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Conformational specificity of the chaperonin GroEL for the compact folding intermediates of α-lactalbumin

Manajit K.Hayer-Hartl, Jonathan J.Ewbank[1,2], Thomas E.Creighton[1] and F.Ulrich Hartl

Howard Hughes Medical Institute, Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA and [1]European Molecular Biology Laboratory, Postfach 10.2209, Meyerhofstrasse 1, 69012 Heidelberg, Germany
[2]Present address: Department of Biology, McGill University, 1205 Dr Penfield Avenue, Montreal, PQ, Canada H3A 1B1

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The chaperonin GroEL binds unfolded polypeptides, preventing aggregation, and then mediates their folding in an ATP-dependent process. To understand the structural features in non-native polypeptides recognized by GroEL, we have used α-lactalbumin (αLA) as a model substrate. αLA (14.2 kDa) is stabilized by four disulfide bonds and a bound Ca^{2+} ion, offering the possibility of trapping partially folded disulfide intermediates between the native and the fully unfolded state. The conformers of αLA with high affinity for GroEL are compact, containing up to three disulfide bonds, and have significant secondary structure, but lack stable tertiary structure and expose hydrophobic surfaces. Complex formation requires almost the complete αLA sequence and is strongly dependent on salts that stabilize hydrophobic interactions. Unfolding of αLA to an extended state as well as the burial of hydrophobic surface upon formation of ordered tertiary structure prevent the binding to GroEL. Interestingly, GroEL interacts only with a specific subset of the many partially folded disulfide intermediates of αLA and thus may influence *in vitro* the kinetics of the folding pathways that lead to disulfide bonds with native combinations. We conclude that the chaperonin interacts with the hydrophobic surfaces exposed by proteins in a flexible compact intermediate or molten globule state.

Key words: chaperonin/GroEL/α-lactalbumin/molten globule/protein folding

Introduction

Molecular chaperones of the Hsp60 family play an essential role in mediating the folding and assembly of newly synthesized polypeptides (Gething and Sambrook, 1992; Hartl *et al.*, 1994). These 'chaperonins' (Hemmingsen *et al.*, 1988) are large oligomeric ring-complexes containing 14 subunits of ~60 kDa in stacked heptameric rings (Hendrix, 1979; Hohn *et al.*, 1979; Langer *et al.*, 1992a; Saibil *et al.*, 1993). GroEL, the *Escherichia coli* chaperonin, was discovered as a host protein required for bacteriophage capsid assembly (Coppo *et al.*, 1973; Georgopoulos *et al.*, 1973). It interacts with newly synthesized polypeptides in the bacterial cytosol and is required for their folding (Bochkareva *et al.*, 1988; Goloubinoff *et al.*, 1989a; Horwich *et al.*, 1993). The GroEL homologues in chloroplasts and mitochondria are involved in protein folding and assembly reactions within the respective organelles (Barraclough and Ellis, 1980; Cheng *et al.*, 1989; Ostermann *et al.*, 1989).

The basic function of the Hsp60s is to assist the folding of monomeric polypeptide chains (Ostermann *et al.*, 1989; Laminet *et al.*, 1990; Martin *et al.*, 1991; Viitanen *et al.*, 1991; Zheng *et al.*, 1993). Hsp60 binds a partially folded polypeptide, preventing aggregation (Buchner *et al.*, 1991), and releases it in an ATP-dependent reaction that can result in folding (Goloubinoff *et al.*, 1989b; Martin *et al.*, 1991, 1993a). This process depends on the co-chaperonin Hsp10, GroES in *E.coli*, a single heptameric ring of ~10 kDa subunits that forms a complex with GroEL (Chandrasekhar *et al.*, 1986; Viitanen *et al.*, 1990; Saibil *et al.*, 1991; Langer *et al.*, 1992a; Landry *et al.*, 1993). GroES regulates the ATPase activity of the GroEL subunits, coordinating their action to allow the release of bound polypeptide in a manner productive for folding (Gray and Fersht, 1991; Bochkareva *et al.*, 1992; Jackson *et al.*, 1993; Martin *et al.*, 1993a,b; Todd *et al.*, 1993). During folding, the polypeptide is maintained in a shielded environment (Martin *et al.*, 1991), apparently provided by the central cavity of the Hsp60 cylinder (Langer *et al.* 1992a; Braig *et al.*, 1993; Saibil *et al.*, 1993).

While there has been considerable progress in understanding the mechanistic principles of Hsp60-mediated folding, the structural features recognized by the chaperonins in unfolded polypeptides are still undefined. Although GroEL binds an amphiphilic N-terminal peptide of rhodanese with low affinity, stabilizing it in an α-helical conformation (Landry *et al.*, 1992), the chaperonin also interacts with the denatured state of an all-β protein (Schmidt and Buchner, 1992). It is not known whether GroEL recognizes primarily contiguous sequence elements or hydrophobic surfaces typically exposed by partially folded polypeptides. It has been suggested that GroEL binds the compact intermediates of proteins produced early in folding by the partial collapse of hydrophobic residues to the interior of the molecule (Laminet *et al.*, 1990; Höll-Neugebauer, 1991; Martin *et al.*, 1991; van der Vies *et al.*, 1992). These compact intermediates or 'molten globules' (Kuwajima, 1989) appear to be of general significance in the folding of globular proteins (Ptitsyn *et al.*, 1990; Ptitsyn, 1992). They are characterized by a hydrodynamic radius close to that of the native state, the presence of a significant amount of secondary structure and the partial or complete lack of stable tertiary structure interactions (Kuwajima, 1989; Christensen and Pain,

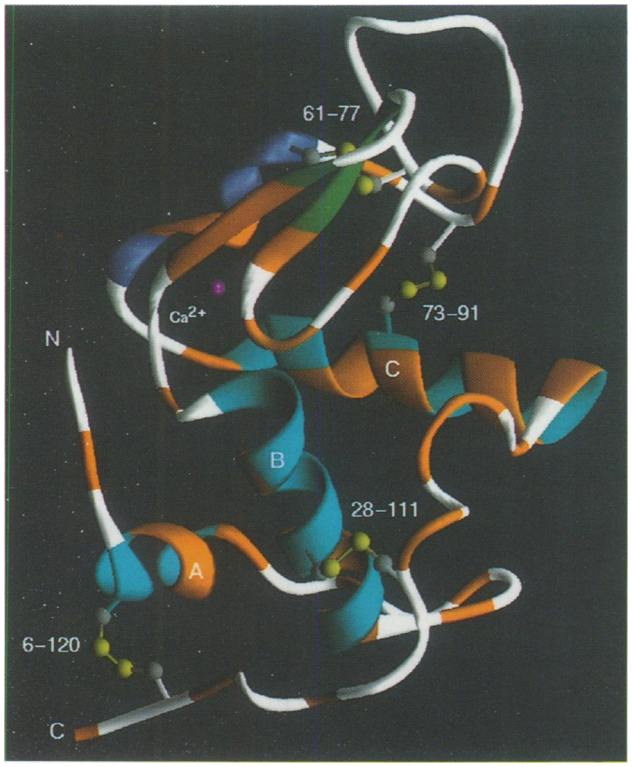

Fig. 1. Ribbon diagram of the three-dimensional structure of baboon αLA, generated using the coordinates 1ALC (Acharya *et al.*, 1989) and the program 'Ribbons' (Carson, 1989). The structure is highly homologous to that of bovine αLA (Shewale *et al.*, 1984). Disulfide bonds are shown in yellow and the bound Ca^{2+} in pink. The major elements of secondary structure include the three α-helices A (residues 5–11), B (23–34) and C (86–99), a 3_{10} helix (76–82) and two β-strands (40–43 and 47–50). The backbone of hydrophobic residues (Ile, Leu, Met, Phe, Trp, Tyr and Val) is shown in gold; all other residues are represented in blue (for α-helices), purple (for the 3_{10} helix), green (for β-strands) or white (for unstructured regions).

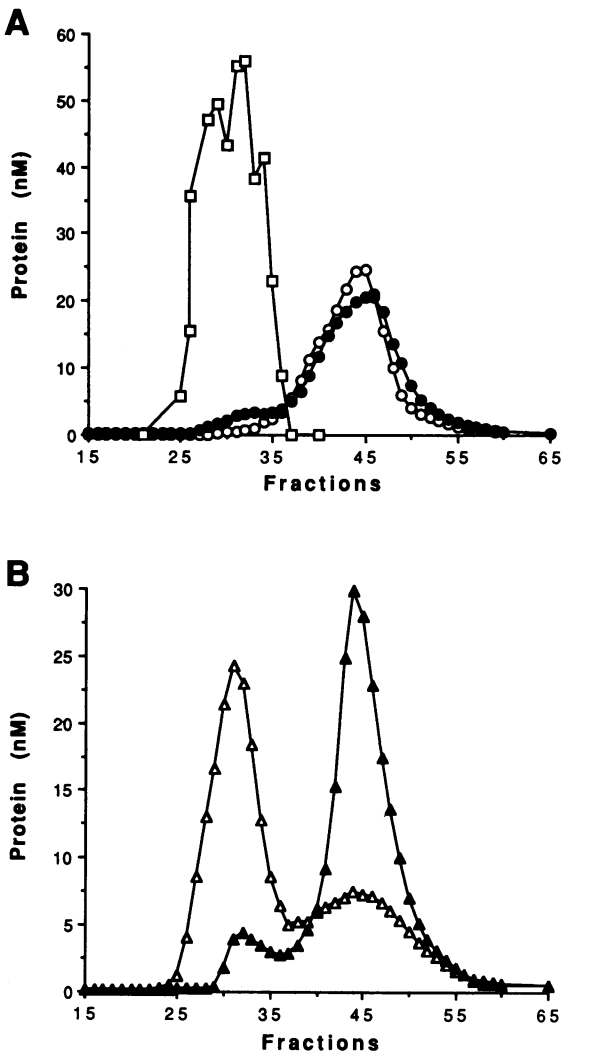

Fig. 2. Binding of holo-αLA, apo-αLA and rearranged [3SS] to GroEL. GroEL and αLA were incubated at 0.31 μM concentrations in buffer A for 15 min and then separated by sizing chromatography on a Sephacryl S300-HR column as described in Materials and methods. To produce rearranged [3SS], αLA was incubated with 0.5 mM DTT for 10 min in buffer A (pH 7.2), diluted 10-fold into buffer A (minus $CaCl_2$) containing 0.5 mM EGTA and incubated for 20 min at 25°C. Then GroEL was added and incubation continued for 15 min. In one case 1 mM ATP/5 mM $MgCl_2$ was added to the binding reaction after this incubation and was then also present in the column buffer. (**A**) GroEL, □; holo-αLA, ○; apo-αLA, ●. (**B**) [3SS], △; [3SS] + Mg-ATP, ▲.

1991). The term 'molten globule' (MG) refers to the presence in these intermediates of a fluctuating hydrophobic core and the transient exposure of hydrophobic surfaces, which is diagnosed by the adsorption of the hydrophobic fluorescent probe 1-anilino-naphthalene-8-sulfonate (ANS) (Ptitsyn *et al.*, 1990; Semisotnov *et al.*, 1991).

The partially folded intermediates of proteins formed early on in the productive folding pathway are short-lived and in rapid equilibrium with the fully unfolded state. This complicates the analysis of the structural properties that GroEL recognizes in a substrate protein, as the conformational equilibrium can be readily shifted by GroEL binding one set of conformations more tightly than others. To circumvent this problem, we have analysed the relative affinities of GroEL for a series of stably trapped disulfide folding intermediates of the 123 residue Ca^{2+} binding protein α-lactalbumin (αLA) (Figure 1). These conformers range from the native to the unfolded state and include MG intermediates generated by selective reduction of one or two of the protein's four disulfide bonds (Kuwajima *et al.*, 1990; Ewbank and Creighton, 1991, 1993a,b). We present evidence that GroEL interacts specifically with a subset of the compact intermediates of αLA that expose hydrophobic surfaces and have a flexible tertiary structure.

Results

Binding to GroEL requires conversion of αLA to a flexible 'molten globule'

The interaction of purified GroEL with different conformers of bovine αLA was routinely analysed at 25°C by size exclusion chromatography at physiological pH and salt concentration. αLA was ^3H-labelled at, on average, two of its 13 lysine residues without affecting its conformation; the far-UV circular dichroism (CD), tryptophan fluorescence and ANS binding of the protein were unchanged (not shown). The Ca^{2+}-bound holo form of αLA with four intact disulfides (native αLA) did not bind to GroEL (Figure 2A). Likewise, only a very small amount of apo-αLA was found to co-fractionate with GroEL on

Table I. Relative binding affinities of αLA and its derivatives for GroEL

	% of total GroEL bound				
	Ca^{2+}	Ca^{2+} + 0.5 mM DTT	EGTA	EGTA + 0.05 mM DTT	EGTA + 2 mM DTT
αLA	0.0	4.0	5.0	69.0	70.0
3SScam	0.0		5.0		67.0
2SScam	15.0		32.0		64.0
[3SS]cam			32.0		
[3SS]Ucam			46.0		
Rcam	72.0		74.0		
Rcm	0.0		0.0		

Binding assays were performed as described in Materials and methods at concentrations of substrate protein and GroEL of 0.31 μM each. Amounts of GroEL-bound protein were quantified and are given as the percentage of total protein eluted from the sizing column. Protein recoveries were ~95%. αLA, four native disulfide bonds; 3SScam, three native disulfide bonds, disulfide bond between residues 6 and 120 reduced and thiols 6 and 120 blocked with iodoacetamide; 2SScam, two native disulfides, disulfide bonds 6–120 and 28–111 reduced and the respective thiols blocked; [3SS]cam and [3SS]Ucam, complex mixtures of three-disulfide forms rearranged under native conditions or in the presence of 8 M urea, respectively, and two free thiols blocked with cam groups; Rcam, fully reduced and iodoacetamide blocked; Rcm, fully reduced and iodoacetic acid blocked.

the sizing column. This is consistent with the known selectivity of the chaperonins for non-native proteins. At moderate ionic strength (200 mM KCl) and at 25°C, removal of Ca^{2+} by EGTA causes little disturbance of the tertiary structure of αLA, although it is markedly destabilized (Kuwajima *et al.*, 1990; Ewbank and Creighton, 1993b). Significantly, after incubation of the αLA with 0.5 mM dithiothreitol (DTT) and EGTA, >70% of the protein bound to an equimolar concentration of chaperonin with an apparent K_d of ~200 nM (Figure 2B). Low concentrations of DTT cause the selective reduction of the hyperreactive disulfide bond between Cys6 and Cys120 of αLA (Iyer and Klee, 1973; Shechter *et al.*, 1973) (Figure 1). The resulting three-disulfide form, 3SS, is native-like in the presence of Ca^{2+} (Ewbank and Creighton, 1991) and does not bind to GroEL (Table I). However, removal of Ca^{2+} converts the protein into an MG state with rearranging disulfide bonds, [3SS], that lacks ordered tertiary structure and the ability to bind Ca^{2+} (Ewbank and Creighton, 1991, 1993b; Creighton and Ewbank, 1994). The three disulfide bonds in the complex mixture of rearranged species maintain the compact shape of the molecule, which retains most of the secondary structure content of the native state (see Figures 5C and 7). The association of GroEL with the [3SS] species was specific, as Mg-ATP caused it to dissociate (Figure 2B). As shown for other small proteins, the release of αLA from GroEL was independent of the cofactor GroES (Laminet *et al.*, 1990; Martin *et al.*, 1991; Viitanen *et al.*, 1991). Addition of Ca^{2+} in the absence of Mg-ATP did not destabilize the [3SS]–GroEL complex (not shown), indicating that the bound species were not in rapid equilibrium with the 3SS isomer containing three native disulfide bonds. When GroEL was incubated with a molar excess of αLA, no more than one molecule of αLA was recovered per GroEL 14mer in the isolated complex (not shown).

To test whether the dynamic rearrangement of disulfides is a requirement for efficient binding of αLA to GroEL, we analysed the relative affinities of GroEL for a series of chemically trapped forms of αLA with blocked free thiols, thereby preventing disulfide rearrangements. All these intermediates remained stable against aggregation under the experimental conditions. The disulfide bond between residues 6 and 120 of αLA was reduced with DTT in the presence of Ca^{2+}, and the Cys6 and Cys120 thiols were blocked with iodoacetamide (holo-3SScam). Upon removal of the Ca^{2+}, apo-3SScam retains the three native disulfides, and adopts some of the features of the MG. This is indicated by the reduced near-UV CD spectrum of the protein, reflecting a decrease of ordered tertiary interactions, and by the native-like secondary structure in the far-UV CD (Ewbank and Creighton, 1993b). Significantly, apo-3SScam showed only very weak affinity for GroEL (Table I). In contrast, apo-2SScam, in which the disulfide bonds between residues 6 and 120, and between 28 and 111 were successively reduced and blocked, bound to GroEL, albeit with lower affinity than rearranged [3SS]. Interestingly, complete reduction of 2SScam by incubation with 2 mM DTT resulted in an increase in binding to the same value measured with the rearranged [3SS] forms. The same highly efficient binding was measured with Rcam, a fully reduced and carboxyamidated αLA that exists in the MG state for part of the time (see below; Ewbank and Creighton, 1993b). These results suggest the importance of structural flexibility for complex formation with GroEL. This flexibility is apparently provided by the disulfide rearrangements in the compact [3SS] species. In contrast, the MG forms of αLA with two and three native disulfide bonds presumably are less flexible and bind to GroEL with lower affinity.

αLA conformers with high affinity for GroEL have collapsed secondary structure and expose hydrophobic surfaces

To differentiate structurally between the various MG forms of αLA, we analysed the extent to which they expose hydrophobic surfaces that adsorb the hydrophobic fluorescent probe ANS. Both native and completely denatured αLA showed low ANS binding (Figure 3). The same was true of Ca^{2+}-bound 3SS in which the disulfide bond between residues 6 and 120 had been selectively reduced. A significant enhancement in ANS adsorption was detected only with partially folded intermediates that have lost the stable tertiary interactions of the native state, but retain a large amount of the native secondary structure content.

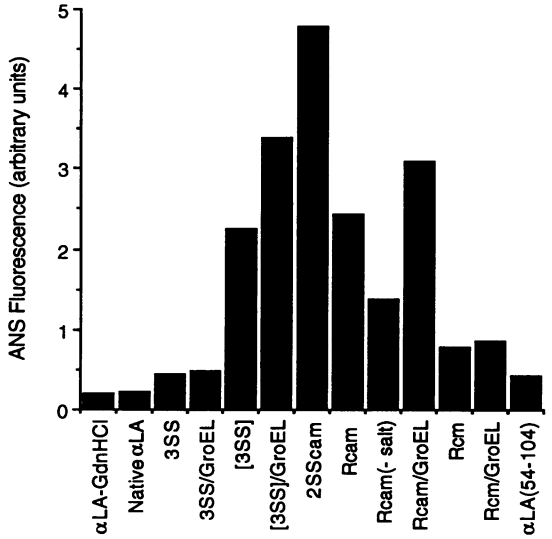

Fig. 3. ANS binding of αLA and its derivatives in the presence and absence of GroEL. Measurements were performed at 1.4 μM αLA protein and 1.7 μM GroEL in buffer A (native αLA; 2SScam), in buffer A containing 0.5 mM DTT (3SS; 3SS/GroEL); buffer A containing 0.5 mM EGTA (apo2SScam; Rcam; Rcam/GroEL; Rcm; Rcm/GroEL) or in buffer A containing 0.05 mM DTT/0.5 mM EGTA {[3SS]; [3SS]/GroEL; αLA (54–104)}. Rcam was also analysed in buffer A lacking KCl. αLA was denatured in buffer A, 0.5 mM EGTA containing 6 M guanidine–HCl for 60 min at 25°C. The concentration of ANS was 43 μM. ANS binding was linearly dependent on the concentration of αLA or αLA/GroEL, indicating the absence of protein aggregation. Spectra were corrected for background fluorescence measured in the absence of αLA protein.

Removal of Ca^{2+} from 3SS, initiating disulfide rearrangement and allowing efficient binding to GroEL, resulted in an ~8-fold increase in ANS adsorption. Even greater ANS binding was measured with 2SScam, although this intermediate associated with GroEL with lower affinity than the rearranged [3SS]. Apparently, there is no simple quantitative relationship between the extent of ANS adsorption by the αLA conformers and their affinity for the chaperonin.

However, the exposure of ANS-accessible surfaces in the MG state reflects a structural property that is critical for the association with the chaperonin. This could be substantiated by the analysis of two forms of fully reduced αLA whose thiols were blocked with either iodoacetamide (Rcam) or iodoacetic acid (Rcm). As noted above (Table I), Rcam associated with GroEL with the same apparent affinity as [3SS]. At neutral pH Rcam possesses ~40% of the secondary structure of the native state (see Figure 5C) and adsorbs ANS as efficiently as [3SS] (Figure 3). In contrast, the iodoacetic acid blocked Rcm showed only 25% of the ANS adsorption (Figure 3) and did not detectably interact with GroEL (Table I). Presumably, due to the repulsive forces resulting from the introduction of eight additional negative charges, Rcm has lost most of the characteristic properties of the MG. Specifically, it has the tryptophan fluorescence spectrum of the fully denatured protein (Figure 4B), contains less secondary structure (Figure 5C) and has a hydrodynamic volume similar to that of the reduced protein in 8 M urea (Ewbank and Creighton, 1993b). We conclude that the MG conformers of αLA that possess collapsed secondary structure and a highly flexible tertiary structure, exposing

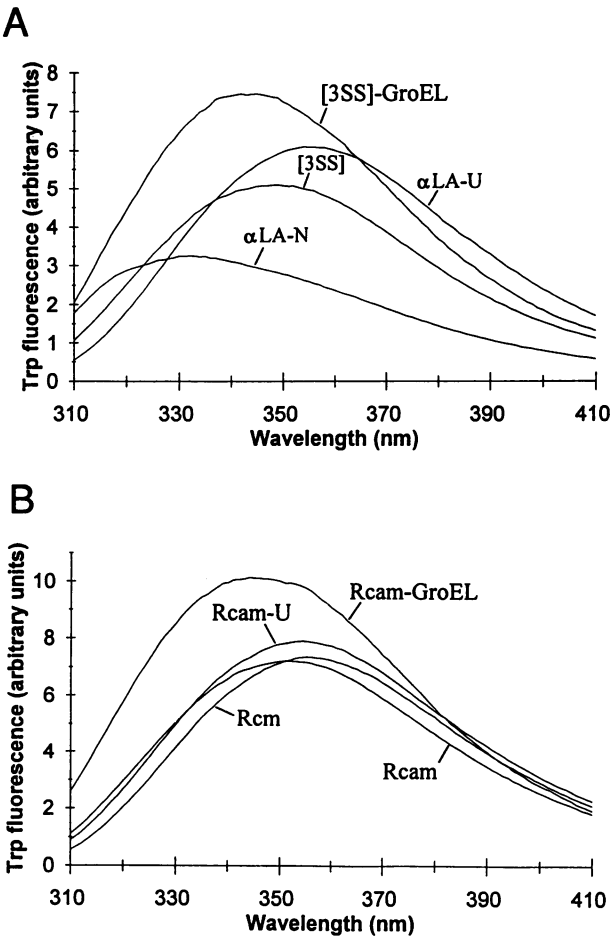

Fig. 4. Effect of GroEL on the tryptophan fluorescence of αLA and its derivatives. Fluorescence was measured at 1.4 μM αLA protein and 1.7 μM GroEL as described in Materials and methods. (**A**) αLA-N, native αLA in buffer A; rearranged [3SS] and [3SS]-GroEL in buffer A containing 0.05 mM DTT/0.5 mM EGTA; αLA-U, denatured αLA in buffer A containing 6 M guanidine–HCl. (**B**) Rcam, Rcam-GroEL and Rcm in buffer A containing 0.5 mM EGTA; Rcam-U, denatured Rcam in buffer A containing 6 M guanidine–HCl. Spectra were corrected for background fluorescence measured in the absence of αLA protein.

hydrophobic surfaces, bind to the chaperonin most efficiently.

GroEL provides a hydrophobic environment for bound αLA

The hydrophobic surfaces exposed by the MG forms of αLA may be directly involved in the association with the chaperonin. We analysed the effect of GroEL binding on the tryptophan and ANS fluorescence of αLA, since a hydrophobic interaction should cause characteristic changes in these parameters. This analysis was possible because GroEL lacks Trp residues (Hemmingsen *et al.*, 1988; Hayer-Hartl and Hartl, 1993). Bovine αLA contains four Trp residues that are spaced evenly throughout its sequence (Shewale *et al.*, 1984). Unfolding of holo-αLA to [3SS] by selective reduction of the disulfide bond between residues 6 and 120 and removal of Ca^{2+} is accompanied by a red-shift in Trp fluorescence from 333 nm to 349 nm and by an 80% increase in fluorescence intensity (Figure 4A). The red-shift reflects a change of the Trp residues to a more polar environment, while the

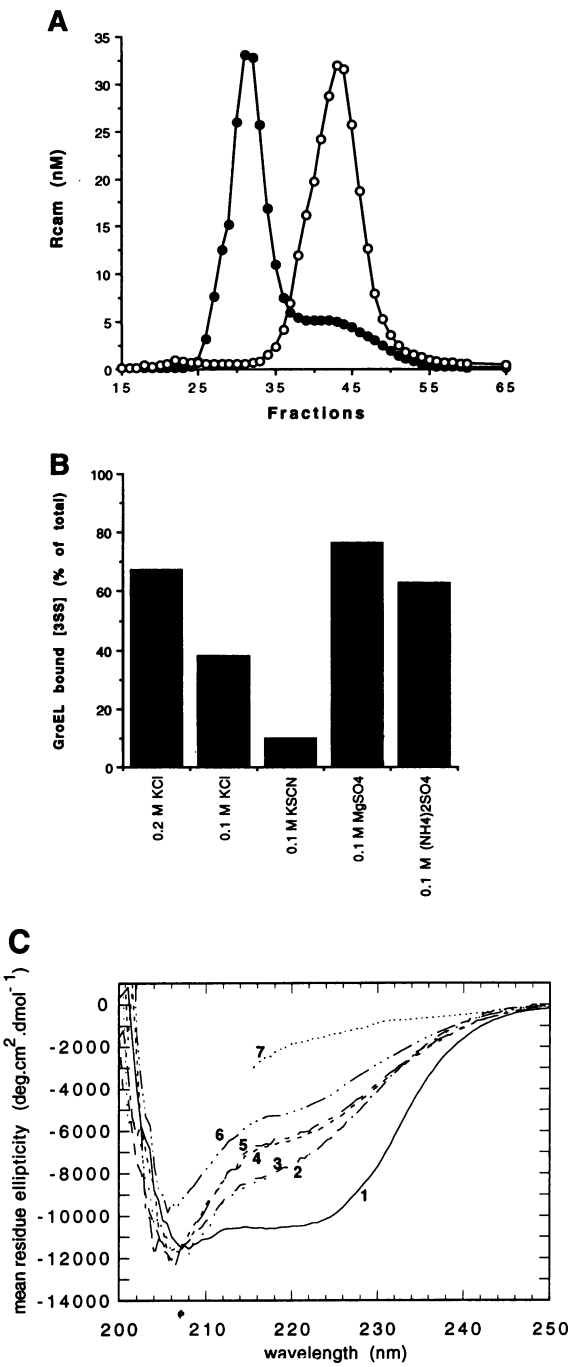

Fig. 5. Effect of salts on the interaction of Rcam and [3SS] αLA with GroEL and on the CD spectra of αLA and derivatives. Binding to GroEL was analysed as described in the legend to Figure 2 at 0.31 μM ^{3}H-labelled αLA derivatives and 0.31 μM GroEL in 20 mM MOPS–KOH pH 7.2, 100 μM $CaCl_2$ containing the indicated additions of salt. (**A**) Binding of Rcam in the presence (●) and absence (○) of 200 mM KCl. (**B**) Binding of rearranged [3SS] in the presence of 0.05 mM DTT/0.5 mM EGTA and the indicated salt concentrations. GroEL-bound ^{3}H-labelled [3SS] was quantified and expressed as a percentage of total protein eluted from the sizing column. Protein recoveries were ~95% in the absence or presence of salt. (**C**) Far-UV CD spectra of native-αLA (1); rearranged [3SS] (2 and 3); Rcam (4 and 5); Rcm (6); fully denatured αLA (7). Spectra were taken in 20 mM MOPS–KOH pH 7.2 containing 200 mM KCl and 100 μM $CaCl_2$ (1), in buffer lacking $CaCl_2$ (2, 4 and 6) or lacking KCl and $CaCl_2$ (3 and 5), and in buffer containing 6 M guanidine-HCl (7). Reactions 2 and 3 contained 0.05 mM DTT/0.5 mM EGTA. Results are expressed as mean residue ellipticity (deg·cm^2·dmol^{-1}).

increase in intensity suggests the removal of tertiary interactions that quench the fluorescence in the native state. Binding of [3SS] to the chaperonin resulted in the opposite, a blue-shift of the Trp fluorescence to 344 nm, indicating the transfer of some or all of the Trp residues into a more hydrophobic environment (Figure 4A). This, however, was not accompanied by a decrease in fluorescence intensity, which would be expected if native-like structure was formed in the GroEL-bound protein. Rather, the fluorescence intensity of GroEL-associated [3SS] increased further to a value greater than that measured with fully unfolded αLA. This would be expected if GroEL were to shield the hydrophobic surfaces exposed by the MG intermediates of αLA. Essentially the same observations were made upon GroEL binding of Rcam, even though this protein is not conformationally restricted by disulfide bonds. Free Rcam has the same emission maximum of Trp fluorescence as [3SS] (Figure 4B). Its fluorescence intensity, however, is almost as high as that of fully unfolded αLA, presumably reflecting greater tertiary structure flexibility. Complex formation with GroEL resulted in a blue-shift of the Trp fluorescence of Rcam to 344 nm and in an increase in fluorescence intensity very similar to the observations made with GroEL-bound [3SS].

Binding of [3SS] and Rcam to GroEL was also accompanied by a 20–30% increase in the intensity of ANS fluorescence as compared with the free proteins (Figure 3), and by a blue-shift in the maximum of ANS fluorescence emission from 476 to 467 nm (not shown). This suggests that the ANS-accessible surfaces of αLA are transferred into a more hydrophobic environment (Semisotnov *et al.*, 1991), consistent with them being shielded by GroEL. However, this analysis was complicated by the necessity to subtract a high background fluorescence (~60% of total) due to the 65-fold mass excess of GroEL protein over αLA. Thus, conformational changes in GroEL upon αLA association may affect the contribution of the chaperonin to total ANS binding.

Hydrophobic protein–protein interactions may exhibit a dependence on the salt concentration of the solution according to the lyotropic series (Collins and Washabaugh, 1985). Complex formation between the MG forms of αLA and GroEL was indeed strictly dependent on salt. While efficient binding of Rcam was seen in a 20 mM MOPS–KOH buffer (pH 7.2) containing 200 mM KCl or NaCl, almost no binding was detected in buffer alone (Figure 5A). This excludes electrostatic interactions as the major attractive force between αLA and the chaperonin and suggests that electrostatic repulsions may need to be screened for binding to occur. The same effect was observed with [3SS] (Figure 5B). Intermediate levels of binding were measured with 100 mM KCl. As predicted based on the potential of different salts to increase the surface tension of water, and thus to stabilize hydrophobic interactions (Collins and Washabaugh, 1985), $MgSO_4$ and $(NH_4)_2SO_4$ were more efficient than KCl or NaCl in promoting αLA binding to GroEL. Almost no binding was detected in the presence of KSCN, which strongly destabilizes hydrophobic interactions (Figure 5B). There was very little influence of the presence or absence of 0.2 M KCl on the secondary structure content of the αLA derivatives measured by CD (Figure 5C), and on their

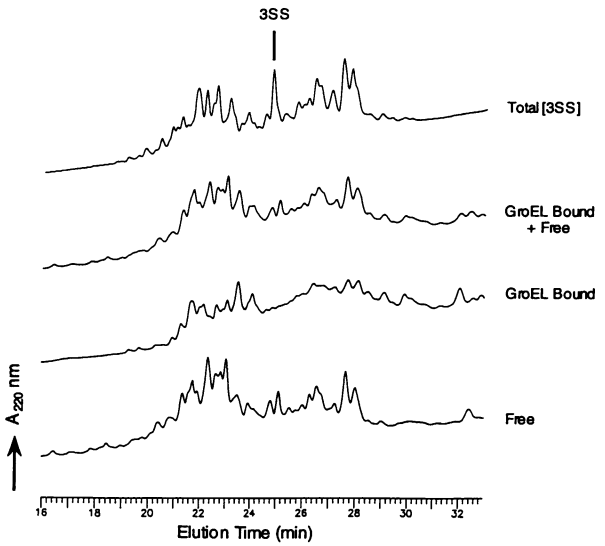

Fig. 6. Analysis of rearranged, unblocked [3SS] species bound to GroEL. Acid trapping of [3SS] and HPLC separation were performed as described in Materials and methods. Rearranged [3SS] was produced by incubation of 30 μM αLA in buffer A containing 0.5 mM DTT for 10 min at 25°C. Half of the reaction was then diluted 5-fold into buffer A (minus $CaCl_2$) containing 0.5 mM EGTA (total) and the other half into the same buffer containing 1 μM GroEL. Both reactions were incubated for 30 min at 25°C. Total [3SS] was acidified at this point to stop further rearrangement (the residual 3SS form with native disulfides is indicated). The GroEL-containing reaction was separated into GroEL-bound and free [3SS] by size exclusion HPLC. Fractions eluted from the sizing column were immediately acid trapped but identical results were obtained when the fractions were acidified after further incubation for 30 min. About 14% of total [3SS] bound to GroEL. Approximately equal amounts of GroEL-bound, free and total material were analysed by reversed phase HPLC. Protein elution was monitored at 220 nm. The traces of GroEL-bound and free [3SS] were added graphically and the scale adjusted to that of the other traces.

tryptophan fluorescence properties (not shown). However, the ANS binding of Rcam was reduced by ~40% in the absence of salt, suggesting that its conformation is less MG-like at low ionic strength of the solvent (Figure 3). Notably, GroEL was not generally defective in protein binding in buffer solutions lacking salt. For example, the ability of the chaperonin to interact with unfolded rhodanese and dihydrofolate reductase diluted from denaturant was not detectably diminished in the absence of salt (not shown; see also Martin *et al.*, 1991). We conclude that the salt dependence of αLA binding to GroEL reflects to a large extent the hydrophobic character of the interaction. The effect of salt may only be revealed with small substrate proteins, which have a relatively low affinity for GroEL.

GroEL binds a spectrum of compact three-disulfide forms of αLA

The presence of three disulfide bonds in the rearranged [3SS] species limits the conformational flexibility of the molecules, preventing total unfolding upon binding to GroEL. Disulfide rearrangements can generate a large number of [3SS] disulfide isomers that can be partially resolved by reversed phase HPLC (Ewbank and Creighton, 1991). To analyse the spectrum of [3SS] isomers bound by GroEL, a mixture of [3SS] was generated by partial reduction of αLA and incubation with EGTA in the absence (total [3SS]) or presence of chaperonin (Figure 6). Further disulfide rearrangement in the total mixture was prevented by acid quenching, whereas the GroEL-containing reaction was first separated by HPLC size exclusion chromatography under native conditions. Both GroEL-bound and free [3SS] pools were then acid trapped and analysed by reversed phase HPLC at low pH. Acid treatment also dissociated the interaction between GroEL and bound [3SS]. A complex set of [3SS] isomers was found to be associated with GroEL (Figure 6), different from the free forms at equilibrium of disulfide rearrangement. The composition of the GroEL-bound [3SS] species was unchanged whether the protein was acid-trapped immediately after size fractionation or 30 min later (not shown). Together with the finding that addition of Ca^{2+} did not cause the [3SS]–GroEL complex to dissociate, this suggests that disulfide rearrangements are slowed or blocked in the GroEL-bound state. In the absence of GroEL, the addition of Ca^{2+} results in the conversion of [3SS] to 3SS and by oxidation back to native αLA (Ewbank and Creighton, 1993a). These data confirm that binding to the chaperonin is compatible with the presence of three intact disulfides in αLA but occurs more readily with a specific subset of isomers. Interestingly, GroEL does not stabilize the substrate protein in a single, defined conformation.

The [3SS] forms of αLA that bind to GroEL were further analysed with respect to their compactness by native PAGE (Ewbank and Creighton, 1991). A chemically stable mixture of [3SS] that had been trapped by reaction with iodoacetamide, [3SS]cam, was used as substrate, thus preventing any effect of GroEL on the equilibrium disulfide pairings of the [3SS] mixture. Native PAGE of the total pool revealed two major populations of [3SS]cam, one comparable in compactness with the native state and the other with 3SScam with three native disulfide bonds (Figure 7A). The 2SScam and Rcam standards migrated more slowly in this gel system, consistent with their less compact structures. Analysis of the GroEL-bound [3SS]cam forms showed only conformers that ranged in compactness between 3SScam and 2SScam, whereas the species with native-like compactness were excluded (Figure 7A). The complexity of the mixture of [3SS]cam forms was revealed by HPLC analysis (Figure 7C). In this experiment, disulfide rearrangement had been stopped by adding iodoacetamide before reaching equilibrium, explaining the presence of a large amount of 3SScam with native disulfides. The composition of the bound [3SS]cam was significantly different from that of the total mixture and appeared less complex (Figure 7C). Specifically, 3SScam was completely excluded from GroEL binding. Upon incubation with GroEL at equimolar concentrations, 32% of an equilibrium mixture of [3SS]cam bound to GroEL as compared with 70% of the non-blocked [3SS] (see Table I). This indicates that GroEL normally shifts the equilibrium of disulfide rearrangement to a subset of [3SS] species, which optimally expose the structural elements recognized by the chaperonin.

The effect of the disulfide bonds on the hydrodynamic flexibility of the GroEL-bound intermediates was analysed by electrophoresis on polyacrylamide gels containing 8 M urea (Ewbank and Creighton, 1991). A mixture of [3SS] whose disulfide bonds had been rearranged in 8 M

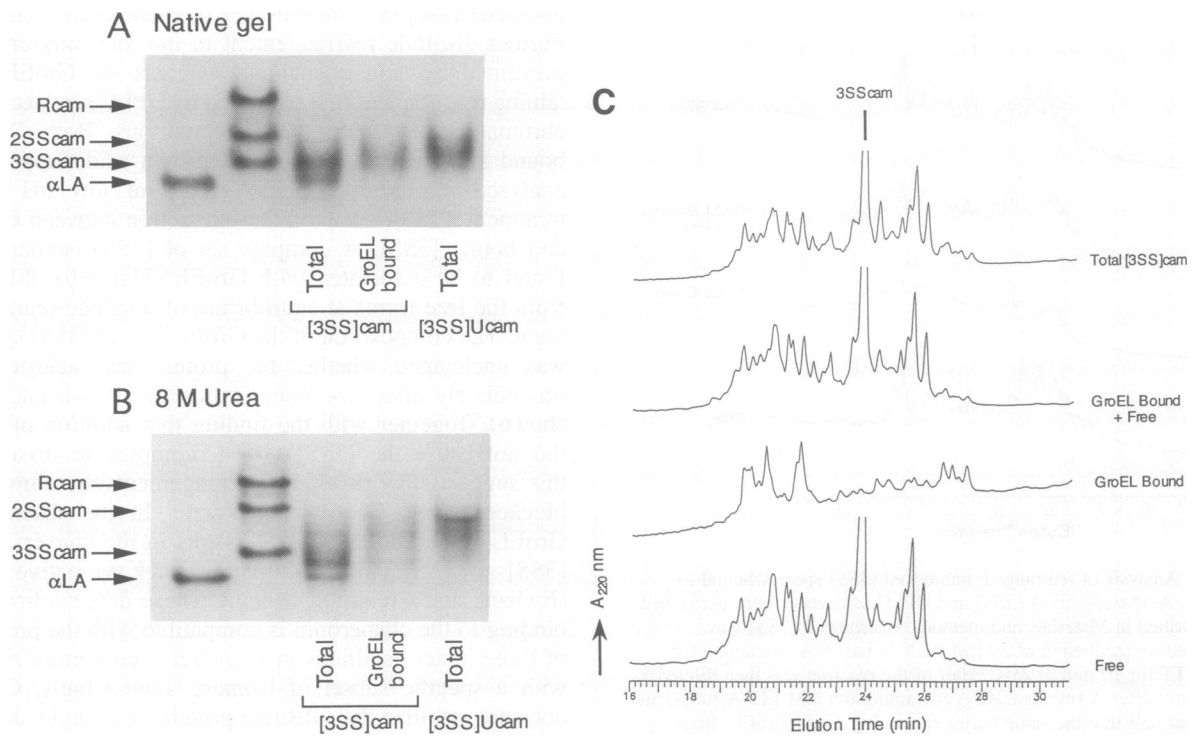

Fig. 7. Analysis of iodoacetamide-blocked [3SS] species bound to GroEL. Compactness of total [3SS]cam, GroEL-bound [3SS]cam and total [3SS]Ucam analysed by native PAGE (**A**) and on denaturing polyacrylamide gels containing 8 M urea (**B**). Native αLA, 3SScam, 2SScam and Rcam were run as standards. Iodoacetamide-blocked [3SS] was generated by partial reduction of holo-αLA in 0.1 M Tris (pH 8.7), 200 mM KCl, 0.5 mM DTT for 2 min at 25°C and 15-fold dilution into 1 mM EDTA-containing buffer with or without 8 M urea. After rearrangement for 2 min at 25°C, the protein was trapped with 0.6 M iodoacetamide (resulting in [3SS]Ucam and [3SS]cam, respectively) and purified from excess blocking reagent by reversed phase HPLC (Ewbank and Creighton, 1993b). GroEL-bound [3SS]cam was isolated as described in Materials and methods, and was lyophilized and prepared for gel electrophoresis in gels containing 1 mM EDTA (Ewbank and Creighton, 1993a). (**C**) Analysis of GroEL-bound and free [3SS]cam by reversed phase HPLC. Disulfide rearrangement was stopped after 1 min incubation to retain a large amount of 3SScam with native disulfide bonds. Binding of [3SS]cam (1.0 μM) to GroEL (2.0 μM) was performed in buffer A (minus $CaCl_2$) containing 0.5 mM EGTA. About 10% of total [3SS]cam bound to GroEL. The HPLC trace of the bound material is shown at three times the scale used for total and free material to visualize the absence of the 3SScam with native disulfides. The trace labelled 'GroEL bound + free' represents a graphical addition of the traces of bound and free material at their correct proportions.

urea and then blocked ([3SS]Ucam) served as a reference for the most expanded [3SS] forms possible (Figure 7B). Rearrangement in urea favours the formation of disulfide bonds between cysteines close in the sequence, resulting in more slowly migrating [3SS]U forms with increased hydrodynamic volume. A large fraction of these species, in the presence of 8 M urea, allowed expansion to an extent comparable with that of a 2SScam standard. In contrast, [3SS]cam that had been rearranged under non-denaturing conditions and then isolated from the complex with GroEL was more compact on the urea-containing gel. Apparently, GroEL does not selectively bind the expanded [3SS]U isomers that are more populated upon rearrangement in the presence of denaturant, although a somewhat larger fraction of the total [3SS]Ucam interacted with GroEL as compared with the [3SS]cam species (Table I).

The complete αLA protein is required for binding to the chaperonin

It is not known whether GroEL recognizes contiguous sequence elements or perhaps the surfaces of partially folded proteins. We attempted to identify peptide regions in αLA that confer the ability of binding to the chaperonin. A soluble peptide comprising residues 54–104 of the 123 amino acid protein was produced by chymotryptic cleavage. This region of αLA contains the Ca^{2+} binding site and the disulfide bonds 61–77 and 73–91. αLA (54–104) was unable to bind to GroEL, independent of the presence of DTT and Ca^{2+} (Figure 8A). In the absence of Ca^{2+}, αLA(54–104) did not retain stable secondary structure (not shown) and exhibited very low adsorption of ANS (Figure 3). Apparently, the presence of secondary structure and the exposure of ANS binding surfaces, typical properties of the MG state, are only acquired in context with additional parts of the protein. Consistent with this, a larger chymotryptic fragment spanning residues 17–108 (containing the Cys61–77 and Cys73–91 disulfide bonds) associated normally with GroEL upon reduction with DTT and removal of Ca^{2+} (Figure 8A). Thus, the N-terminal helix A (residues 5–11) and the C-terminal unstructured region (residues 110–123) of αLA (Figure 1) are dispensable for GroEL binding.

Indeed, we were able to demonstrate that the complete core region of αLA is required for the association with the chaperonin. Incubation of radiolabelled apo-αLA with proteinase K in the absence of DTT resulted in cleavage of ~50% of the protein into two main fragments of ~6 kDa and ~8 kDa (starting with residues 1 and 54, respectively) and some smaller degradation products. SDS–PAGE in the absence of mercaptoethanol revealed that the two major fragments remained connected via

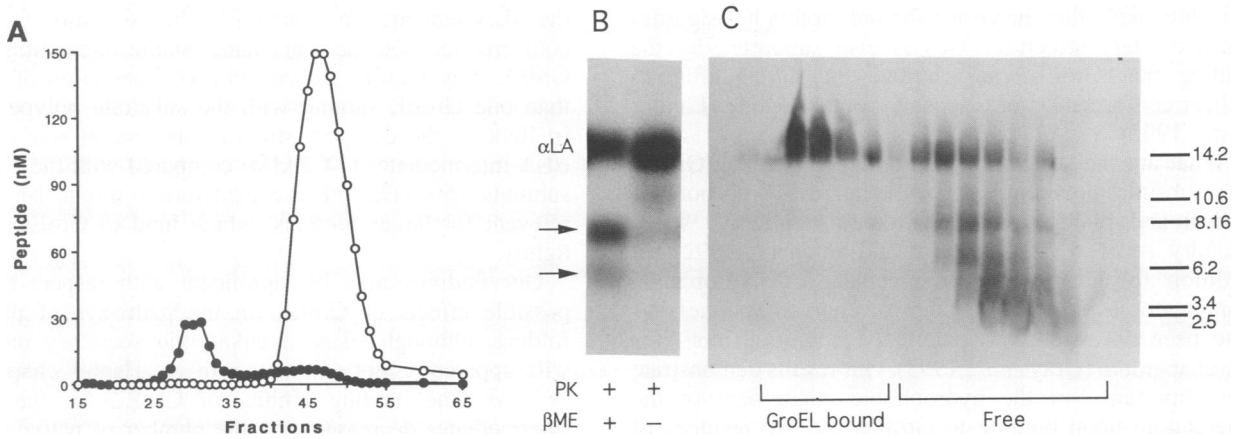

Fig. 8. Interaction of GroEL with proteolytic fragments of αLA. (**A**) Binding of 0.31 μM ^{3}H-labelled αLA(17–108) (●) and 1.55 μM αLA(54–104) (○) to 0.31 μM GroEL was analysed by Sephacryl S300-HR sizing chromatography in buffer A containing 0.05 mM DTT and 0.5 mM EGTA. No binding was detected with holo-αLA(17–108). αLA(54–104) did not detectably interact with GroEL under any condition including the presence of Ca^{2+} or upon complete reduction. (**B**) Proteinase K treatment of ^{3}H-labelled apo-αLA was carried out as described in Materials and methods. The protein was analysed by SDS–PAGE in the presence or absence of β-mercaptoethanol and visualized by autoradiography. The arrows mark the two major fragments of αLA of ~8 kDa and ~6 kDa. (**C**) Binding of proteinase K treated apo-αLA (0.31 μM) to GroEL (0.46 μM) was analysed in buffer A containing 0.5 mM DTT and 0.5 mM EGTA. There was no detectable aggregation of proteolytic fragments. An overexposed autoradiograph is shown, demonstrating the absence of αLA fragments in the GroEL fractions of the sizing column.

disulfide bonds (Figure 8B). In the absence of DTT, the cleaved but disulfide-connected protein did not associate with GroEL. Upon addition of DTT, only the non-cleaved full-length αLA bound to GroEL, whereas all the proteolytic products were recovered in the low molecular weight fractions of the sizing column (Figure 8C). We conclude that the complete αLA protein, with the exception of the flexible tail regions, is required for efficient binding to GroEL. Apolar amino acid residues of αLA, non-contiguous in sequence, may have to cooperate in forming a hydrophobic surface that associates with the chaperonin.

Discussion

Employing a series of well characterized disulfide folding intermediates of αLA, we have defined structural properties of non-native polypeptides that are recognized by the chaperonin GroEL. The conformers of αLA with high affinity for GroEL are compact, contain 40–60% of the secondary structure of the native state, lack ordered tertiary structure and expose hydrophobic surfaces that adsorb the fluorescent probe ANS. Unfolding to an extended state prevents the interaction. Complex formation with GroEL is compatible with a number of distinct conformations that have the above structural properties in common. These results demonstrate the ability of GroEL to interact with an ensemble of collapsed, MG-like intermediates produced early in the folding of globular proteins.

The stable accumulation of specific disulfide folding intermediates of bovine αLA allowed us to control the possible effects of GroEL on the conformation of the bound protein. In the complex with the chaperonin the rearranged [3SS] forms of αLA maintain three intact disulfide bonds and preserve their overall Trp and ANS fluorescence properties. Importantly, imposing compactness by the presence of rearrangeable disulfides does not limit the extent of binding to GroEL. Both the [3SS] forms and the conformationally unrestricted Rcam assume almost identical Trp fluoresence spectra when bound to

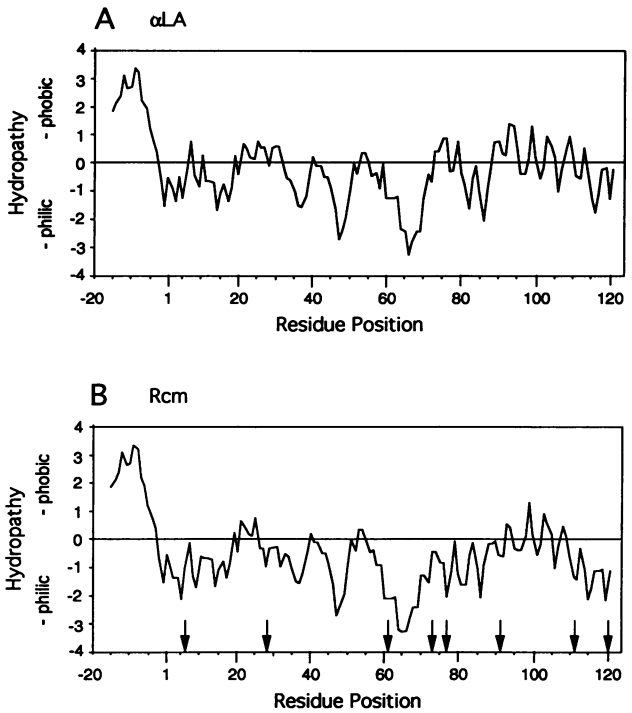

Fig. 9. Hydropathy profiles of bovine pre-αLA and its modelled Rcm derivative. (**A**) Hydropathy plot of pre-αLA showing the hydrophobic signal sequence of the αLA precursor (residues –1 to –19) and the absence of major hydrophobic segments from the mature protein part. A pH of 7.0 is assumed where Cys thiols are non-ionized and the influence of the bound Ca^{2+} ion is neglected. (**B**) The hydropathy plot of Rcm was simulated by introducing glutamic acid residues at the positions of the eight cysteines (indicated by arrows). The University of Wisconsin Genetics Computer Group program, Pepwindow, was used with a window size of seven residues, the preferred length of peptide bound by the chaperone Hsp70 (Flynn *et al.*, 1991).

GroEL, suggesting that the polypeptide chain is naturally stabilized by the chaperonin in a compact conformation. The criteria of ANS binding correlates with the presence of collapsed secondary structure in the αLA intermediates.

It is thus likely that the GroEL-bound protein has significant secondary structure. This is also supported by the finding that GroEL can stabilize the amphiphilic α-helical conformation of a loosely bound peptide (Landry *et al.*, 1992).

What are the structural features recognized by GroEL in a substrate polypeptide? GroEL interacts with both α-helical and β-sheet proteins in their non-native states (Landry *et al.*, 1992; Schmidt and Buchner, 1992). In addition to the possible involvement of hydrophobic binding forces, a certain pattern of charged residues has also been discussed as a potential recognition motif for the chaperonin (Gray *et al.*, 1993). Our results demonstrate the importance of the hydrophobic component of the interaction: upon binding to GroEL, the Trp residues of αLA are transferred into a more hydrophobic environment. Moreover, complex formation is strictly dependent on the presence of salts in the solution, which stabilize hydrophobic binding according to the order established in the lyotropic series (Collins and Washabaugh, 1985). Since the native conformation of αLA is unstable in the absence of native disulfides and bound Ca^{2+}, the most efficient way for the MG intermediates to bury hydrophobic surface would be their association with a putative hydrophobic binding region of the chaperonin. As shown in Figure 9A, mature αLA does not contain any contiguous sequences of substantial hydrophobicity. In contrast, the presumably α-helical signal sequence of pre-αLA is highly hydrophobic. The presence of such sequences increases the affinity of unfolded proteins for GroEL (Laminet *et al.*, 1990; Koll *et al.*, 1992). Regions of similar hydrophobicity in mature αLA may be the hydrophobic faces of its three amphiphilic α-helices (Figure 1), which, although transiently exposed, are expected to be stabilized in the compact folding intermediates. The chaperonin may bind the hydrophobic faces of one or several amphiphilic α-helical segments, but the requirement for binding of most of the αLA polypeptide chain would suggest the interaction with a hydrophobic surface resulting from the clustering of residues more distant in the linear sequence.

The relevance of a compact conformation of αLA for chaperonin binding is stressed by the inability of the Rcm derivative to interact with GroEL. Presumably due to the repulsive forces resulting from the introduction of eight negative charges, Rcm has a rather extended conformation (Ewbank and Creighton, 1993b). Notably, Rcm is able to associate with other molecular chaperones. It forms a stable complex with Hsp70 (Palleros *et al.*, 1991; Langer *et al.*, 1992b) that recognizes extended heptameric sequences containing several hydrophobic residues (Flynn *et al.*, 1991; Landry *et al.*, 1992; Blond-Elguindi *et al.*, 1993). Apparently, the introduction of negative charges in αLA does not disrupt these sequences, although, due to the modification of cysteines, Rcm has lost several moderately hydrophobic segments of eight or more residues (Figure 9B). Given that αLA interacts with GroEL as a compact three-disulfide intermediate, it seems unlikely, however, that in the unmodified protein these cysteine-containing sequences would be available for chaperonin binding in an extended conformation. In contrast to polypeptide recognition by Hsp70, GroEL (Hsp60) requires almost the complete αLA molecule for efficient binding. This may be due to the fact that neither half of the αLA sequence in isolation is able to assume an MG conformation. On the other hand, stable interaction with GroEL may require the cooperative interaction of more than one GroEL subunit with the substrate polypeptide. In light of the compact structure and small size of the αLA intermediates (14.2 kDa) compared with the GroEL subunits (60 kDa), this consideration might be more relevant for larger proteins, which bind to GroEL more tightly.

Our findings may be significant with respect to the possible effects of GroEL on the pathways of protein folding, although αLA, a eukaryotic secretory protein, will apparently not interact with an Hsp60 chaperone *in vivo*. The binding affinity of GroEL for the αLA intermediates decreases with the number of native disulfides present. 2SScam, containing two native disulfides, is bound less efficiently than the rearranging [3SS] isomers. 3SScam with three native disulfides is excluded from GroEL binding. The rearranging [3SS] form and the blocked 2SScam and 3SScam forms have been characterized as MG states with decreasing conformational flexibility. As pointed out by Dobson and colleagues (Redfield *et al.*, 1994), it may be appropriate to distinguish between more 'ordered' MG states, representing late intermediates preceding the native state, and 'disordered' MG states, which are important early in folding. According to these considerations, GroEL would interact preferentially with the more flexible MG intermediates of αLA, including those with three non-native disulfide bonds. The highest free energy barriers in the disulfide folding pathway of αLA are the steps involving disulfide bond rearrangement to 2SS and 3SS. The formation of the 3SS intermediate seems to be most important for regaining the four native disulfide bonds (Ewbank and Creighton, 1993a). Disulfide rearrangement and folding, up to the formation of at least two native disulfides, could proceed through cycles of ATP-dependent protein release from the chaperonin followed by rebinding. Given that GroEL interacts only with a subset of the possible disulfide intermediates, the chaperonin would be likely to influence the kinetics of the normal random walk-like folding pathways that lead to the 3SS intermediate *in vitro*.

Materials and methods

αLA and derivatives

Bovine αLA was obtained from Sigma (type III, Ca^{2+}-depleted); iodoacetamide/iodoacetate trapped reduced and partially reduced forms of αLA (3SScam, 2SScam, Rcam and Rcm), and rearranged iodoacetamide blocked forms ([3SS]cam and [3SS]Ucam) were prepared as described (Ewbank and Creighton, 1993b). Protein concentrations were determined using the extinction coefficients for the various αLA forms at 280 nm given by Ewbank and Creighton (1993b). The various forms of αLA were radiolabelled by reductive methylation using $NaB[^3H]_4$ (10.6 Ci/mmol; NEN). The respective protein was dissolved at 100–600 μM in 0.2 M $Na_2B_4O_7$ pH 8.9, 100 μM $CaCl_2$ and formaldehyde was added to a concentration corresponding to a 2-fold excess over lysine residues. After addition of $NaB[^3H]_4$ at half the concentration of lysine residues, the sample was incubated for 15 min at 4°C. The reaction was stopped by addition of a large excess of $(NH_4)_2SO_4$. Labelled protein was isolated on a PD10 column (Pharmacia) equilibrated with buffer A (20 mM MOPS–KOH pH 7.2, 200 mM KCl, 100 μM $CaCl_2$). The specific activity of the labelled protein was between 0.8 and 1.3×10^6 c.p.m./μg, corresponding to one or two lysine residues modified per molecule of αLA.

GroEL purification

GroEL was purified from an *E.coli* strain containing the plasmid pOF39 by a modification of published procedures (Fayet *et al.*, 1989; Viitanen *et al.*, 1990). Cells were grown at 34°C until stationary phase and frozen in liquid nitrogen as a suspension in 50 mM Tris pH 8.0, 10% sucrose. Thawed cells (0.25 g/ml) were lysed for 30 min on ice in 50 mM Tris pH 8.0, 10 mM EDTA, 10 mM $Mg(OAc)_2$, 50 mM KCl, 2 mM DTT, 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mg/ml of 150 000 U/mg lysozyme and 50 mg/ml of 3000 U/mg DNase I by sonication with a tip sonicator (Branson sonifier, position 7 at 40% duty in pulse mode, 4 × 10 pulses). After removal of cell debris and membranes by centrifugation at 4°C (20 min, 48 000 *g*, followed by 1 h at 257 000 *g*), the supernatant was adjusted to 20 mg/ml in 30 mM Tris pH 7.8, 50 mM NaCl, 1 mM EDTA, 1 mM DTT and fractionated by successive steps of chromatography on DE52 in the same buffer, DE52 in 25 mM histidine pH 5.5, 50 mM NaCl, 1 mM DTT (final pH of the loaded sample ~6.0), phenyl-Sepharose CL-4B (Pharmacia) in 20 mM MOPS pH 7.2, 400 mM NaCl, 1 mM DTT and Sephacryl S300-HR (Pharmacia) in 10 mM MOPS pH 7.2, 50 mM KCl, 1 mM DTT. GroEL protein eluting at ~800 kDa was pooled. Protein concentration was based on quantitative amino acid analysis.

Binding of αLA to GroEL

αLA or derivatives were incubated with GroEL at 25°C in buffer A containing 0.05–2 mM DTT and/or 0.5 mM EGTA as indicated in the figure legends and then loaded onto a 1 × 30 cm Sephacryl S300-HR column equilibrated with the corresponding buffer containing 0.008% Tween 20 (Sigma). Rearranged ^{3}H-labelled [3SS] was generated by partial reduction of 0.31 μM Ca^{2+}-bound ^{3}H-labelled αLA in buffer A containing 0.5 mM DTT for 10 min, followed by 10-fold dilution into buffer A containing 0.5 mM EGTA (final volume 400 μl) (Ewbank and Creighton, 1993a). After incubation for 20 min at 25°C, 0.31 μM GroEL was added for another 15 min prior to column chromatography. For the other derivatives, GroEL and αLA were incubated for 15 min at 25°C. Fractions of 0.5 ml were collected from the column and 100 μl removed for tritium counts. The rest was trichloroacetic acid-precipitated and analysed by SDS–PAGE. The amounts of GroEL on Coomassie-stained gels were determined by laser densitometry. The lack of significant absorbance above 320 nm and the absence of time-dependent absorbance changes indicated the absence of aggregation of the αLA derivatives under the conditions used.

HPLC analysis of GroEL-bound [3SS]

Rearranged and blocked [3SS]cam (1 μM) was incubated for 15 min at 25°C with 2 μM GroEL in 400 μl buffer A lacking $CaCl_2$ and containing 0.5 mM EGTA. GroEL-bound protein was separated from free [3SS]cam on an HPLC-TSK sizing column (type G3000SW, 7.5 × 600 mm; flow rate 0.5 ml/min), equilibrated with the same buffer containing 0.008% Tween 20. Free protein was lyophilized, resuspended in 10 mM HCl and applied onto a C_4 reversed phase column (Vydac 214TP 5 micron C_4, 4.5 × 250 mm), eluted with a linear gradient from 25% to 50% acetonitrile/0.1% (v/v) TFA over 35 min (flow rate 1.0 ml/min). GroEL-bound protein was adjusted to 0.2 M HCl to cause the dissociation of the GroEL–[3SS]cam complex and then applied onto a C_4 reversed phase column equilibrated with 25% acetonitrile/0.1% (v/v) TFA. [3SS]cam was more than 90% eluted with 45% acetonitrile/0.1% TFA while GroEL was retained on the column. The eluted protein was lyophilized, resuspended in 10 mM HCl and analysed by reversed phase HPLC as for the free species.

Unblocked GroEL-bound [3SS] was analysed by acid trapping. [3SS] (6 μM) was generated as described above and incubated with 1 μM GroEL in buffer A containing 0.5 mM EGTA. Bound and free [3SS] were separated by size exclusion HPLC. Fractions were collected directly by 1.25-fold dilution into 1 M HCl (0.2 M HCl, final concentration) followed by reversed phase HPLC as above.

CD measurements

CD spectra were acquired at 25°C on a Jobin Yvon CD6 spectrometer essentially as described (Ewbank and Creighton, 1993b), except that αLA and derivatives were dissolved in buffer A as indicated in the figure legend.

Tryptophan fluorescence

Fluorescence spectra were recorded on a Spex Fluorolog-2. Protein–GroEL complexes were formed at 1.7 μM GroEL 14mer and 1.4 μM substrate protein. The absorbance at the excitation wavelength of 295 nm was <0.05. Background fluorescence of chemically identical reactions lacking substrate protein (15–30% of total fluorescence due to minor impurities of the GroEL preparation) was subtracted. Analysis was at 25°C in micro-cuvettes with a path length of 5 mm. A scan range of 310–410 nm with bandwidths of 4.9 nm was used.

ANS binding

Various forms of αLA or αLA–GroEL complexes were incubated at 25°C for 5–10 min in 20 mM MOPS–KOH pH 7.2 with the additions indicated in the figure legends and a 25-fold molar excess of ANS over GroEL (1.7 μM GroEL and 1.4 μM αLA). The absorbance at the excitation wavelength of 390 nm was <0.05. Emission spectra (430–530 nm) were corrected for background fluorescence caused by ANS in reactions lacking the protein; background subtracted in the presence of GroEL was ~60% of total ANS fluorescence intensity. The concentration of ANS was determined using an extinction coefficient of 5000 M^{-1} cm^{-1} at 350 nm (Weber and Young, 1964).

Proteolysis of αLA

^{3}H-labelled αLA (3.5 μM) was incubated for 10 min on ice in buffer A containing 0.5 mM EGTA and 10 μg/ml proteinase K. Protease action was stopped by adding PMSF to 1 mM from a 100 mM stock solution in ethanol, followed by incubation on ice for 3 min. Binding to GroEL in the presence or absence of DTT was analysed on a Sephacryl S300-HR column as described above using a 1.5-fold molar excess of GroEL over αLA (based on the concentration of αLA prior to proteinase K treatment). Column fractions were lyophilized, resuspended in SDS-sample buffer and analysed by highly resolving SDS–PAGE (16.5% T/6% C; Schägger and von Jagow, 1987).

Miscellaneous

The following procedures were carried out according to published methods: TCA precipitation (Bensadoun and Weinstein, 1976); native PAGE and denaturing polyacrylamide gels containing 8 M urea (Ewbank and Creighton, 1993a).

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