C/k_B) \times I_B}{1 + k_C/k_B} \tag{13}$$

If the relative signal intensities at the beginning ($I_0 = 0$) and at the end of the labeling pulse (I_X, calculated with equations (10) to (13)) are known, the rate constant for proton exchange in GroEL-bound CypA, k_{exp}, can be calculated using equation (14), where t_p is the length of the labeling pulse.

$$I_X(t_p) = I_{eq} + (I_0 - I_{eq})e^{-k_{exp}t_p} \tag{14}$$

To relate k_{exp} to tabulated values, it needs to be converted from the experimental conditions used (mixed solvent) to standard conditions (H_2O, 30°C, pH 6.0) using equation (5). The ratio $k_{H_2O}/k_{^2H_2O}$ can be calculated from the isotope effect as described by Connelly *et al.* (1993), and equation (5) can thus be solved for k_{H_2O}. To determine the protection factors of the individual protons, equation (8) was used. The deprotection factor (*DF*), which is a measure for the destabilization compared to the native state, is given by equation (15):

$$DF = k_{EL}/k_{nat} \tag{15}$$

In the calculations of *PF* and *DF*, all rates have been corrected for the experimental conditions used (pH, temperature, isotope effects), using equations (5) to (7).

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References

Bai, Y., Milne, J. S., Mayne, L. & Englander, S. W. (1993). Primary structure effects on peptide group hydrogen exchange. *Proteins: Struct. Funct. Genet.* **17**, 75–86.

Bai, Y., Milne, J. S., Mayne, L. & Englander, S. W. (1994). Protein stability parameters measured by hydrogen exchange. *Proteins: Struct. Funct. Genet.* **20**, 4–14.

Bartels, C., Güntert, P. & Wüthrich, K. (1995a). IFLAT—a new automatic baseline-correction method for multidimensional NMR spectra with strong solvent signals. *J. Magn. Reson. A*, **117**, 330–333.

Bartels, C., Xia, T., Billeter, M., Güntert, P. & Wüthrich, K. (1995b). The program XEASY for computer-supported NMR spectral analysis of biological macromolecules. *J. Biomol. NMR*, **6**, 1–10.

Bodenhausen, G. & Ruben, D. J. (1980). Natural abundance Nitrogen-15 NMR by enhanced heteronuclear spectroscopy. *Chem. Phys. Letters*, **69**, 185–189.

Braig, K., Otwinowski, Z., Hegde, R., Boisvert, D. C., Joachimiak, A., Horwich, A. L. & Sigler, P. B. (1994). The crystal structure of the bacterial chaperonin GroEL at 2.8 Å. *Nature*, **371**, 578–586.

Braig, K., Adams, P. D. & Brunger, A. T. (1995). Conformational variability in the refined structure of the chaperonin GroEL at 2.8 Å resolution. *Nature Struct. Biol.* **2**, 1083–1094.

Braun, W., Kallen, J., Mikol, V., Walkinshaw, M. D. & Wüthrich, K. (1995). Three-dimensional structure and actions of immunosuppressants and their immunophilins. *FASEB J.* **9**, 63–72.

Buchner, J., Schmidt, M., Fuchs, M., Jaenicke, R., Rudolph, R., Schmid, F. X. & Kiefhaber, T. (1991). GroE facilitates refolding of citrate synthase by suppressing aggregation. *Biochemistry*, **30**, 1586–1591.

Burston, S. G., Ranson, N. A. & Clarke, A. R. (1995). The origins and consequences of asymmetry in the chaperonin reaction cycle. *J. Mol. Biol.* **249**, 138–152.

Chen, S., Roseman, A. M., Hunter, A. S., Wood, S. P., Burston, S. G., Ranson, N. A., Clarke, A. R. & Saibil, H. R. (1994). Location of a folding protein and shape changes in GroEL-GroES complexes imaged by cryo-electron microscopy. *Nature,* **371**, 261–264.

Connelly, G. P., Bai, Y., Jeng, M.-F. & Englander, S. W. (1993). Isotope effects in peptide group hydrogen exchange. *Proteins: Struct. Funct. Genet.* **17**, 87–92.

Corrales, F. J. & Fersht, A. R. (1996). Toward a mechanism for GroEL·GroES chaperone activity: an ATPase-gated and -pulsed folding and annealing cage. *Proc. Natl Acad. Sci. USA,* **93**, 4509–4512.

Englander, S. W. & Kallenbach, N. R. (1984). Hydrogen exchange and structural dynamics of proteins and nucleic acids. *Quart Rev. Biophys.* **16**, 521–655.

Englander, S. W., Sosnik, T. R., Englander, J. J. & Mayne, L. (1996). Mechanisms and uses of hydrogen exchange. *Curr. Opin. Struct. Biol.* **6**, 18–23.

Fenton, W. A., Kashi, Y., Furtak, K. & Horwich, A. L. (1994). Residues in chaperonin GroEL required for polypeptide binding and release. *Nature,* **371**, 614–619.

Fischer, G., Bang, H. & Mech, C. (1984). Determination of enzymatic catalysis for the *cis-trans*-isomerization of peptide binding in proline-containing peptides. *Biomed. Biochim. Acta,* **43**, 1101–1111.

Gervasoni, P., Staudenmann, W., James, P., Gehrig, P. & Plückthun, A. (1996). β-Lactamase binds to GroEL in a conformation highly protected against hydrogen/deuterium exchange. *Proc. Natl Acad. Sci. USA,* **93**, 12189–12194.

Gething, M. J. & Sambrook, J. (1992). Protein folding in the cell. *Nature,* **355**, 33–45.

Glasoe, P. K. & Long, F. A. (1960). Use of glass electrodes to measure acidity in deuterium oxide. *J. Phys. Chem.* **64**, 188–190.

Goloubinoff, P., Christeller, J. T., Gatenby, A. A. & Lorimer, G. H. (1989). Reconstitution of active dimeric ribulose bisphosphate carboxylase from an unfolded state depends on two chaperonin proteins and MgATP. *Nature,* **342**, 884–889.

Gray, T. E. & Fersht, A. R. (1993). Refolding of barnase in the presence of GroE. *J. Mol. Biol.* **232**, 1197–1207.

Groß, M., Robinson, C. V., Mayhew, M., Hartl, F. U. & Radford, S. E. (1996). Significant hydrogen exchange protection in GroEL-bound DHFR is maintained during iterative rounds of substrate cycling. *Protein Sci.* **5**, 2506–2513.

Güntert, P., Dötsch, V., Wider, G. & Wüthrich, K. (1992). Processing of multi-dimensional NMR data with the new software PROSA. *J. Biomol. NMR,* **2**, 619–629.

Hunt, J. F., Weaver, A. J., Landry, S. J., Gierasch, L. & Deisenhofer, J. (1996). The crystal structure of the GroES co-chaperonin at 2.8 Å resolution. *Nature,* **379**, 37–45.

Hvidt, A. & Nielsen, S. O. (1966). Hydrogen exchange in proteins. *Advan. Protein Chem.* **21**, 287–386.

Kallen, J., Spitzfaden, C., Zurini, M. G. M., Wider, G., Widmer, H., Wüthrich, K. & Walkinshaw, M. D. (1991). Structure of human cyclophilin and its binding site for cyclosporin A determined by X-ray crystallography and NMR spectroscopy. *Nature,* **353**, 276–279.

Kiefhaber, T. & Baldwin, R. L. (1995). Kinetics of hydrogen bond breakage in the process of unfolding of ribonuclease A measured by pulsed hydrogen exchange. *Proc. Natl Acad. Sci. USA,* **92**, 2657–2661.

Koradi, R., Billeter, M. & Wüthrich, K. (1996). MOLMOL: a program for display and analysis of macromolecular structures. *J. Mol. Graphics,* **14**, 52–55.

Laminet, A. A., Ziegelhoffer, T., Georgopoulos, C. & Plückthun, A. (1990). The *Escherichia coli* heat shock proteins GroEL and GroES modulate the folding of the β-lactamase precursor. *EMBO J.* **9**, 2315–2319.

Lin, Z., Schwarz, F. P. & Eisenstein, E. (1995). The hydrophobic nature of GroEL-substrate binding. *J. Biol. Chem.* **270**, 1011–1014.

Marion, D., Ikura, K., Tschudin, R. & Bax, A. (1989). Rapid recording of 2D NMR spectra without phase cycling: application to the study of hydrogen exchange in proteins. *J. Magn. Reson.* **85**, 393–399.

Martin, J., Mayhew, M., Langer, T. & Hartl, F. U. (1993). The reaction cycle of GroEL and GroES in chaperonin-assisted folding. *Nature,* **366**, 228–233.

Mayhew, M., da Silva, A. C. R., Martin, J., Erdjument-Bromage, H., Tempst, P. & Hartl, F. U. (1996). Protein folding in the central cavity of the GroEL-GroES chaperonin complex. *Nature,* **379**, 420–426.

Messerle, B. A., Wider, G., Otting, G., Weber, C. & Wüthrich, K. (1989). Solvent suppression using a spin lock in 2D and 3D NMR spectroscopy with H_2O solutions. *J. Magn. Reson.* **85**, 608–613.

Ottiger, M., Zerbe, O., Güntert, P. & Wüthrich, K. (1997). The NMR solution conformation of unligated cyclophilin A. *J. Mol. Biol.* In the press.

Perrett, S., Clarke, J., Hounslow, A. M. & Fersht, A. R. (1995). Relationship between equilibrium amide proton exchange behavior and the folding pathway of barnase. *Biochemistry,* **34**, 9288–9298.

Robinson, C. V., Groß, M., Eyles, S. J., Ewbank, J. J., Mayhew, M., Hartl, F. U., Dobson, C. M. & Radford, S. E. (1994). Conformation of GroEL-bound α-lactalbumin probed by mass spectrometry. *Nature,* **372**, 646–651.

Roder, H. & Wüthrich, K. (1986). Protein folding kinetics by combined use of rapid mixing techniques and NMR observation of individual amide protons. *Proteins: Struct. Funct. Genet.* **1**, 34–42.

Roder, H., Wagner, G. & Wüthrich, K. (1985). Amide proton exchange in proteins by EX1 kinetics: studies of BPTI at variable pD and temperature. *Biochemistry,* **24**, 7396–7407.

Roder, H., Elöve, G. A. & Englander, S. W. (1988). Structural characterization of folding intermediates in cytochrome *c* by H-exchange labeling and proton NMR. *Nature,* **335**, 700–704.

Roseman, A. M., Chen, S., White, H., Braig, K. & Saibil, H. R. (1996). The chaperonin ATPase cycle: Mechanism of allosteric switching and movements of substrate-binding domains in GroEL. *Cell,* **87**, 241–251.

Saibil, H. R., Zheng, D., Roseman, A. M., Hunter, A. S., Watson, G. M. F., Chen, S., auf der Mauer, A., O'Hara, B. P., Wood, S. P., Mann, N. H., Barnett, L. K. & Ellis, R. J. (1993). ATP induces large quaternary rearrangements in a cage-like chaperonin structure. *Curr. Biol.* **3**, 265–273.

Todd, M. J., Viitanen, P. V. & Lorimer, G. H. (1994). Dynamics of the chaperonin ATPase cycle: Implications for facilitated protein folding. *Science,* **265**, 659–666.

Todd, M. J., Lorimer, G. H. & Thirumalai, D. (1996). Chaperonin-facilitated protein folding: Optimization of rate and yield by an iterative annealing

mechanism. *Proc. Natl Acad. Sci. USA,* **93**, 4030–4035.
Udgaonkar, J. B. & Baldwin, R. L. (1988). NMR evidence for an early framework intermediate on the folding pathway of ribonuclease A. *Nature,* **335**, 694–699.
Walter, S., Lorimer, G. H. & Schmid, F. X. (1996). A thermodynamic coupling mechanism for GroEL-mediated unfolding. *Proc. Natl Acad. Sci. USA,* **93**, 9425–9430.
Weber, C. (1991). Ermittlung der räumlichen Struktur von an Cyclophilin A gebundenem Cyclosporin A mittels isotopengefilterter Protonenresonanzspektroskopie in wässriger Lösung. Dissertation Nr. 9343, ETH, Zürich.
Weber, C., Wider, G., von Freyberg, B., Traber, R., Braun, W., Widmer, H. & Wüthrich, K. (1991). The NMR structure of cyclosporin A bound to cyclophilin in aqueous solution. *Biochemistry,* **30**, 6563–6574.
Weissman, J. S., Kashi, Y., Fenton, W. A. & Horwich, A. L. (1994). GroEL-mediated protein folding proceeds by multiple rounds of binding and release of nonnative forms. *Cell,* **78**, 693–702.
Weissman, J. S., Hohl, C. M., Kovalenko, O., Kashi, Y., Chen, S., Braig, K., Saibil, H. R., Fenton, W. A. & Horwich, A. L. (1995). Mechanism of GroEL action: productive release of polypeptide from a sequestered position under GroES. *Cell,* **83**, 577–587.
Weissman, J. S., Rye, H. S., Fenton, W. A., Beechem, J. M. & Horwich, A. L. (1996). Characterization of the active intermediate of a GroEL-GroES-mediated protein folding reaction. *Cell,* **84**, 481–490.
Wüthrich, K. (1986). *NMR of Proteins and Nucleic Acids,* Wiley, New York.
Wüthrich, K., Spitzfaden, C., Memmert, K., Widmer, H. & Wider, G. (1991). Protein secondary structure determination by NMR: application with recombinant human cyclophilin. *FEBS Letters,* **285**, 237–247.
Yifrach, O. & Horovitz, A. (1996). Allosteric control by ATP of non-folded protein binding to GroEL. *J. Mol. Biol.* **255**, 356–361.
Zahn, R. & Plückthun, A. (1992). GroE prevents the accumulation of early folding intermediates of pre-β-lactamase without changing the folding pathway. *Biochemistry,* **31**, 3249–3255.
Zahn, R. & Plückthun, A. (1994). Thermodynamic partitioning model for hydrophobic binding of polypeptides by GroEL, II. GroEL recognizes thermally unfolded mature β-lactamase. *J. Mol. Biol.* **242**, 165–174.
Zahn, R., Axmann, S. E., Rücknagel, K.-P., Jaeger, E., Laminet, A. A. & Plückthun, A. (1994a). Thermodynamic partitioning model for hydrophobic binding of polypeptides by GroEL, I. GroEL recognizes the signal sequences of β-lactamase precursor. *J. Mol. Biol.* **242**, 150–164.
Zahn, R., Spitzfaden, C., Ottiger, M., Wüthrich, K. & Plückthun, A. (1994b). Destabilization of the complete secondary structure on binding to the chaperone GroEL. *Nature,* **368**, 261–265.
Zahn, R., Perrett, S., Stenberg, G. & Fersht, A. R. (1996a). Catalysis of amide proton exchange by the molecular chaperones GroEL and SecB. *Science,* **271**, 642–645.
Zahn, R., Perrett, S. & Fersht, A. R. (1996b). Conformational states bound by the molecular chaperones GroEL and SecB: a hidden unfolding (annealing) activity. *J. Mol. Biol.* **261**, 43–61.

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