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Thermodynamic Stability and Folding of GroEL Minichaperones

Ralph Golbik[1], Ralph Zahn[1], Stephen E. Harding[2] and Alan R. Fersht[1]*

[1]*MRC, Centre for Protein Engineering, Hills Road Cambridge CB2 2QH, England*

[2]*National Centre for Macromolecular Hydrodynamics, University of Nottingham, Sutton Bonnington, LE12 5RD England*

The apical domain of GroEL (residues 191 to 376) and its C-terminally truncated fragment GroEL(191-345) are expressed with high yield in *Escherichia coli* to give functional monomeric minichaperones. Owing to the reversible folding behaviour of the minichaperones we can analyse the folding of the polypeptide binding domain of the multidomain GroEL protein, the folding of which is known to be irreversible. The apical domain shows two reversible temperature transitions with transition midpoints at 35°C and at 67°C that can be attributed to the unfolding of the C-terminal helices and the domain core, respectively. The native state of the domain core is stabilized by 5.5 kcal mol^{-1} relative to the unfolded state. The rate constant of folding of the apical domain core is independent of the minichaperone concentration and the presence of the C-terminal α-helices. A folding intermediate on the folding pathway is destabilized relative to the native state by 1.6 kcal mol^{-1}, which is also detected by equilibrium and kinetic binding of the dye bis-ANS. Reversible folding of the polypeptide domain of GroEL guarantees highly efficient chaperonin activity within the GroEL toroid.

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*Corresponding author

Introduction

The molecular chaperone GroEL facilitates protein folding by prevention of protein aggregation and by correction of protein misfolding (Zahn *et al.*, 1996a,b; Fenton & Horwich, 1997). The chaperonin is composed of 14 identical subunits of 57 kDa which form a cylinder containing a central cavity. The GroEL subunit is composed of three domains (Braig *et al.*, 1994, 1995). The equatorial domain (residues 6 to 133 and 409 to 523) has been shown to contain the binding site for ATP (Boisvert *et al.*, 1996), and forms most of the intersubunit contacts.

Abbreviations used: bis-ANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulphonic acid, dipotassium salt; CD, circular dichroism; [D], denaturant (urea) concentration; H, helix; IPTG, isopropyl-1-thio-β-galactoside; T_m, the temperature at the midpoint of the thermal unfolding transition; UV, ultraviolet; X_{aa}, N-terminally preceding amino acid.

The intermediate domain (residues 134 to 190 and 377 to 408) connects the equatorial domain with the apical domain (residues 191 to 376), which has been identified as the polypeptide binding domain of GroEL from mutagenesis studies (Fenton & Horwich, 1997). Binding of ATP decreases substrate affinity of GroEL. Very recently, we have expressed the apical domain and its fragments in *Escherichia coli* (Zahn *et al.*, 1996c). The apical domain, GroEL(191-376), and its C-terminally truncated fragment GroEL(191-345) show high chaperone activity, i.e. they catalyse unfolding of barnase and facilitate refolding of cyclophilin and rhodanese. GroEL must, therefore, have an intrinsic chaperone activity that is independent of the central cavity and its allosteric behaviour. This unexpected chaperone activity inspired the name minichaperones (Zahn *et al.*, 1996c). The three-dimensional structure of the minichaperones has been solved (Zahn *et al.*, 1996c; Buckle *et al.*, 1997). The N-terminal tail of the molecule in the 1.7 Å resolution structure of GroEL(191-376) binds in the active site of a neighbouring molecule in the crystal. Seven residues of the polypeptide are bound in a relatively extended conformation, mainly by hydrophobic interactions and hydrogen bonding. The dynamic behaviour of α-helices H8 and H9

(see Figure 1) and surrounding loops allows accommodation of a wide range of substrate proteins and protein conformations to the binding sites of the apical domains within the central cavity.

The minichaperones GroEL(191-345) and GroEL(191-376) denature reversibly. The thermodynamic stability of the fragments is not influenced by the N-terminal 17-residue histidine tag (Zahn *et al.*, 1996c). There is only one transition during the thermal unfolding of GroEL(191-345), while there are two transitions for GroEL(191-376). The transition of GroEL(191-376) at the lower temperature is attributed to the low stability of the C-terminal α-helices (H11 and H12) (see Figure 1).

Intact GroEL denatures irreversibly above 68°C (Zahn & Plückthun, 1994) or when subjected to high concentrations of urea or guanidinium chloride (Price *et al.*, 1993; Gorovits *et al.*, 1995). GroEL renatured from urea shows secondary but no native tertiary structure. There is some recovery of activity of GroEL after chemical denaturation only in the presence of ATP and ammonium sulphate (Ybarra & Horowitz, 1995). The binding of bis-ANS at 3.1 M urea, under conditions where the majority of GroEL has already unfolded, indicates the existence of hydrophobic residual structure (Gorovits *et al.*, 1995). Analysis of photolabelled chymotryptic fragments of GroEL with bis-ANS suggests that residual structure is located at residues 203 to 249 of the apical domain and might act as a nucleation site for folding.

Here, we analyse the equilibrium unfolding and folding kinetics of the two minichaperones using various techniques. The equilibrium unfolding of the GroEL(191-345) and of GroEL(191-376) fits a two-state model when measured by circular dichroism or fluorescence. Kinetic stopped-flow measurements reveal two phases during unfolding and refolding consistent with the presence of a folding intermediate. Binding studies of the fluorescent dye bis-ANS confirm the formation of intermediates with exposure of hydrophobic surfaces.

Results

Equilibrium unfolding

Monitored by fluorescence

The intrinsic fluorescence of GroEL(191-345) originates from two tyrosine residues, Tyr199 in a loop and Tyr203 in a 3_{10}-helix. There is an additional tyrosine residue in α-helix H12 of GroEL(191-376) (Figure 1). The purified minichaperones are not contaminated with the tryptophan-
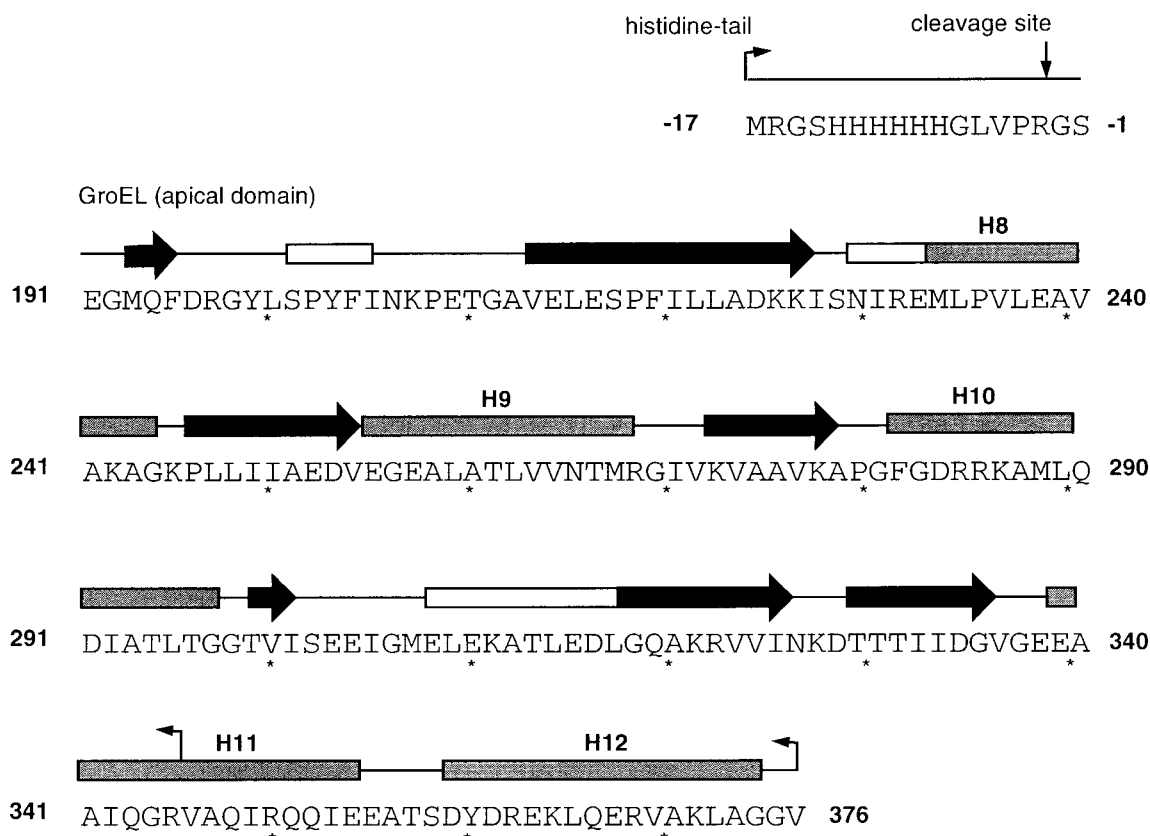

Figure 1. Secondary structure map of the apical domain of the chaperonin GroEL. The N and C-terminal ends of the generated fusion proteins GroEL(191-345) and GroEL(191-376) are indicated by rectangular arrows. Secondary structure is indicated by boxes and arrows for α-helix (grey) or 3_{10}-helix (white) and β-sheet structure, respectively. Assignment of secondary structure was according to the methods of Zahn *et al.* (1996c) and Buckle *et al.* (1997).

containing peptides that are usually observed in GroEL preparations (Hayer-Hartl & Hartl, 1993). The intrinsic fluorescence of the tyrosine residues of both fragments decreases on unfolding by temperature or urea. There is a continuous decrease in fluorescence during temperature unfolding until about 60°C followed by a sharp unfolding transition, with a midpoint temperature of denaturation of about 67°C (Figure 2A and B). The value for $[D]_{50\%}$, the concentration, at which half of the protein is unfolded, is 2.7 M urea for both GroEL fragments when measured by fluorescence and analysed using a two-state model (see Figure 4A and B). Denaturation by temperature or urea of both fragments is fully reversible when measured by fluorescence. Thermodynamic parameters derived from temperature and urea unfolding measurements are summarized in Tables 1 and 2, respectively.

Monitored by circular dichroism

The temperature unfolding curve of GroEL(191-345) measured by far UV circular dichroism at 222 nm (secondary structure, helicity) reveals a single transition (Figure 2E), whereas that of GroEL(191-376) reveals a double transition (Figure 2F). Circular dichroism spectra of the proteins at various temperatures are shown in Figure 3. The values for $[D]_{50\%}$ are 2.9 M urea for both proteins when measured by far UV circular dichroism (Figure 4C and D). The temperature unfolding monitored by near UV circular dichroism (tertiary structure) at 275 nm shows a single transition in the melting curve for both proteins (Figure 2C and D). Thermodynamic parameters derived from temperature and urea unfolding measurements are summarized in Tables 1 and 2, respectively.

Folding kinetics

Unfolding and refolding kinetics using intrinsic tyrosine fluorescence

The unfolding and folding of GroEL fragments were examined using kinetic fluorescence stopped-flow measurements and the data fitted to double exponential first-order kinetics. The fragments each contain seven proline residues, all of which are *trans*. The chevron curves determined from the fast, main refolding/unfolding
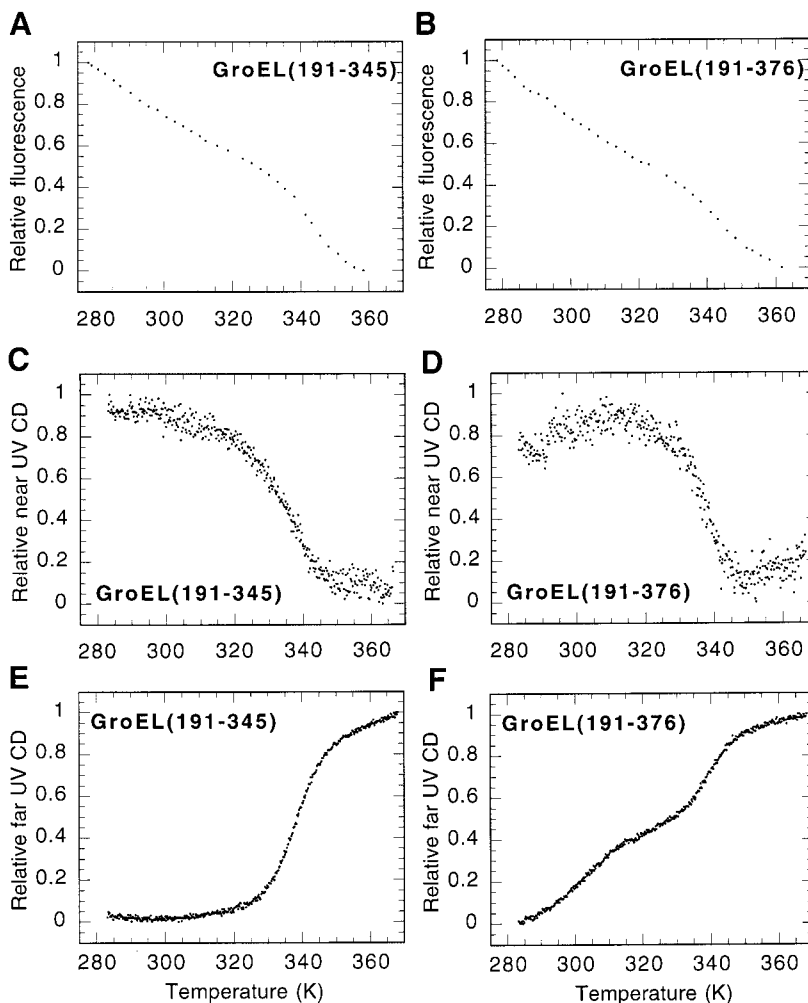

Figure 2. Thermal denaturation of apical domain monitored by tyrosine fluorescence at 303 nm (A and B), circular dichroism at 275 nm (C and D), and circular dichroism at 222 nm (E and F). All traces are normalized.

Table 1. Temperature midpoint of denaturation

Protein	Method	T_M (K)
GroEL(191-345)	Intrinsic fluorescence[a]	343.7 ± 0.7
	Near UV CD[b]	337.7 ± 0.6
	Far UV CD[c]	340.2 ± 0.1
GroEL(191-376)	Intrinsic fluorescence[a]	343.8 ± 1.0
	Near UV CD[b]	339.7 ± 0.8
	Far UV CD (1.transition)[c]	307.5 ± 1.0
	Far UV CD (2.transition)[c]	340.4 ± 0.2

All experiments were performed in 10 mM sodium hydrogen phosphate buffer (pH 7.0) at 25°C. The experimental curves were fitted with the data analysis program Kaleidagraph® (Abelbeck Software) and standard errors represent 1σ.

[a] Temperature unfolding was monitored on excitation of the intrinsic tyrosine fluorescence at $\lambda_{ex} = 275$ nm and emission $\lambda_{em} = 300$ to 303 nm at a protein concentration of 2.7 μM.

[b] Determined at 275 nm at a protein concentration of 18.8 μM.

[c] Determined at 222 nm at a protein concentration of 18.8 μM.

kinetic phases of the GroEL fragments that are not connected with isomerization events show a transition midpoint at about 3.2 M urea (Figure 5A and B). Rate constants of unfolding and refolding were determined by extrapolating the respective parts of the chevron curve to zero denaturant (Table 3). The folding amplitudes of GroEL(191-376) are about 30% larger than those of the shorter fragment, indicating that the environment of tyrosine 360 in α-helix H12 changes upon folding. The dependence of the rate constant of unfolding and refolding on denaturant concentration fit equations (1) to (3):

$$k_{obs} = k_u + k_f \tag{1}$$

$$\log k_u = \log k_u^{H_2O} + m_u[D] \tag{2}$$

$$\log k_f = \log k_f^{H_2O} + m_f[D] \tag{3}$$

where k_u and $k_u^{H_2O}$ are the rate constants of unfolding at the apparent urea concentration and in water, respectively, and k_f and $k_u^{H_2O}$ are the respective rate constants of refolding. The m_u and m_f values represent the slopes for unfolding and refolding in the chevron plot and are proportional to the increase in exposure of the protein on going from the folded to the transition state or the decrease in exposure on going from

the unfolded state to the transition state (Fersht, 1993). The rate constants determined for the second, slow phase both in unfolding and refolding kinetics also show a urea dependence with a transition midpoint at about 2.4 M urea (Figure 5C). There is no dependence of the rate constants on protein concentration.

Measurements on testing a self-chaperoning activity were performed with GroEL(191-345). The protein was unfolded in 5 M urea and refolded by 1 + 5 dilution in refolding buffer containing different concentrations of native GroEL(191-345). The final urea concentration was 0.83 M, therefore, in the pretransition region of the equilibrium unfolding curve. A molar relationship native/denatured GroEL(191-345) of 1:2 and 1:1 was measured and related to the refolding rate constant without additives. The first-order rate constant of the first phase in refolding slightly increases from 1.2 s⁻¹ to 1.6 s⁻¹, that of the second phase slightly decreases from 0.4 s⁻¹ to 0.1 s⁻¹. Higher molar relationships were not studied because of reabsorbance and of less sensitivity of the tyrosine fluorescence of GroEL(191-345). The relationship of the amplitudes of the respective phases does not change.

Binding studies on bis-ANS

The binding capacity of the minichaperones of the dye bis-ANS was determined by fluorescence

Table 2. Thermodynamic values of equilibrium unfolding by urea[a]

Protein	Monitored by fluorescence[a]			Monitored by circular dichroism[b]		
	$[D]_{50\%}$ (mol l⁻¹)	$m(\partial\Delta G/\partial[D])$ (kcal l mol⁻²)	$\Delta G_{D-N}^{H_2O}$ (kcal mol⁻¹)	$[D]_{50\%}$ (mol l⁻¹)	$m(\partial\Delta G/\partial[D])$ (kcal l mol⁻²)	$\Delta G_{D-N}^{H_2O}$ (kcal mol⁻¹)
GroEL(191-345)	2.68 ± 0.06	2.0 ± 0.2	5.4 ± 0.6	2.91 ± 0.02	2.0 ± 0.1	5.7 ± 0.3
GroEL(191-376)	2.68 ± 0.06	1.9 ± 0.3	5.2 ± 0.9	2.90 ± 0.06	2.0 ± 0.3	5.7 ± 0.9

All experiments were performed in 10 mM sodium hydrogen phosphate buffer (pH 7.0) at 25°C and a protein concentration of 2.7 μM. The experimental curves were fitted with the data analysis program Kaleidagraph® (Abelbeck Software) and standard errors represent 1σ.

[a] Unfolding was monitored on excitation of the intrinsic tyrosine fluorescence at $\lambda_{ex} = 275$ nm and emission $\lambda_{em} = 300$ to 302 nm.

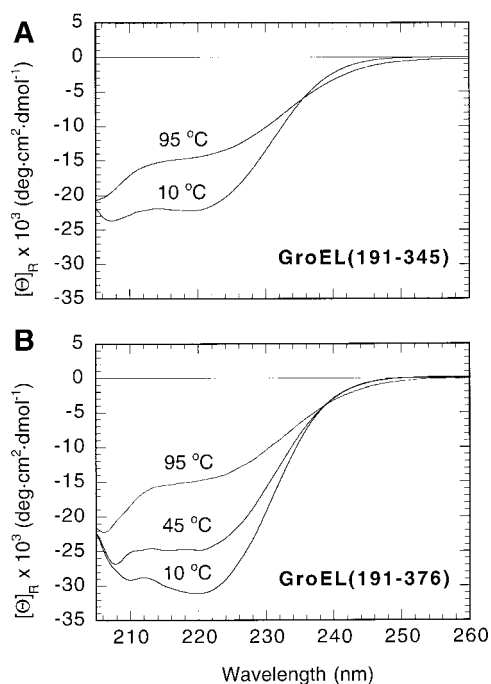[b] Unfolding was monitored at 222 nm (far UV).

Figure 3. Far UV circular dichroism spectra of apical domain. Both GroEL fragments are native at 10°C, and denatured at 95°C. The 45°C spectrum in B is similar to the 10°C spectrum in A, indicating that the C-terminal helices of GroEL(191-376) (see Figure 1) are unfolded while the domain core is still folded.

15 mol (Figure 6A and B), indicating a different extent of exposure of hydrophobic surface in both proteins. bis-ANS fluorescence of both minichaperones decreases with increasing urea concentration, owing to repulsion of the dye by the denaturant, but shows a plateau between 2.0 M and 2.5 M urea (Figure 6C and D). bis-ANS interaction with GroEL(191-345) was studied by stopped-flow kinetics at different urea concentrations. Both unfolding and refolding experiments show a first very fast binding phase of the dye (reaction of second-order kinetics) to the protein and afterwards two phases with an increase in intensity on unfolding and a decrease on refolding (data not shown). The progress curves can be fitted to double exponential first-order kinetics, which is similar to that determined in kinetics monitored by intrinsic tyrosine fluorescence. The amplitude of the first phase has a minimum at about 2.5 M urea, where the amplitude of the second phase is at maximum (Figure 7), in agreement with the ANS equilibrium experiments.

Analytical ultracentrifugation

Results from analytical ultracentrifugation measurements are summarized in Table 4. The M_r values of the fragments confirm their monomeric state in solution at both low and high ionic strength.

Discussion

Equilibrium unfolding of the minichaperones

There are two transitions during the equilibrium unfolding of GroEL(191-376), which are best seen

titration. bis-ANS is known to increase its fluorescence on binding to hydrophobic surfaces. The fluorescence maximum shifts from 550 nm to 500 nm on binding to the native apical domain proteins. The GroEL(191-345) binds 8 mol fluorescent dye per mol protein and GroEL(191-376)
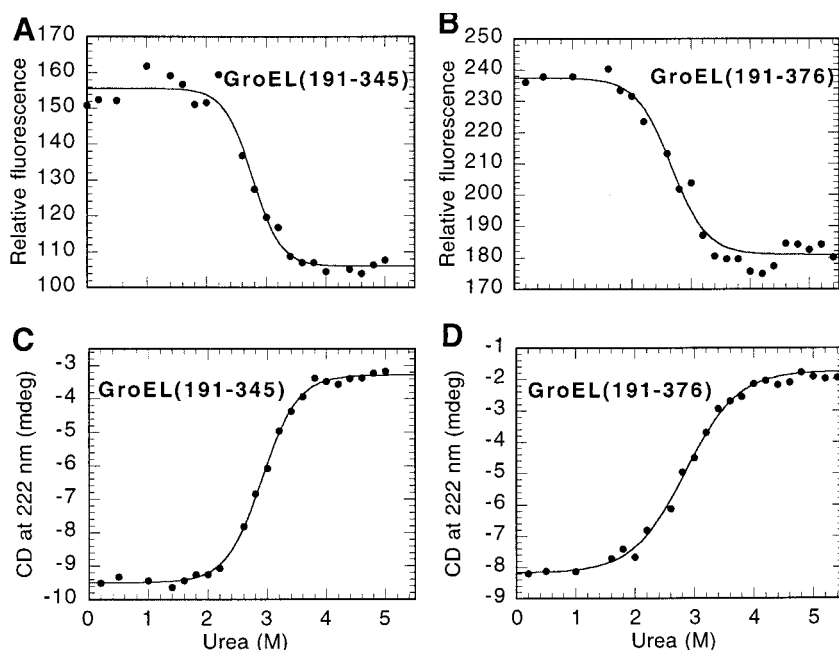

Figure 4. Urea equilibrium denaturation of apical domain monitored by fluorescence at 303 nm (A and B), and circular dichroism at 222 nm (C and D). Data are fitted to equation (7). In D, there is a burst phase between 0 and 0.1 M urea, which has not been taken into account for data fitting.
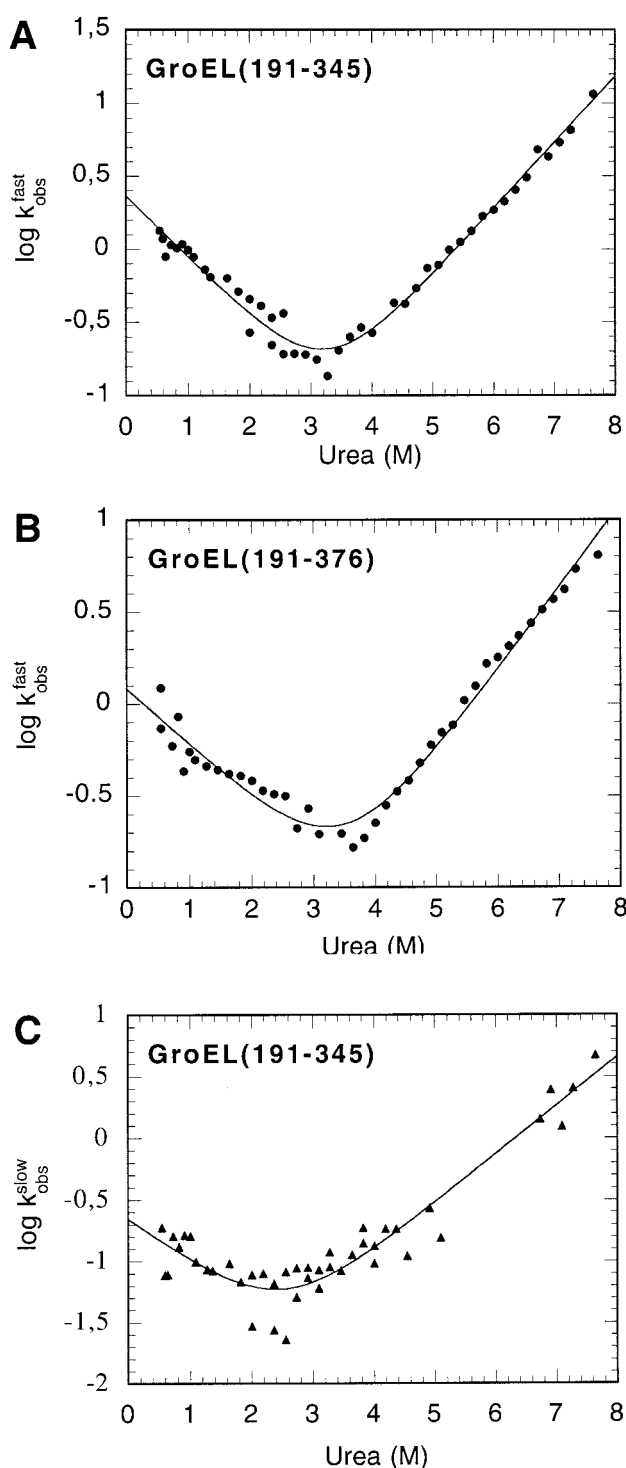
Figure 5. Chevron curves of the main folding phase of GroEL(191-345) (A), GroEL(191-376) (B) and of the slow folding phase of GroEL(191-345) (C) monitored by intrinsic tyrosine fluorescence.

in thermal denaturation studies (Zahn *et al.*, 1996c). Both transitions are reversible and fit a two-state model. The C-terminal helices and loops of GroEL(191-376) melt at the lower temperature, but its core melts at the higher temperature, and is represented by the N-terminal fragment GroEL(191-345).

Domain core

There are similar temperature midpoints of denaturation for GroEL(191-345) and GroEL(191-376) when measured by fluorescence (Table 1), indicating that the tertiary structures of the two fragments melts exactly at the same temperature. Unfolding of the domain core is fully reversible in the presence or absence of C-terminal helices. The ΔG value for unfolding of the apical domain core is about 5.5 kcal mol^{-1} when calculated from the urea denaturation curves measured by fluorescence or far UV circular dichroism using the two-state model (Table 2). The temperature midpoint of denaturation determined by circular dichroism is about 3 degrees lower than the t_m determined by fluorescence. The different melting temperatures may result from the intrusion of a small fraction of a folding intermediate. Studies of the binding of the fluorescent dye bis-ANS to urea-unfolded GroEL showed that in the post-transition region an intermediate with exposed hydrophobic surface is populated (Gorovits *et al.*, 1995). This intermediate has been attributed to the apical domain. We determined that 15 mol bis-ANS bind per mol apical domain protein, 8 mol bis-ANS per mol apical domain core under native conditions (Figure 6A and B). In the course of unfolding of bis-ANS saturated apical domain the binding capacity for the dye shows an optimum in the pretransition region at about 2.5 M urea, indicating exposed hydrophobic surface (Figure 6C and D). Thus, an intermediate accumulates during equilibrium unfolding of the minichaperone core, which is observed by bis-ANS fluorescence, but not by intrinsic fluorescence or circular dichroism.

C-terminal helices

There is a well separated low-temperature transition during equilibrium denaturation of the minichaperone, which is discerned by far UV circular dichroism (Figure 2F). This is in agreement with a small "burst" between 0 M and 0.1 M urea in the denaturation curve of GroEL(191-376) measured by far UV circular dichroism (data not shown in Figure 4D) and a different slope in the pretransition baseline of its temperature denaturation curve compared with GroEL(191-345) (Figure 2C and D). The far UV circular dichroism spectrum of GroEL(191-376) at 45°C is similar to that of GroEL(191-345) at 10°C (Figure 3). Thus, the lower temperature transition can be attributed to the melting of α-helices H11 and H12 at the C terminus of the minichaperone.

Table 3. Thermodynamic data derived from kinetic measurements of folding and unfolding

Protein	$k_u^{H_2O}$ (s^{-1})	m_u (kcal l mol^{-2})	$k_f^{H_2O}$ (s^{-1})	m_f (kcal l mol^{-2})	$\Delta G_{D-I}^{H_2O}$ (kcal mol^{-1})[a]	$\Delta G_{I-N}^{H_2O}$ (kcal mol^{-1})[b]
GroEL(19-345)	0.004 ± 0.0008	0.45 ± 0.02	2.3 ± 0.2	0.42 ± 0.03	3.8 ± 0.3	1.6 ± 0.6
GroEL(191-376)	0.003 ± 0.0009	0.45 ± 0.02	1.2 ± 0.1	0.30 ± 0.03	3.5 ± 0.2	1.6 ± 0.9

Rate constants and *m* values of the fast phase were derived from the data in Figure 5A and B, and defined by equations (1) to (3).
[a] Energy differences between the denatured state D and the intermediate state I were calculated according to equation (9).
[b] $\Delta G_{I-N}^{H_2O} = \Delta G_{D-N}^{H_2O} - \Delta G_{D-I}^{H_2O}$. Energy differences between the denatured state and the native state were determined from equilibrium urea denaturation curves.

Folding kinetics of the minichaperones

The progress curves in unfolding and refolding experiments of the GroEL fragments can be fitted to double exponential first-order kinetics. The folding amplitudes of GroEL(191-376) are about 30% larger than those of the shorter fragment indicating that the flexible C terminus containing an additional tyrosine at position 360 can only refold when the apical domain core has already been formed.

The analysis of the chevron curves of the fast, main folding phase (Figure 5A and B) gives the respective rate constants of unfolding and refolding in water (Table 3). There is a slight difference

in the slope of the refolding part ($m_f = 0.30$ compared with $m_f = 0.45$) for the chevron curves of GroEL(191-376) and GroEL(191-345), respectively, which extrapolates to difference folding rate constants of about a factor of 2 in water. The kinetic *m* values of the unfolding part of the chevron curves are the same for both fragments ($m_u = 0.42$), indicating that helices H11 and H12 do not influence unfolding. The free energy for unfolding of GroEL(191-345) derived from the kinetic experiments (Table 3) is about 1.6 kcal mol^{-1} smaller than that derived from the equilibrium experiments (Table 2). We can conclude that the fast phase in refolding and unfolding can be attributed to the conversion of a folding intermediate of the
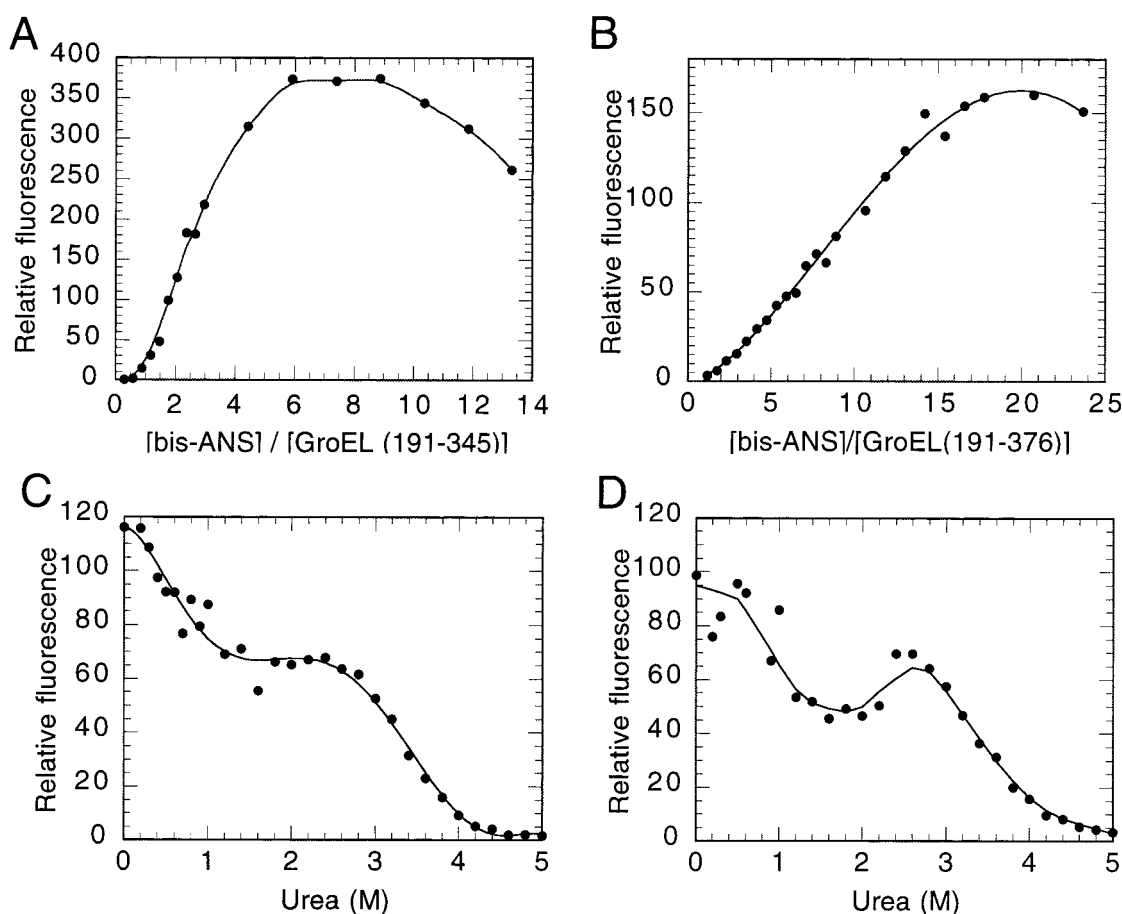

Figure 6. Fluorescence titration curve of bis-ANS to GroEL(191-345) (A) and to GroEL(191-376) (B). Change of bis-ANS fluorescence dependent on urea for GroEL(191-345) (C) and GroEL(191-376) (D) after incubation with saturation concentrations of the dye.
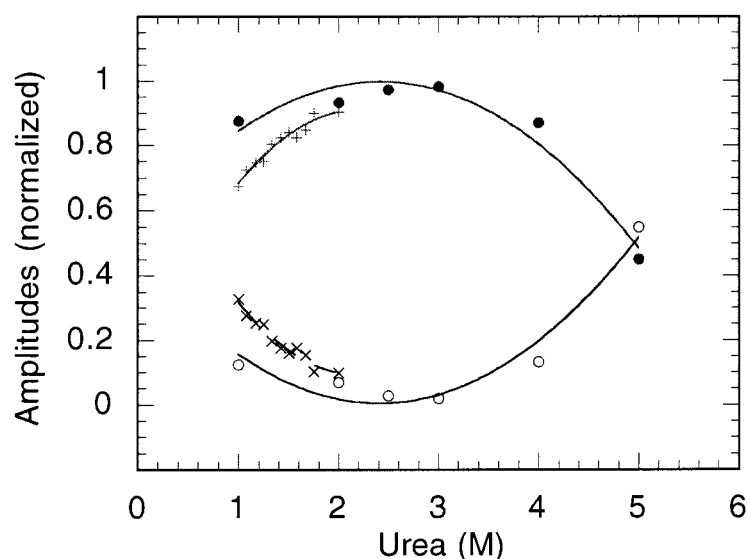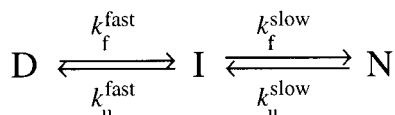
Figure 7. Kinetics of bis-ANS interaction with GroEL(191-345). Amplitudes of the fast phase in refolding (X) and unfolding (○), amplitudes of the slow phase in refolding (+) and unfolding (●).

minichaperone (Matouschek *et al.*, 1990). The slower folding phase can be attributed to the decay of the intermediate (Scheme 1):

$$D \underset{k_{u}^{fast}}{\overset{k_{f}^{fast}}{\rightleftarrows}} I \underset{k_{u}^{slow}}{\overset{k_{f}^{slow}}{\rightleftarrows}} N$$

Scheme 1.

Depending on the urea concentration, the amplitude of the slow phase is between 30 and 40% of the total change. The proteins studied contain seven peptidyl-prolyl bonds. All of them are known to be in the *trans* conformation in the native state (Zahn *et al.*, 1996c; Buckle *et al.*, 1997). Assuming a concentration relationship of ≈30% *cis* and ≈70 % *trans* conformation of the respective peptidyl–prolyl bonds in the denatured state, this phase might be attributed to proline isomerization processes known as slow folding phases (Brandts *et al.*,

1975; Schmid, 1992). But, the observed rate constants clearly show a dependence on denaturant concentration, unlike that of prolyl-isomerization in model peptides (Dyson *et al.*, 1988; Schmid, 1992). This is consistent with there being an intermediate on the folding pathway of the minichaperone that is destabilized relatively to the native state by about 1.6 kcal mol⁻¹. Self-chaperoning was tested for GroEL(191-345), the yield of refolding is not influenced for the minichaperone in the concentration range used; the rate constants of the two phases change slightly in opposite manners. Kinetic experiments with bis-ANS show that the intermediate has highly exposed hydrophobic surfaces.

Monomeric state

NMR experiments have shown that GroEL(191-345) (without the N-terminal tag tail) has a tendency for fast association and dissociation (Zahn

Table 4. Analytical ultracentrifugation experiments

Protein	$s_{20,w}$ (Svedberg)	M_r (kDa)	M_r calculated (kDa)	Association number
GroEL(191-345)	1.1 ± 0.1[a]	15.0 ± 4.0[a]		1[a]
	0.8 ± 0.3[b]	16.0 ± 3.0[b]	18.5	1[b]
GroEL(191-376)	1.19 ± 0.03[a]	23.0 ± 2.0[a]		1[a]
	0.9 ± 0.2[b]	n.d.[b]	22.0	n.d.[b]
GroEL(191-345)	1.1 ± 0.2[a]	15.0 ± 4.0[a]		1[a]
(cleaved)	0.8 ± 0.3[b]	20.5 ± 2.0[b]	16.7	1[b]

[a] Data set was derived from measurements in deionized distilled water (ionic strength ≈0). Sedimentation velocity was done at 50,000 rev/min and scans were recorded at 220 nm or 276 nm. Sedimentation equilibrium was done at 18,000 rev/min for GroEL(191-345), 15,000 rev/min for GroEL(191-376) and 22,000 rev/min for GroEL(191-345) (cleaved). Scans were recorded at 276 nm for GroEL(191-345) and GroEL(191-376) and at 220 nm for GroEL(191-345) (cleaved).
[b] Data set was derived from measurements in sodium/potassium phosphate, sodium chloride buffer (pH 7.0; ionic strength = 0.1) according to Green (1933). Sedimentation velocity was done at 50,000 rev/min. Scans were recorded at 276 nm for GroEL(191-345) (cleaved) and GroEL(191-345) and at 220 nm for GroEL(191-376). Sedimentation equilibrium was done at 15,000 and 18,000 rev/min for GroEL(191-345) and at 15,000 rev/min for GroEL(191-345) (cleaved histidine tail, Zahn *et al.* (1996c)). Scans were recorded at 276 nm.

et al., 1996c). There could be a small fraction of multimeric GroEL fragment from self-aggregation. However, the molecular masses of both GroEL(191-345) and GroEL(191-376) determined from ultracentrifugation experiments (Table 4) fit well with the theoretical molecular masses of the monomeric states. Thus, both fragments are populated mainly as monomers in solution. There is no dependence of the rate constants for folding and unfolding on the protein concentration. In agreement with the crystal structure of intact GroEL (Braig *et al.*, 1994, 1995) the apical domain of GroEL is obviously not responsible for association of the subunits in native GroEL.

The apical domain in intact GroEL

Intact GroEL can be renatured from urea to some extent only in the presence of ammonium sulphate and ATP or ADP (Gorovits *et al.*, 1995; Ybarra & Horowitz, 1995). Only once monomeric GroEL has formed, can it assemble spontaneously to tetradecameric GroEL and association is facilitated by the substrate protein (Mendoza *et al.*, 1994). The urea concentration, where half of GroEL is denatured when monitored by fluorescence or far UV circular dichroism, at about 2.5 M urea (Gorovits *et al.*, 1995), is lower than the $[D]_{50\%}$ value of the GroEL fragments (Table 1). The temperature at which GroEL starts to denature irreversibly and to form aggregates, at 68°C (Zahn & Plückthun, 1994), is close to the T_m of the minichaperones. In contrast, folding of GroEL (191 to 376) is fully reversible on changing urea concentration or temperature. Thus, the intermediate or equatorial domains are most probably responsible for the inability of GroEL to refold. Most importantly, reversible folding of the minichaperones is independent of its intrinsic chaperone activity, as unfolding and refolding rate constants are independent of protein concentration. The reversible folding of the polypeptide binding domain of GroEL protects the chaperone against self-recognition, and thus guarantees efficient chaperone activity of the flexibly ordered apical domains within the GroEL toroid.

Materials and Methods

Protein expression and purification

The DNA for the GroEL fragment was amplified by PCR from the pOF plasmid (Fayet *et al.*, 1989) and subcloned into a pRSET-vector (Invitrogen) as described (Zahn *et al.*, 1996c). The vector codes for an N-terminal histidine-tail of 17 amino acid residues, containing a thrombin cleavage site. Proteins were expressed in *E. coli* JM109 cells using the XPRESS SYSTEM® Protein Expression, Version 1.1, supplied by Invitrogen Corporation. The protein purification was performed as described using Ni²⁺-NTA agarose supplied by Qiagen (Zahn *et al.*, 1996c). Additional purification of GroEL(191-376) was performed on SOURCE® 15Q. The protein was applied in 50 mM Tris-HCl (pH 7.0). Impuri-

ties were separated with a discontinous NaCl gradient (8%) and the GroEL(191-376) eluted with 10% NaCl. The protein concentration was determined by absorbance at 280 nm with a molar extinction coefficient of 6300 M⁻¹ cm⁻¹ for GroEL(191-345) and 9270 M⁻¹ cm⁻¹ for GroEL(191-376) determined according to the method of Gill & von Hippel (1989) and using a correction factor derived from amino acid analysis.

Equilibrium unfolding studies

Urea unfolding of GroEL fragments was monitored by fluorescence and circular dichroism. The intrinsic tyrosine fluorescence on excitation at 275 nm was recorded for emission from 285 nm to 350 nm at different urea concentrations using a F-4500 Hitachi fluorimeter at 25°C. The bandpass for both the excitation and the emission monochromators was set at 5 nm, the scan speed at 240 nm per min and the response time automatically adapted by the device. All fluorescence spectra are corrected according to the supplier's recommendation using Rhodamine B as standard. The relative fluorescence intensity at 303 nm was used for monitoring urea unfolding (Demchenko, 1986; Eftink, 1994). The final protein concentration was about 5 μM, in 10 mM sodium hydrogen phosphate buffer (pH 7.0). Temperature unfolding measurements were performed by increasing the temperature in two-degree steps from 10°C to 85°C every five minutes. The temperature dependence of the fluorescence of N-Ac-Tyr-NH₂ was determined under the same conditions as internal standard for the intrinsic chromophores of the apical domains and found to decrease linearly with temperature (data not shown).

Circular dichroism spectra of the GroEL fragments were recorded with a Jasco J720 spectropolarimeter using cuvettes of pathlength 0.1 cm (far UV) and 1 cm (near UV) and a buffer of 10 mM sodium hydrogen phosphate (pH 7.0) at 25°C. Spectra were acquired at a scan speed of 20 nm per min, a slit width of 2 nm and a response time of four seconds. The ellipticity at 222 nm was used for monitoring urea unfolding. Temperature unfolding measurements were performed in thermostated cuvettes of pathlength 0.1 cm (far UV) and 1 cm (near UV) using a temperature gradient of 50 degrees per hour.

Urea unfolding curves were fitted to a two-state model, in which the native (N) and denatured (D) states are present, with the program Kaleidagraph® and a Macintosh computer. The values for $[D]_{50\%}$ (concentration of denaturant, where 50% of the protein is unfolded) and $\Delta G_{D-N}^{H_2O}$ (free energy of unfolding in the absence of denaturant) were determined by the linear extrapolation method:

$$\Delta G_{D-N} = \Delta G_{D-N}^{H_2O} - m[D] \qquad (4)$$

$$\Delta G_{D-N} = -RT \ln K_{D/N} \qquad (5)$$

$$K_{D/N} = \frac{(X_N - X)}{(X - X_D)} \qquad (6)$$

where ΔG_{D-N} is the free energy of unfolding at a given denaturant concentration, m is the slope of the plot in the transition region, X_N is the value of the specified optical signal (fluorescence F_{303} at 303 nm or ellipticity θ_{222} at 222 nm) of the folded protein, X_D of the denatured protein and X of the protein at a given dena-

turant concentration. $K_{D/N}$ represents the equilibrium ratio of unfolded/native protein species at any specified denaturant concentration, T is the absolute temperature and is the gas constant (Bolen & Santoro, 1988; Santoro & Bolen, 1988; Clarke & Fersht, 1993).

Evaluation of equilibrium constants in the transition region requires extrapolation of the pre- and post-unfolding baselines into the transition region. Considering the intercepts (n_N, n_D) and the slopes (m_N, n_D) of the pre- and post-unfolding regimes, the following equation was used for fitting urea denaturation curves (Clarke & Fersht, 1993):

$$X = \frac{(n_N + m_N[D]) + (n_D + m_D)[D] \cdot e^{\frac{-m([D]_{50\%} - [D])}{RT}}}{1 + e^{\frac{-m([D]_{50\%} - [D])}{RT}}} \quad (7)$$

Optically detected kinetic experiments

Tyrosine residues are the intrinsic chromophores of the apical domain of GroEL. From steady state fluorescence spectra the emission maximum has been determined at 303 nm. Kinetic experiments were performed with an Applied Photophysics BioSequential DX.17 MV stopped-flow spectrometer (Leatherhead, UK). The change in total fluorescence above 320 nm (cutoff filter) on excitation at 275 nm was recorded at 25°C. The photomultiplier was set between 640 and 670 V in all experiments. The pathlength of the observation chamber was 0.2 cm. All kinetic folding studies were carried out in 10 mM sodium hydrogen phosphate buffer, (pH 7.0) and a final protein concentration of about 4 μM. Protein folding occurs as a multistep transition and can be described as:

$$y(t) = \Sigma y_i \cdot e^{-k_i t} + y(\infty) \quad (8)$$

where $y(t)$ and $y(\infty)$ are amplitudes of the optical probe at time t and at equilibrium, respectively, while y_i is the amplitude at zero time and k_i is the first-order rate constant of phase i (Schmid, 1992; Kim *et al.*, 1994). Data were fitted to a double exponential first-order reaction. Single-jump unfolding experiments were performed at 25°C by an 11-fold dilution of folded apical domain with unfolding buffer containing different amounts of urea. Single-jump refolding experiments performed by an 11-fold dilution of 7 M urea-unfolded apical domain with refolding buffer containing different amounts of urea. At least seven traces were used for averaging. The final protein concentration was 4.1 μM for GroEL(191-345) and 4.4 μM for GroEL(191-376).

The rate constants of unfolding and refolding derived from the chevron curves were used to determine the energy difference between unfolded state and intermediate state according to:

$$\Delta G_{D-I}^{H_2O} = -RT \cdot \ln \frac{k_u^{H_2O}}{k_f^{H_2O}} \quad (9)$$

where $k_u^{H_2O}$ is the fast phase rate constant of unfolding and $k_f^{H_2O}$ that of refolding in water.

Binding of the fluorescent dye bis-ANS

The dipotassium salt of the fluorescent dye bis-ANS was dissolved in water giving a stock concentration of 1.48 mM solution. Fluorescence titration was performed with 5 μM GroEL (191 to 345) and 2.5 μM GroEL (191 to 376) in 10 mM sodium phosphate buffer (pH 7.0) at 25°C. The same concentration of the proteins and buffer conditions were used for monitoring bis-ANS fluorescence at different urea concentrations.

Kinetic experiments with GroEL (191 to 345) were performed with an Applied Photophysics BioSequential DX.17MV stopped-flow spectrometer (Leatherhead, UK). The change in total fluorescence above 460 nm (cutoff filter) on excitation at 382 nm was recorded at 25°C. The photomultiplier was set between 350 and 400 V in all experiments. The pathlength of the observation chamber was 0.2 cm. Single-jump unfolding experiments were performed by a sixfold dilution of folded apical domain with unfolding buffer containing bis-ANS at saturation concentration and different amounts of urea. Single-jump refolding experiments were performed by a sixfold dilution of 6 M urea-unfolded apical domain with refolding buffer containing bis-ANS at saturation concentration. The final concentration of the protein was 2.5 μM and of the fluorescent dye 20 μM (eightfold molar excess). Investigation of the progress curves was done according to the procedure described above. The first very fast phase, representing the initial binding of bis-ANS, is not related to folding and was not considered for data investigation.

Analytical ultracentrifugation measurements

In order to determine the association state of the apical domain of GroEL, analytical ultracentrifugation measurements were performed on an XL-A (Beckman, Palo Alto, USA) ultracentrifuge (Giebeler, 1992). Sedimentation velocity boundaries and sedimentation equilibrium distributions were recorded using the scanning absorption optical system at both 220 nm (peptide bond chromophore) and 276 nm (aromatic chromophore). Samples were run at a protein concentration of ≈1.5 mg/ml at pH 7.0 and a temperature of 20.0°C and two different ionic strengths ($I = 0.0$ and 0.1). Weight average molecular masses were evaluated from sedimentation equilibrium distributions according to the MSTAR procedure (Cölfen & Harding, 1997). Sedimentation coefficients were corrected to standard solvent conditions (the viscosity and density of water at 20°C) yielding $s_{20,w}$ according to the method of van Holde (1971).

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