The chaperonin cycle cannot substitute for prolyl isomerase activity, but GroEL alone promotes productive folding of a cyclophilin-sensitive substrate to a cyclophilin-resistant form

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The chaperonin GroEL and the peptidyl-prolyl *cis-trans* isomerase cyclophilin are major representatives of two distinct cellular systems that help proteins to adopt their native three-dimensional structure: molecular chaperones and folding catalysts. Little is known about whether and how these proteins cooperate in protein folding. In this study, we have examined the action of GroEL and cyclophilin on a substrate protein in two distinct prolyl isomerization states. Our results indicate that: (i) GroEL binds the same substrate in different prolyl isomerization states. (ii) GroEL-ES does not promote prolyl isomerizations, but even retards isomerizations. (iii) Cyclophilin cannot promote the correct isomerization of prolyl bonds of a GroEL-bound substrate, but acts sequentially after release of the substrate from GroEL. (iv) A denatured substrate with all-native prolyl bonds is delayed in folding by cyclophilin due to isomerization to non-native prolyl bonds; a substrate that has proceeded in folding beyond a stage where it can be bound by GroEL is still sensitive to cyclophilin. (v) If a denatured cyclophilin-sensitive substrate is first bound to GroEL, however, productive folding to a cyclophilin-resistant form can be promoted, even without GroES. We conclude that GroEL and cyclophilin act sequentially and exert complementary functions in protein folding.

Keywords: cyclophilin/GroEL/peptidyl-prolyl *cis-trans* isomerase/protein folding

Introduction

After synthesis at the ribosome and translocation across biological membranes, proteins have to find folding pathways which eventually direct them to their native structures. All cells and cellular compartments contain high concentrations of different proteins which interfere with this process (Rothman, 1989; Fischer and Schmid, 1990; Ellis and van der Vies, 1991; Gething and Sambrook, 1992; Seckler and Jaenicke, 1992; Georgopoulos and Welch, 1993; Rowling and Freedman, 1993; Ellis, 1994; Rassow and Pfanner, 1995; Hartl, 1996). Two functions of these systems can be distinguished. (i) Some proteins involved in protein folding reactions are enzymes which accelerate rate-limiting steps in folding pathways. Protein disulfide isomerases (PDIs) and peptidyl-prolyl *cis-trans*

isomerases (PPIases, also named 'rotamases') are well characterized members of this group. (ii) A different function is exerted by molecular chaperones. They can bind non-native folding intermediates, keep them in a folding-competent state and provide a means to prevent or reverse unproductive side reactions. This can considerably increase the yield of protein folding reactions.

One of the best-characterized chaperone proteins is GroEL, an abundant protein in the cytosol of *Escherichia coli*. GroEL belongs to a subclass of chaperone proteins, termed chaperonins (Ellis, 1994). The subunits of chaperonins form two heptameric ring complexes that associate back to back. The GroEL complex cooperates with GroES, a protein which forms single heptameric complexes. GroES can bind to one or both sides of the GroEL toroid. The function of the GroEL-ES system (and its mitochondrial homolog Hsp60/Cpn10) has been investigated in a series of detailed studies (reviewed by Hartl, 1996; Fenton and Horwich, 1997). Substrate proteins bind in the central cavity of the GroEL toroids on the tips of the apical domains (Fenton *et al.*, 1994). After binding of GroES, the substrate is released in a nucleotide-dependent reaction cycle. Folding of the substrate is not directly coupled to hydrolysis of nucleotides, since GroES binding and release can initiate a folding reaction in the absence of ATP hydrolysis (Hayer-Hartl *et al.*, 1996). Depending on the substrate protein, the folding reaction is completed at GroEL-ES or proceeds after release (Burston *et al.*, 1996; Corrales and Fersht, 1996; Mayhew *et al.*, 1996; Weissman *et al.*, 1996). Interaction with GroES is not essential for all substrate proteins. Some proteins can fold in a reaction cycle in the absence of GroES (Martin *et al.*, 1991; Viitanen *et al.*, 1991; Schmidt and Buchner, 1992; Höhfeld and Hartl, 1994; Corrales and Fersht, 1995). In this case the protein folds spontaneously after one or several cycles of release from GroEL. Although the cage-like structure is regarded as an intrinsic property of chaperonins in providing a sheltered environment for aggregation-prone folding intermediates (Hartl, 1996), some chaperone activities have already been observed with monomeric fragments of GroEL subunits (Zahn *et al.*, 1996a).

Peptidyl prolyl *cis-trans* isomerases (PPIases) catalyze the *cis-trans* isomerization of peptide bonds preceding proline residues (Schmid, 1993, 1995; Fischer, 1994). If the conformation of a prolyl bond is not stabilized externally the *cis* and *trans* forms have very similar free energies. To attain the native folding state, a protein must obviously isomerize all its prolyl bonds into distinct conformations. The uncatalyzed isomerization is very slow, therefore *cis-trans* isomerizations are often the rate-limiting steps in protein folding pathways. Enzymes with PPIase activity belong to three different groups: cyclophilins which bind cyclosporin A (CsA), FKBP which bind the drug FK506

and parvulins which do not bind to CsA or FK506. As binding proteins for the immunosuppressant drugs CsA and FK506, some PPIases are of particular interest for clinical immunology and are named immunophilins (Schreiber and Crabtree, 1992). A physiological role for PPIases in cellular protein folding reactions and a possible relation to molecular chaperones has been suggested by increased expression of some PPIases as a response to heat shock and accumulation of unfolded proteins (Davis *et al.*, 1992; Partaledis and Berlin, 1993; Sykes *et al.*, 1993) and has been shown for the mitochondrial cyclophilin with a substrate protein imported into the matrix (Matouschek *et al.*, 1995; Rassow *et al.*, 1995; Rospert *et al.*, 1996).

Typical PPIases are abundant single-domain proteins of 10–20 kDa. Some special PPIases are larger and contain additional domains besides the PPIase domain. Large PPIases, such as trigger factor (Kandror *et al.*, 1995; Hesterkamp and Bukau, 1996; Rassow and Pfanner, 1996; Stoller *et al.*, 1996; Kandror *et al.*, 1997; Scholz *et al.*, 1997), NinaA (Baker *et al.*, 1994), RanBP2 (Ferreira *et al.*, 1996), cyclophilin A (Franke *et al.*, 1994) and the Hsp90-associated PPIases (Bose *et al.*, 1996; Duina *et al.*, 1996; Freeman *et al.*, 1996) function as chaperone proteins, most likely by their additional domains. In contrast, the single-domain PPIases seem to function solely as catalysts of peptidyl-prolyl isomerization without involvement of a chaperone activity (Kern *et al.*, 1994); it is unknown whether these abundant PPIases and molecular chaperones cooperate in protein folding.

Here we investigated a possible cooperation of GroEL and the cyclophilin CyP20 (Tropschug *et al.*, 1988, 1989) by using the same substrate in two different states of prolyl bond conformation (all-native bonds or non-native bonds). We show that GroEL, even in the presence of GroES, cannot substitute for a PPIase function, but releases a substrate in the same prolyl isomerization state as it was bound to the chaperonin. Cyclophilin does not promote the correct isomerization of prolyl bonds in a GroEL-bound substrate, but acts sequentially after release of the substrate from GroEL. Strikingly, GroEL can promote productive folding of a denatured substrate with all-native prolyl bonds (cyclophilin-sensitive) to a cyclophilin-resistant form even in the absence of GroES.

Results

Su9-DHFR is a substrate for both cyclophilin CyP20 and GroEL

A number of unfolded proteins were tested for their suitability to investigate interactions with isolated CyP20 (Figure 1A) as well as with GroEL. We found that folding of the hybrid protein Su9-DHFR, consisting of a mitochondrial presequence fused to dihydrofolate reductase (DHFR) (Pfanner *et al.*, 1987), revealed a clear dependence on both folding helpers. We used this protein to set up a model reaction to monitor peptidyl-prolyl *cis-trans* isomerizations in a GroEL-mediated protein folding reaction. DHFR is a protein of 187 residues, 13 of which are proline residues. DHFR has already been used in several studies on the principles of the GroEL reaction cycle (Martin *et al.*, 1991; Viitanen *et al.*, 1991; Engel *et al.*, 1995; Groß *et al.*, 1996; Mayhew *et al.*, 1996; Goldberg *et al.*, 1997). For the *in vitro* assay, Su9-DHFR

was synthesized in rabbit reticulocyte lysate, denatured in 6 M GdmCl (guanidinium chloride) and subsequently diluted into buffer to start the refolding reaction. The folding kinetics of the DHFR domain were monitored by determining its resistance against proteinase K (Pfanner *et al.*, 1987; Ostermann *et al.*, 1989; Matouschek *et al.*, 1995; Rassow *et al.*, 1995; Rospert *et al.*, 1996). If the rate of refolding was dependent on the isomerization state of prolyl bonds, Su9-DHFR should refold in a fast reaction after denaturation under conditions which keep the native conformations of the prolyl bonds intact. This can be achieved by short periods of denaturation at low temperatures. Denaturation at higher temperatures should lead to isomerization of the prolyl bonds and a reduction in the rate of the refolding reaction (Brandts *et al.*, 1975; Fransson *et al.*, 1992; Kern *et al.*, 1995). We tested Su9-DHFR for this effect by denaturation for 15 min at different temperatures and comparison of the folding kinetics (Figure 1B). We found that after denaturation at 2°C, refolding was significantly faster than after denaturation at 10, 20 or 30°C. This result is in agreement with a prolyl isomerization in a rate-limiting step in the refolding reaction. To reduce the uncatalyzed thermal isomerizations of prolyl bonds and keep the conformations of prolyl bonds in a defined state, experiments on PPIases are often performed at low temperatures (10°C–0°C) (Brandts *et al.*, 1975; Lang *et al.*, 1987; Liu *et al.*, 1990; Kofron *et al.*, 1991). We therefore tested the refolding reactions of Su9-DHFR at 4°C. We first compared the folding kinetics of Su9-DHFR in the presence and in the absence of CyP20. In the presence of CyP20 the rate of folding was accelerated, demonstrating the PPIase activity of the enzyme on the substrate (Figure 1C). To test for aggregation, denatured Su9-DHFR was diluted into buffer and subjected to centrifugation. Less than 5% of Su9-DHFR was pelleted after a 1.5 h incubation both in the absence and in the presence of CyP20, confirming that Su9-DHFR remained soluble (not shown). We then tested for binding of Su9-DHFR to GroEL (Viitanen *et al.*, 1991; Rospert *et al.*, 1996). The chaperonin was preincubated in buffer and the substrate protein was added from 6 M GdmCl. As shown in Figure 1D, folding of Su9-DHFR was efficiently retarded by GroEL in the absence of ATP. The folding reaction could be re-started by addition of MgATP (Figure 1E). These effects confirm that Su9-DHFR is not only a substrate for the PPIase CyP20 but also for the chaperonin GroEL.

Su9-DHFR containing non-native prolyl bonds is a substrate for CyP20 after release from GroEL

If Su9-DHFR has been bound to GroEL in a state containing prolyl bonds in non-native conformation, will GroEL release the substrate in a native state or will GroEL preserve the prolyl bonds in their non-native conformation? To obtain prolyl bonds in non-native conformation, Su9-DHFR was denatured for 45 min at room temperature (allowing partitioning of the *cis-trans* conformations according to their thermodynamic equilibrium; Schmid, 1993). After this treatment, Su9-DHFR_{ld} (ld = long denaturation) was diluted into buffer containing GroEL. The reaction was first monitored at 4°C; release of the GroEL-bound protein was started by addition of AMP-PNP together with CyP20 or in the absence of CyP20

Fig. 1. Interaction of Su9-DHFR_{ld} with CyP20 and with GroEL. (A) Isolation of CyP20 from *Neurospora crassa*. CyP20 was isolated as described in Materials and methods; proteins were separated by SDS-PAGE and stained by Coomassie dye. Lane 2, total cell extract after centrifugation at 100 000 g; lane 3, flow through of Q-Sepharose column; lane 4, pooled fractions from hydroxyapatite column; lane 5, isolated CyP20 (20 µg) after elution from Mono-S column. (B) Refolding of Su9-DHFR after denaturation at different temperatures. ³⁵S-labeled Su9-DHFR was denatured in 6 M GdmCl for 15 min at the indicated temperatures and diluted 40-fold into buffer for refolding at 4°C (see Materials and methods). At different time points of refolding, fractions were removed and the folding state of Su9-DHFR was determined by treatment with proteinase K. Indicated is the amount of native (protease-resistant) DHFR. The relative amount of protease-resistant DHFR (percent of total) after 1 h of refolding of the 4°C sample was set to 100% (control). (C) CyP20 accelerates refolding of Su9-DHFR_{ld}. ³⁵S-labeled Su9-DHFR was denatured in 6 M GdmCl for 45 min (ld = 'long denaturation') at room temperature and diluted into buffer at 4°C for refolding in the presence of 2 µM CyP20 (+CyP20) or in the absence of CyP20 (-CyP20) (see Materials and methods). After the indicated time points fractions were taken for treatment with proteinase K. Indicated is the amount of native (protease-resistant) DHFR. The relative amount of protease-resistant DHFR (percent of total) after 1 h of refolding in the absence of CyP20 was set to 100% (control). (D) Folding of Su9-DHFR_{ld} in the presence of GroEL. Su9-DHFR was denatured in 6 M GdmCl for 45 min at room temperature and diluted into buffer in the presence of 0.7 µM GroEL (+GroEL) or in the absence of GroEL (-GroEL). Fractions were removed after the indicated time points and folding of Su9-DHFR_{ld} was monitored by treatment with proteinase K. Indicated is the amount of protease-resistant DHFR. The relative amount of protease-resistant DHFR (percent of total) after 1 h of refolding in the absence of GroEL was set to 100% (control). (E) Folding of Su9-DHFR_{ld} after release from GroEL in the presence of ATP. Su9-DHFR was denatured for 45 min at room temperature and diluted into buffer in the presence of 0.7 µM GroEL. After 10 min MgATP was added at a final concentration of 5 mM. Fractions were removed after the indicated time points and folding of Su9-DHFR_{ld} was monitored by treatment with proteinase K. Indicated is the amount of native (protease-resistant) DHFR. The relative amount of protease-resistant DHFR (percent of total) after 1 h of refolding in the absence of GroEL was set to 100% (control).

(Figure 2A). The non-hydrolyzable ATP analog AMP-PNP is sufficient to release the DHFR from GroEL (Viitanen *et al.*, 1991). We found that under these conditions CyP20 significantly accelerated the rate of folding of Su9-DHFR_{ld}. This effect of CyP20 demonstrates that Su9-DHFR_{ld} is not released in a native folding state but as a folding intermediate which has retained incorrectly isomerized prolyl bonds. GroEL seems to preserve the prolyl bonds of a bound substrate protein in those conformations which are present during binding; it does not induce the formation of native prolyl bonds.

AMP-PNP blocks GroEL for further binding of substrate proteins and only permits a single cycle of substrate binding and release. In contrast, several reaction cycles are possible in the presence of ATP (Jackson *et al.*, 1993; Todd *et al.*, 1994; Weissman *et al.*, 1994; Lilie and Buchner, 1995; Mayhew *et al.*, 1996). We tested for an effect of CyP20 on Su9-DHFR_{ld} in an ATP-mediated reaction and found that the effect of the cyclophilin with ATP was similar to

that with the non-hydrolyzable analog at both 4°C (Figure 2B) and 25°C (not shown). We conclude that GroEL does not promote the formation of native prolyl bonds in the bound substrate protein.

Does the presence of GroES promote the release of Su9-DHFR from GroEL in a native state? We tested for the effect of CyP20 on the folding Su9-DHFR_{ld} after release from the complex of GroEL together with its partner protein GroES, both at 4°C and at 25°C (Figure 2C and D). At 4°C, CyP20 again enhanced the folding reaction to a similar extent as in the absence of GroES (Figure 2B) or GroEL-ES (Figure 1C). Binding of GroES to GroEL was confirmed by a protease protection assay (Martin *et al.*, 1993; Weissman *et al.*, 1995). By binding of GroES, half of the GroEL monomers were protected against proteinase K, in agreement with the structure of the GroEL-ES complex (Figure 2C, insert). Essentially the same effect of CyP20 on Su9-DHFR was observed at 25°C (Figure 2D). The experiments indicate that GroEL-

ES does not promote the transformation of non-native prolyl bonds into their native state. GroEL–ES rather seems to retain the prolyl bonds in their current conformation and release the substrate protein for subsequent isomerizations. The prolyl bonds which isomerize after release from GroEL are accessible for CyP20.

Non-native conformations of prolyl bonds are retained in GroEL-bound Su9-DHFR in the presence of CyP20

Does CyP20 act on prolyl bonds of GroEL-bound proteins? CyP20 is a very compact protein of only 20 kDa which should easily have access to GroEL-bound Su9-DHFR. Substrates of up to 60 kDa can be placed within the chaperonin cavity (Langer *et al.*, 1992; Braig *et al.*, 1994; Chen *et al.*, 1994) and the molecular weight of Su9-DHFR is only 29 kDa. To test for an interaction with the cyclophilin, Su9-DHFR_{Id} was accumulated at GroEL, then CyP20 was added and the solution was incubated for 15 min. Eventually the folding state of Su9-DHFR was tested by addition of 5 mM ATP and monitoring the rate of folding. A parallel sample was first incubated without cyclophilin and received CyP20 only together with the ATP. If the incubation with CyP20 had increased the ratio of correctly isomerized prolyl bonds, folding of the DHFR domain in the first sample should be accelerated. However, this was not observed. After addition of ATP, the folding reaction of both samples was identical (Figure 3A). We modified the experiment including a reisolation of the Su9-DHFR_{Id}–GroEL complex by size exclusion chromatography prior to addition of ATP. The folding reactions now proceeded in the absence of CyP20. However, the folding rates were again indistinguishable (Figure 3B). Also an increase in the temperature to 25°C did not lead to a difference in the folding rate (Figure 3C). The incubation of GroEL-bound Su9-DHFR_{Id} with CyP20 apparently did not increase the number of correctly isomerized prolyl bonds. With respect to the conformation of prolyl bonds, the folding state of Su9-DHFR_{Id} was conserved by GroEL even in the presence of an active PPIase.

Fig. 2. Su9-DHFR_{Id} is a substrate for CyP20 after release from GroEL. **(A)** Su9-DHFR was denatured in GdmCl for 45 min (Id = 'long denaturation') at room temperature and diluted into buffer containing 0.7 μ M GroEL. After an incubation for 5 min at 4°C, the sample was split into halves. Both halves received 5 mM MgAMP-PNP; only one half was mixed with CyP20 (final concentration 2 μ M). Both samples were kept at 4°C. At different time points aliquots were taken and treated with proteinase K to determine the amount of native DHFR. The amount of DHFR which was already folded at the starting point (time 0) was less than 7% and was subtracted. Folding was quantified as described in the legend to Figure 1. **(B)** The experiment was carried out as described in (A) except that ATP was added instead of AMP-PNP. **(C)** Su9-DHFR was denatured for 45 min and diluted into buffer containing 0.6 μ M GroEL and 1.2 μ M GroES. After an incubation for 5 min at 4°C, the sample was split into halves and 5 mM MgATP were added to both samples. One half additionally received 2 μ M CyP20. To determine the amount of native DHFR, aliquots were treated with proteinase K at different time points. The amount of DHFR already folded at the starting point (time 0) was set to 0. Insert: complex formation of GroEL and GroES. GroEL and GroES treated with 0.2 μ g/ml proteinase K for 10 min at 4°C. Parallel samples were left without GroES or without proteinase K (Prot. K). Proteolysis was stopped by addition of PMSF. The products were analyzed by SDS-PAGE and Coomassie staining. GroEL, intact GroEL; GroEL*, proteinase K-resistant fragment of GroEL. **(D)** The experiment followed the same protocol as in (C) except that the entire experiment was carried out at 25°C.

Fig. 3. Incubation of GroEL-bound Su9-DHFR_{ld} with CyP20 does not change the characteristics of folding after release. **(A)** Su9-DHFR was denatured for 45 min (ld = 'long denaturation') at room temperature and diluted into buffer containing 0.7 μ M GroEL at 4°C. Two samples were run in parallel. One received 2 μ M CyP20 after 5 min (sample a); the other sample was left without CyP20 (sample b). After an incubation for 15 min at 4°C, 5 mM MgATP were added to both samples; additionally 2 μ M CyP20 were added to sample b. To test for folding, fractions were collected at different time points, treated with proteinase K and quantified as described in the legend to Figure 1. **(B)** Su9-DHFR_{ld} was bound to GroEL as in experiment (A) and incubated in the presence or in the absence of CyP20 for 15 min. Both samples were subsequently subjected to size exclusion chromatography to separate the Su9-DHFR–GroEL complex from CyP20 (see Materials and methods). Fractions containing the purified Su9-DHFR–GroEL complex were pooled and 5 mM MgATP were added to start the folding reaction. Folding of Su9-DHFR_{ld} was monitored by addition of proteinase K. **(C)** The experiment followed the same protocol as in (A) except all incubations were carried out at 25°C.

Su9-DHFR can be bound to GroEL in different states of prolyl bond isomerization

We then investigated the fate of two different folding states of Su9-DHFR when bound to GroEL. Is it possible to stably bind Su9-DHFR to GroEL in different states of prolyl isomerization? Proteins which are denatured only for a short time retain their prolyl bonds in their native conformation (Brandts *et al.*, 1975). Therefore, after dilution of the denaturant, they refold faster. We applied this principle to Su9-DHFR and found that after a denaturation of 10 s, the initial folding rate of Su9-DHFR_{sd} (sd = short denaturation) was strongly increased as compared with long denaturation (Figure 4A). This effect was not due to incomplete unfolding since the denatured Su9-DHFR_{sd} was completely protease sensitive (Figure 4A, $t = 0$).

Upon dilution of Su9-DHFR_{sd} in the presence of GroEL, folding was efficiently retarded (Figure 4B). Since it was possible to bind Su9-DHFR to GroEL in two different folding states (after long and after short denaturation) we could now test whether these different states were conserved by binding to GroEL. A conservation of prolyl bond conformations should lead to a conservation of the characteristics of the folding reactions of both forms of Su9-DHFR. In contrast, if GroEL functions by binding substrate proteins of different folding states and directing them on a common folding pathway, both forms of Su9-DHFR (after long and after short denaturation) should fold similarly after release from the chaperonin. We bound Su9-DHFR_{ld} and Su9-DHFR_{sd} to GroEL in parallel samples and tested for folding after release from the chaperonin (Figure 4C). To reduce thermal isomerization, the experiment was performed at 4°C. We found that after release in the presence of ATP both proteins again showed their different behavior, fast folding in the case of Su9-DHFR_{sd} and delayed folding in the case of Su9-DHFR_{ld}. GroEL

can thus bind and retain a substrate protein in different patterns of prolyl bond conformations.

We then investigated the behavior of GroEL-bound Su9-DHFR_{sd} at different temperatures (Figure 4D). Su9-DHFR was denatured in GdmCl for 10 s at low temperature and bound to GroEL at 4°C. The sample was divided into aliquots for incubation at 2, 10, 20 or 30°C. After 15 min at the respective temperature the samples were cooled to 4°C. ATP was added to initiate the folding reaction. We observed fast folding of Su9-DHFR in the samples which had been incubated at 2–20°C; slow folding was only observed with the sample which had been incubated at 30°C. This result demonstrates that GroEL can retain the prolyl isomerization state of most of the prolyl bonds of the substrate for 15 min at temperatures up to 20°C. At 30°C the native conformations of Su9-DHFR_{sd} were rapidly lost, leading to a reduction in the rate of folding. This pattern can be compared directly with the result of the experiment in Figure 1B. Incubations of Su9-DHFR in the absence of GroEL cause rapid isomerization of prolyl bonds already at 10°C (Figure 1B). The conservation of the conformations of the prolyl bonds in GroEL-bound Su9-DHFR is thus due to the interaction with the chaperone protein. We then investigated the conservation of prolyl bonds by GroEL at 25°C for different periods of incubation (Figure 4E). Within 7 min the prolyl bonds were mainly retained in their native conformation, while after 15 min considerable isomerization was observed.

These results demonstrate that GroEL does not facilitate prolyl isomerizations, but retards the isomerization of prolyl bonds in the bound substrate protein.

Different sensitivity against CyP20 of Su9-DHFR_{sd} before and after binding to GroEL

Does GroEL only play a passive role in the cooperation with PPIases? To address this question we studied the

Fig. 4. Su9-DHFR can stably bind to GroEL in two different states of prolyl bond conformations. (A) Su9-DHFR was denatured in 6 M GdmCl for 45 min (Su9-DHFR_{ld}, long denaturation) or 10 s (Su9-DHFR_{sd}, short denaturation) and diluted into buffer. Aliquots were taken at different time points and treated with proteinase K to determine the amount of native (protease-resistant) DHFR. (B) Su9-DHFR was denatured for 10 s (sd = 'short denaturation') and diluted into buffer containing 0.7 μ M GroEL (+ GroEL) or no GroEL (- GroEL). The folding state of Su9-DHFR was determined by treatment with proteinase K. (C) Su9-DHFR was denatured in 6 M GdmCl for 10 s (Su9-DHFR_{sd}, short denaturation; sample a) or 45 min (Su9-DHFR_{ld}, long denaturation; sample b) and diluted into buffer containing 0.7 μ M GroEL. After an incubation for 5 min at 4°C, 5 mM MgATP was added to initiate release and folding of the bound proteins. The samples were further incubated at 4°C; the folding reaction was monitored by treatment of aliquots with proteinase K. (D) Su9-DHFR was denatured in 6 M GdmCl for 10 s (Su9-DHFR_{sd}, short denaturation) and diluted into buffer containing 0.7 μ M GroEL at 4°C. The sample was split and the aliquots were incubated for 15 min at the temperatures indicated. All samples were then cooled to 4°C and release of Su9-DHFR from GroEL was initiated by addition of ATP. After different time points the folding state of Su9-DHFR was determined by treatment with proteinase K. (E) Su9-DHFR was denatured in 6 M GdmCl for 10 s (Su9-DHFR_{sd}, short denaturation) and diluted into buffer containing 0.7 μ M GroEL at 4°C. The sample was split into halves for incubation at 25°C. After 7 min or 15 min, respectively, the samples were then cooled to 4°C and ATP was added to initiate folding of Su9-DHFR. Folding was monitored by treatment with proteinase K.

interaction of non-native Su9-DHFR with GroEL and Cyp20 under conditions which provide the substrate protein with all prolyl bonds in their correct conformation.

After short denaturation of a substrate protein, PPIases do not mediate an acceleration in folding but they can cause a reduced rate of the folding reaction by facilitating the formation of non-native proline isomers (Brandts *et al.*, 1975; Kern *et al.*, 1995). The isomerization of a prolyl bond is accelerated by PPIases even if it is already in the correct conformation as long the conformation is not stabilized by the native state of the protein. We tested this by dilution of denatured Su9-DHFR_{sd} into buffer in the presence and in the absence of Cyp20. As expected, we observed a delay in the folding reaction by Cyp20 after short denaturation of the substrate protein (Figure 5A). Interaction of Su9-DHFR with Cyp20 can thus have

opposite effects on the kinetics of folding, depending on the period of denaturation. The same effect was also observed with human cyclophilin A (not shown), indicating that the reduction in the rate of folding was not caused by a specific property of Cyp20 protein, but was due to the PPIase activity of the enzyme. Using this system we investigated the effect of GroEL on the folding pathway of Su9-DHFR containing all prolyl bonds in their native conformation.

We first tested whether GroEL-bound Su9-DHFR_{sd} is resistant against isomerization by Cyp20. With Su9-DHFR_{sd} (after short denaturation) a substrate protein could be bound to GroEL as a homogeneous population of molecules which contain all their prolyl bonds in their native conformation. How did Cyp20 interfere with this state?

Fig. 5. Sensitivity of Su9-DHFR_{sd} against CyP20 is different before and after interaction with GroEL. **(A)** After short denaturation (sd), folding of Su9-DHFR_{sd} is delayed in the presence of CyP20. Su9-DHFR was denatured in 6 M GdmCl for 10 s and immediately diluted into buffer for refolding in the presence of 2 μ M CyP20 (sample b) or in the absence of CyP20 (sample a). At different time points, aliquots were removed and tested for folded DHFR by treatment with proteinase K. Folding was quantified as described in the legend to Figure 1. **(B)** Incubation of GroEL-bound Su9-DHFR_{sd} with CyP20. Su9-DHFR was denatured in GdmCl for 10 s and diluted into buffer containing 0.7 μ M GroEL. After incubation for 5 min at 4°C the sample was split into halves; one half received CyP20 at a final concentration of 2 μ M (sample a) and the other sample was left without CyP20 (sample b). The Su9-DHFR–GroEL complexes were isolated by size exclusion chromatography and folding of Su9-DHFR_{sd} was initiated by addition of ATP. After different time points, aliquots were removed and the folding state of Su9-DHFR_{sd} was determined by treatment with proteinase K. **(C)** Folding of Su9-DHFR_{sd} after release from GroEL in the presence of CyP20. Su9-DHFR was denatured in GdmCl for 10 s and diluted into buffer containing GroEL. Release of the bound Su9-DHFR_{sd} was initiated by addition of AMP-PNP. Two samples were run in parallel; in one sample 2 μ M CyP20 were added together with AMP-PNP (a); no CyP20 was included in the other sample (b). Aliquots were taken at different time points and tested for folding of Su9-DHFR. **(D)** The experiment was carried out as in (C), using ATP instead of AMP-PNP. The result was the same using CyP20 at lower or higher concentrations (up to 10 μ M, not shown).

Su9-DHFR_{sd} was bound to GroEL and incubated with CyP20 for 15 min; a parallel sample was incubated in the absence of CyP20. The Su9-DHFR–GroEL complex was reisolated by size exclusion chromatography and the folding reaction was monitored after addition of ATP (Figure 5B). The kinetics of folding were found to be

independent of the preincubation with CyP20. This was not due to stabilization of the prolyl bonds by a native folding state of the entire protein structure, since at the time of addition of the ATP (time 0), all GroEL-bound Su9-DHFR was still sensitive against proteinase K and hence in a non-native state. We conclude that the native conformations of the prolyl bonds of Su9-DHFR_{sd} were conserved by interaction with GroEL and protected against isomerization by CyP20, in agreement with the data obtained with Su9-DHFR_{ld} (Figure 3).

If GroEL interacted with Su9-DHFR_{sd} in analogy to Su9-DHFR_{ld}, it should be expected that after release from GroEL, folding of Su9-DHFR_{sd} is again delayed by CyP20 (as in Figure 5A). We bound denatured Su9-DHFR_{sd} to GroEL and added AMP-PNP to start the folding reaction. In one sample, CyP20 was added together with the nucleotide; a parallel sample was left without CyP20 (Figure 5C). In contrast to the analogous experiment with Su9-DHFR_{ld} (Su9-DHFR after long denaturation, Figure 2), the cyclophilin showed no effect on the folding reaction. Prior to binding to GroEL, folding of Su9-DHFR_{sd} had been delayed in the presence of CyP20; after release from GroEL the same substrate had lost this property. Similarly we performed the experiment with ATP to initiate release of Su9-DHFR_{sd} (Figure 5D); CyP20 again had no effect on folding. We also tested CyP20 at higher concentrations (Figure 5, legend) but did not observe a reduction in the rate of folding. GroEL seems to play an active role in transforming Su9-DHFR_{sd} containing all-native prolyl bonds into a cyclophilin-resistant protein.

GroEL mediates resistance of Su9-DHFR_{sd} against induction of non-native prolyl bonds by CyP20 after release

Does GroEL only bind a small subfraction of Su9-DHFR_{sd} which has been CyP20-resistant already during dilution into the GroEL buffer? This possibility can be excluded because the effect was observed, even though >90% of the denatured Su9-DHFR had been bound to the chaperonin. However, it has not been tested whether CyP20 can interfere with all stages of the folding pathway of Su9-DHFR_{sd} or if possibly it can only interact during the first and initial stages of folding. In the latter case, GroEL could preferentially accumulate Su9-DHFR_{sd} in a late and thereby CyP20-resistant state.

We therefore examined whether CyP20 can reduce the rate of folding if added after dilution of the denatured Su9-DHFR_{sd} (Figure 6A). We found that CyP20 added after 15 s delayed folding of Su9-DHFR_{sd} nearly to the same extent as CyP20 which had been present during dilution of the substrate protein. This result is in agreement with a previous study demonstrating that folding intermediates in the compact state are substrates for PPIases (Kern *et al.*, 1995). Most of the Su9-DHFR_{sd} was also affected by CyP20 after 30 s (Figure 6B). We next investigated whether the sensitivity of Su9-DHFR_{sd} against CyP20 changes with the same kinetics as the sensitivity against GroEL (Figure 6C and D). Su9-DHFR_{sd} was diluted into buffer in the absence of GroEL which was added after 15 or 30 s, respectively. We found that Su9-DHFR_{sd} loses its sensitivity against GroEL at a different rate than against CyP20; at time point 15 s, nearly all the

Fig. 6. Sensitivity of Su9-DHFR_{sd} folding intermediates against CyP20 and GroEL. (A) Sensitivity of Su9-DHFR_{sd} against CyP20, added 15 s after the start of the folding reaction. Su9-DHFR was denatured in 6 M GdmCl for 10 s (sd = 'short denaturation') and diluted 40-fold into buffer to start the folding reaction at 4°C. Three samples were run in parallel. In one sample Su9-DHFR_{sd} was diluted in the presence of 2 μM CyP20 (sample a). In the second sample Su9-DHFR_{sd} was diluted in the absence of CyP20. 15 s after the start of the folding reaction, CyP20 was added at a final concentration of 2 μM (sample b). The third sample was left without CyP20 (sample c). After different time points aliquots were removed and tested for folding of Su9-DHFR_{sd} by treatment with proteinase K. Folding of Su9-DHFR was quantified as described in the legend to Figure 1. (B) Sensitivity of Su9-DHFR against CyP20 30 s after the start of the folding reaction. The experiment was carried out as in (A) except that in one sample CyP20 was added after 30 s instead of 15 s. (C) Sensitivity of Su9-DHFR_{sd} against GroEL 15 s after the start of the folding reaction. Su9-DHFR was denatured for 10 s and diluted into buffer for folding under three different conditions. In the first sample Su9-DHFR_{sd} was diluted in the presence of 0.7 μM GroEL (sample a). In the second sample (b) GroEL was added 15 s after the start of the folding reaction; the final concentration of GroEL was the same as in sample a. The third sample (c) was left without GroEL. After different time points, aliquots were tested for folding by treatment with proteinase K. (D) Sensitivity of Su9-DHFR against GroEL 30 s after the start of the folding reaction. The experiment was carried out as in (A) except that in one sample GroEL was added after 30 s instead of 15 s. (E) Time course of acquisition of resistance against CyP20 and against GroEL in folding of Su9-DHFR_{sd}. The sensitivity against CyP20 and GroEL was tested as in experiments (A–D). The inhibition of folding was determined by adding CyP20 and GroEL to the assay at different time points (Δt). The folding reactions were allowed to proceed for 6 min and stopped by addition of proteinase K. Shown is the increase of resistance (calculated as described in Materials and methods) against inhibition of folding by GroEL (a) or CyP20 (b), respectively. Maximal inhibition was achieved by addition of CyP20 or GroEL prior to dilution of Su9-DHFR_{sd} (Δt = 0). At early time points sensitivity of Su9-DHFR_{sd} against GroEL quickly decreases while sensitivity against CyP20 still persists. Folding of Su9-DHFR within Δt was determined in parallel by immediate addition of proteinase K (c). The amount of folded Su9-DHFR after a reaction of 6 min without GroEL or CyP20 was set to 100% (control). Curves a and b were corrected for the amount of folded Su9-DHFR that proceeded if GroEL or CyP20 were present from the beginning for 6 min – Δt.

Su9-DHFR_{sd} is still CyP20-sensitive but nearly half of the protein has lost its ability to bind to GroEL (Figure 6A versus C). A much higher sensitivity of the substrate against CyP20 than against GroEL is also detectable after 30 s (Figure 6B versus D). This is not due to a lower activity of GroEL, because if added at time 0, the effect of GroEL against Su9-DHFR_{sd} is much more pronounced than the effect of CyP20 (Figure 4B versus Figure 5A). We conclude that Su9-DHFR_{sd}, when diluted from denaturant, quickly loses its affinity for GroEL but retains its sensitivity against CyP20 for a longer time.

The sensitivity of Su9-DHFR_{sd} against CyP20 and against GroEL was similarly tested for at additional time points in the folding pathway of Su9-DHFR_{sd} (Figure 6E). The extent of inhibition was determined in comparison with the inhibition that was achieved if the respective mediator (CyP20 or GroEL) was added before addition

of the denatured substrate protein. The data confirm that GroEL and CyP20 differ in their effect on the successive folding intermediates of Su9-DHFR_{sd}. Sensitivity of Su9-DHFR against GroEL is rapidly lost while its sensitivity against CyP20 is retained for a longer time. These results indicate that GroEL only binds early folding intermediates of Su9-DHFR_{sd} and that these intermediates are still substrates for CyP20. We conclude that it is due to the interaction with GroEL that folding intermediates of Su9-DHFR_{sd} that are released from GroEL in the presence of nucleotides have lost their sensitivity to the PPIase CyP20.

Discussion

We show that both chaperonin GroEL and cyclophilin CyP20 can interact with the same substrate in two different states of prolyl isomerization. Binding of different folding

intermediates of a substrate protein has been reported previously, yet not with respect to prolyl bonds (Martin *et al.*, 1991; Hayer-Hartl *et al.*, 1994; Lilie and Buchner, 1995; Smith and Fisher, 1995; Goldberg *et al.*, 1997). Other data have suggested that upon rebinding to GroEL, a protein always adopts the same folding state (Weissman *et al.*, 1994). Most models of chaperonin function predict that within a single reaction cycle, only a fraction of the substrate is converted to the native state whereas the residual non-native protein is subjected to an additional reaction cycle (Hartl, 1996; Todd *et al.*, 1996; Fenton and Horwich, 1997; Ranson *et al.*, 1997). An affinity of GroEL for different folding intermediates of the same substrate should facilitate such iterative cycles of binding and release.

Our results indicate that GroEL with or without GroES does not promote the isomerization of non-native prolyl bonds into the conformations of the native state, demonstrating that the interaction with the chaperonin cannot substitute for interaction with the prolyl isomerase. If the substrate protein requires a prolyl isomerization for progress on the folding pathway, it has to leave GroEL for this step. CyP20 does not induce native conformations in the GroEL-bound substrate, although access is not prevented by GroES, and the size of Su9-DHFR (29 kDa) and CyP20 (20 kDa) should permit the simultaneous presence of GroEL [substrates of 60 kDa can be placed within GroEL (Langer *et al.*, 1992; Braig *et al.*, 1994; Chen *et al.*, 1994)].

In parallel we found that by binding to GroEL, the thermal isomerization of prolyl bonds in the substrate protein is also impeded. In comparison with the free state, isomerizations at GroEL require higher temperatures and longer times. How is this conservation of prolyl bond conformation achieved at GroEL? GroEL-bound substrates predominantly adopt a compact or molten globule state which has been shown for DHFR and other proteins (Groß *et al.*, 1996; Hartl, 1996; Goldberg *et al.*, 1997). It is therefore likely that Su9-DHFR is also bound in a molten globule state. Free in solution, folding intermediates of this type are still substrates for PPIases (Kern *et al.*, 1995). The molten globule state thus cannot account for the stability of prolyl conformations at GroEL. In principle PPIases can only interfere with the isomerization of prolyl bonds which are exposed at the outer surface of a protein (Schmid, 1993; Fischer, 1994). Similarly, proteins in a compact state will bind to GroEL by residues of the outer surface. A possible explanation for the apparent lack of interaction between GroEL-bound Su9-DHFR and CyP20 could be that the same residues (or structures in the vicinity) are recruited for binding to GroEL and for isomerization by CyP20.

A denatured protein with all-native prolyl bond conformations is a substrate for both GroEL and CyP20; the interaction with CyP20 leads to formation of non-native prolyl bonds and thus causes a delay in folding. In spontaneous folding, resistance of the substrate to binding by GroEL is achieved at an earlier folding stage than the resistance to interaction with CyP20. Preferential binding of GroEL to early folding intermediates has recently also been observed with β -lactamase (Gervasoni *et al.*, 1996; Gervasoni and Plückthun, 1997). When denatured Su9-DHFR is first bound to GroEL, however, it is resistant to

cyclophilin after nucleotide-dependent release from the chaperonin. The cyclophilin resistance is already acquired by a single round of binding to GroEL (release by a non-hydrolyzable ATP analog). Since in spontaneous folding GroEL resistance precedes CyP20 resistance, it can be excluded that GroEL simply traps CyP20-resistant forms of the substrate. This leads to the unexpected conclusion that GroES-free GroEL is able to promote productive folding of a substrate with all-native prolyl bonds to a CyP20-resistant form. At GroEL or in the moment of release, the substrate proceeds to a new folding state which is closer to the native state. Interaction with GroEL thus prevents the substrate protein from using an unfavorable folding pathway.

It is evident from the work of Mayhew *et al.* (1996) and Weissman *et al.* (1996) that complete folding of a GroEL-bound substrate occurs in a sequestered position under GroES, yet the results presented here with the novel CyP20-resistant form indicate that a productive folding reaction can also occur in the absence of GroES. In this respect it is interesting to note that different views exist about a possible active role of GroEL in protein unfolding. While Walter *et al.* (1996) reported that GroEL does not change the microscopic rate constant of unfolding, suggesting that GroEL can trap non-native folding intermediates but does not serve as an active unfolding enzyme, Itzhaki *et al.* (1995) and Ranson *et al.* (1995) proposed a catalytic function of GroEL in unfolding of the substrate. Complete unfolding of a GroEL-bound substrate was observed even in the absence of GroES (Zahn *et al.*, 1996b).

Under cellular conditions, proteins fold in the presence of high concentrations of PPIases which could direct the folding reactions of proteins onto different folding pathways (Fischer, 1994). Chaperonins seem to function by restricting these pathways to those which are most effective. A substrate containing non-native prolyl bond conformations is released for subsequent folding steps including acceleration by cyclophilin, whereas a substrate with all-native prolyl bond conformations is released in a cyclophilin-resistant state for immediate folding.

Materials and methods

Isolation of CyP20

CyP20 was isolated from *Neurospora crassa* wild-type strain 74A. The cells were grown and harvested as described previously (Tropschug *et al.*, 1988). The cells were ground in 0.05% Triton X-100, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM Tris-HCl pH 7.5. After a centrifugation of 100 000 g for 1 h, floating lipids were removed and the supernatant was applied to a Q-Sepharose FF column (Pharmacia). The flow-through was concentrated by ultrafiltration (Amicon; 10 kDa exclusion membrane) and dialyzed against 1 mM DTT, 1 mM EDTA, 20 mM potassium phosphate pH 7.0. The solution was applied to a hydroxyapatite column (Bio-Rad). CyP20 was eluted by a linear gradient of 0–500 mM potassium phosphate pH 7.0. CyP20-containing fractions were concentrated by ultrafiltration and dialyzed against 1 mM DTT, 1 mM EDTA, 20 mM MES-KOH pH 6.3. The major contaminant of these fractions was FKBP14 (Tropschug *et al.*, 1989) which was removed by Mono-S chromatography (Pharmacia). CyP20 was eluted from the Mono-S column by a 0–100 mM NaCl gradient; CyP20 eluted at ~50 mM NaCl. From 400 g of *Neurospora crassa* cells, 7.6 mg of CyP20 were obtained. After a final concentration by ultrafiltration, the protein was frozen in liquid nitrogen and stored at –20°C.

Preparation and folding of Su9-DHFR

Su9-DHFR (Pfanner *et al.*, 1987) was synthesized in reticulocyte lysate (Amersham) in the presence of [³⁵S]methionine and [³⁵S]cysteine. The lysate was centrifuged for 30 min at 220 000 g to remove the ribosomes. Su9-DHFR was precipitated by ammonium sulfate (40% saturation) at 2°C for separation from globin. The precipitated protein was dissolved in 6 M GdmCl, 2 mM DTT, 100 mM 3-(N-morpholino)propanesulfonic acid (MOPS), adjusted to pH 7.2 with KOH. For 'short denaturation', the protein was dissolved for only 10 s at -4°C and then immediately diluted into buffer for subsequent refolding. For 'long denaturation', the sample was shaken at room temperature for 45 min. The folding reaction was started by 40-fold dilution into 80 mM KCl, 60 mM MOPS-KOH pH 7.2 and rapid mixing. The folding reactions were carried out at 4–30°C, as indicated in the figure legends. To avoid thermal isomerizations of prolyl bonds, most experiments were carried out at 4°C. The temperatures in the samples were determined using an electronic thermometer. CyP20 was added from a solution (2 mg/ml) in 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 20 mM MES-KOH and used at a final concentration of 2 µM. Parallel samples without CyP20 received an equivalent volume of the same buffer. Human cyclophilin A (Boehringer, Mannheim) was used at a final concentration of 1 µM. GroEL (Boehringer, Mannheim) was added from 2 mM EDTA, 50 mM Tris-HCl pH 7.5; the same buffer was added to samples without chaperonin. The final concentration of GroEL 14mers was 0.7 µM. MgATP was added from a 100 mM stock solution and the pH was adjusted to 7.0 by addition of KOH. GroEL-substrate complexes were isolated by size exclusion chromatography using Sephacryl S200 (Pharmacia) in a 0.7×18 cm column. The column was run at a flow rate of 0.05 ml/h.

Folding of Su9-DHFR was monitored by resistance of the DHFR domain to proteinase K (Ostermann *et al.*, 1989; Matouschek *et al.*, 1995; Mayhew *et al.*, 1996). The protease was added at a final concentration of 200 µg/ml. The samples were incubated at 0°C for 10 min. Proteolysis was stopped by addition of 1 µM PMSF. The samples were subjected to precipitation with 10% (w/v) trichloroacetic acid (TCA) in the presence of 0.02% deoxycholate and 20 µg cytochrome *c* and analyzed by SDS-PAGE. Samples of untreated Su9-DHFR were used to determine the total amount of added protein and to calculate the ratio of proteinase K-resistant protein after folding. Quantifications were carried out using a PhosphorImager (Molecular Dynamics).

Resistance of non-native Su9-DHFR against inhibition of folding by GroEL or CyP20 was calculated as $F_{6 \text{ min}} - [(F_{6 \text{ min}} - F_{\Delta t}) \cdot I_{\Delta t} : I_0]$ with $F_{6 \text{ min}}$ = folding within 6 min in the absence of mediators; $F_{\Delta t}$ = folding within Δt ; $I_{\Delta t}$ = % inhibition of folding by addition of mediator (CyP20 or GroEL) at Δt ; I_0 = % inhibition of folding by addition of mediator at $\Delta t = 0$.

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