Affinity of chaperonin-60 for a protein substrate and its modulation by nucleotides and chaperonin-10

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The refolding of lactate dehydrogenase fully unfolded in 4 M guanidinium chloride was initiated by dilution into assay buffer, and the emergence of active enzyme was recorded. This was performed in the presence of the following chaperonin complexes in the refolding medium: chaperonin-60 (cpn60), cpn60–MgATP, cpn60–Mgp[NH]ppA, cpn60–MgADP in both the presence and absence of chaperonin-10 (cpn10). For each nucleotide–chaperonin complex studied, the effect of nucleotide concentration was measured. Dissociation constants (K_d) for unfolded LDH bound to the various chaperonin complexes were derived directly from the ability of the complexes to retard the folding of

the enzyme. Dissociation constants for the different complexes were found to be in the order: cpn60 < cpn60–MgADP–cpn10 (formed at low [MgADP]) < cpn60–MgADP < cpn60–MgADP–cpn10 < cpn60–Mgp[NH]ppA < cpn60–Mgp[NH]ppA–cpn10 < cpn60–MgATP < cpn60–MgATP–cpn10; i.e. the tightest complex is with cpn60 and the weakest with cpn60–MgATP–cpn10. Only when MgATP is the nucleotide do we see the yield of native enzyme increased on the time scale of 1 h. The results provide estimates of the change in binding energy between the chaperonin and a substrate protein through the cycle of MgATP binding, hydrolysis and dissociation.

INTRODUCTION

Constitutive expression of chaperonins in prokaryotic cytoplasm, plastids and mitochondria ensures efficient folding and assembly of newly synthesized and transported proteins. When cells are under stress and the ‘heat-shock’ response is induced, chaperonin concentrations rise in order to salvage unfolded or partially folded protein and assist folding back to the native state, preventing side reactions such as aggregation from occurring [for reviews see Ellis and van der Vies (1991), Zeilstra-Ryalls et al. (1991), Lorimer (1992) and Jaenicke (1993)]. These activities require input of energy from the hydrolysis of MgATP, but little is known about the mechanism of transduction.

These phenomena can be studied *in vitro* by denaturing proteins in chaotropic solvents and allowing them to refold in native conditions in the presence of chaperonins. For proteins that fold inefficiently *in vitro*, the inclusion of chaperonins is seen to increase the yield of the native state (Mendoza et al., 1989; Laminet et al., 1990; Viitanen et al., 1990; Buchner et al., 1991; Martin et al., 1991; Fisher, 1992).

There are two proteins involved in this folding activity, chaperonin-60 (cpn60) and chaperonin-10 (cpn10). The former is composed of 14 subunits of approx. 60 kDa each, arranged in two seven-membered rings; the MgATP site resides on these subunits (Hendrix, 1979; Hohn et al., 1979). The latter exists as a single smaller seven-membered ring, each subunit having a mass of 10 kDa (Tilly et al., 1981; Chandrasekhar et al., 1986). In the presence of MgATP or MgADP, a single ring of cpn10 associates with one of the cpn60 rings, leaving the other open (Saibil et al., 1992; Ishii et al., 1992). The protein-binding site has been shown to lie in the hole formed by the cpn60 subunits (Braig et al., 1993), thus each subunit can contribute a binding interaction with the same folding polypeptide. Through the cata-

lytic cycle of MgATP binding, hydrolysis and release, several chaperonin states are generated. The rates of some steps in this cycle have been measured and the stabilities and conformational properties of constituent complexes have been defined by a combination of kinetic, spectroscopic and electron-microscopic techniques (Jackson et al., 1993; Saibil and Wood, 1993; Todd et al., 1993).

A remarkable property of chaperonins is their lack of specificity for protein substrates; molecules with widely differing secondary and tertiary structures are able to bind to cpn60 (Schmidt and Buchner, 1992), making it unlikely that chaperonins recognize particular structural features of their targets. This can be contrasted with the conventional mode of enzyme catalysis in which the active site undergoes specific stabilizing interactions with the transition state of the reaction. If this were true of chaperonins, the type of interaction made with the ‘transition state’ would have to be much less specific and recognize some general physical property. However, it is also now accepted that chaperonins are not, in a conventional sense, folding catalysts (Lorimer, 1992; Jaenicke, 1993; Miller et al., 1993; Jackson et al., 1993). They have not been seen to accelerate the rate of folding over that recorded in their absence. However, they do reduce the amount of misfolded or aggregated product, and, in doing so, increase the yield of the native state. Therefore they can only be said to be catalytic in that they increase the flux from misfolded to correctly folded states in the overall process.

One aspect of the individual chaperonin–nucleotide complexes which has not been examined in detail is their relative affinity for protein substrates. The protein-binding surface lies on cpn60, but this protein can be ligated with different combinations of nucleotide and cpn10. The following stable complexes have been identified and characterized: cpn60, cpn60–MgATP, cpn60–MgADP, cpn60–MgATP–cpn10 and cpn60–MgADP–cpn10

Abbreviations used: LDH, lactate dehydrogenase; cpn60, chaperonin-60; cpn10, chaperonin-10; GdmCl, guanidinium chloride; p[NH]ppA, adenosine 5′-[β , γ -imido]triphosphate; TEA, triethanolamine hydrochloride.

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(Jackson et al., 1993). It is the aim of the experiments described here to define the binding affinity of each of these states for unfolded protein ligands. This is estimated by their ability to retard the rate of folding of lactate dehydrogenase (LDH) from *Bacillus stearothermophilus*. The tighter the interaction, the slower is the release of LDH and therefore the slower the emergence of enzyme activity (Badcoe et al., 1991; Jackson et al., 1993).

This simple analysis is most valid when the chaperonin complex is 'inert', i.e. it can bind the protein but not undergo hydrolysis; thus in these experiments an unreactive nucleoside triphosphate analogue, adenosine 5'-[β,γ -imido]triphosphate (Mgp[NH]ppA), is used, in addition to MgATP. The information gained from these studies is important for constructing a chaperonin mechanism, as it defines changes in the interaction energy between the chaperonin and the substrate protein during the reaction cycle. In doing so, it helps illuminate the means of energy transduction in these proteins.

MATERIALS AND METHODS

Preparation of proteins

The cloned LDH gene from *B. stearothermophilus* was expressed in *Escherichia coli* TG2 cells from the pKK223-3 vector, as described previously (Barstow et al., 1986), and the protein was purified by using an oxamate affinity column followed by Q-Sepharose ion-exchange chromatography (Clarke et al., 1985a). The *E. coli* groEL and groES genes (Fayet et al., 1986) were co-expressed in *E. coli* MC1061 cells from the pND5 plasmid (Jenkins et al., 1986).

To prepare the protein, cultures were grown for 18 h at 37 °C, followed by 1 h heat-shock treatment at 42 °C and a final 1 h growth at 37 °C. Cells were disrupted by sonication in 50 mM triethanolamine hydrochloride (TEA) buffer at pH 7.5 containing 2 mM EDTA, and the soluble fraction, after removal of cell debris by centrifugation (12000 *g* for 40 min), was treated with 0.1 % (w/v) DNAase and 4 mM MgCl₂ for 30 min. This solution was then adjusted to pH 8.5, further 5 mM EDTA was added and the solution was loaded on to a Q-Sepharose ion-exchange column. Protein fractions were eluted using a 0–0.75 M NaCl gradient; cpn10 was eluted at 0.4 M and cpn60 at 0.6 M NaCl.

Cpn10 was further purified by adding (NH₄)₂SO₄ to a concentration of 1.5 M and loading on to a phenyl-Sepharose column. The protein was eluted with a 1.5–0 M (NH₄)₂SO₄ gradient; cpn10 was eluted at an (NH₄)₂SO₄ concentration of approx. 0.8 M.

Cpn60 was diluted with an equal volume of buffer and rechromatographed on Q-Sepharose. Material from this second ion-exchange step was treated with MgATP and MgCl₂ to release any bound protein before a final gel-filtration step with a Bio-Rad Bio-Sil TSK-250 h.p.l.c. column. After purification, both proteins were stored as precipitates in 70 % satd. (NH₄)₂SO₄. Before use, both proteins were dialysed against 300–500 vol. of 50 mM TEA, pH 7.5, containing 50 mM KCl, 20 mM MgCl₂ and 0.5 mg/ml acid-washed charcoal for 10–18 h. The dialysed proteins were stored at 4 °C and used within 36 h. The concentrations of cpn60 (Badcoe et al., 1991) and cpn10 (Jackson et al., 1993) were determined as described previously.

Standard buffer conditions

The standard buffer for all of the experiments presented in this paper, except where stated, was 50 mM TEA, pH 7.5, containing

50 mM KCl, 20 mM MgCl₂ and 2 mM dithiothreitol and is hereafter referred to as the standard buffer. All experiments were performed at 25 °C.

Measurement of refolding of LDH

LDH was denatured in 4 M guanidinium chloride (GdmCl)/50 mM TEA buffer, pH 7.5, to a final concentration of 15 μ M enzyme subunits. Dithiothreitol was added to a concentration of 5 mM to prevent slow oxidative disulphide-bridge formation which renders LDH denaturation irreversible over a period of hours (Smith et al., 1991). Protein solutions in denaturant were incubated for 1 h to allow the unfolding to reach equilibrium.

Refolding was initiated by a 200-fold dilution of denatured LDH to a final concentration of 75 nM in a 1 ml cuvette containing 10 mM pyruvate and 0.2 mM NADH in the standard buffer. These concentrations are sufficient to saturate LDH with respect to both substrates. The residual concentration of GdmCl (0.02 M) is insufficient to disrupt either LDH (Smith et al., 1991) or cpn60 (Price et al., 1993). To monitor the refolding of LDH in the presence of the chaperonins, 200 nM cpn60 in the presence and absence of 400 nM cpn10 (oligomers) was preincubated in the cuvette for 30 s with different concentrations of MgATP, Mgp[NH]ppA or MgADP (4 h for the slowly formed cpn60–MgADP complex).

A_{340} was monitored as refolding progressed, providing a direct measure of NADH consumption by the refolding enzyme. Tangents to curves of NADH concentration versus time were taken to be proportional to the concentration of active (refolded) LDH; to safeguard against loss of saturation, extra NADH was added periodically to maintain it at a concentration at which product and substrate inhibition are negligible (Clarke et al., 1985b). The data were adjusted for inhibition of LDH activity at high concentrations of MgATP, Mgp[NH]ppA and MgADP.

Analysis of results

The Facsimile numerical integration program (Curtis and Sweetenham, 1985) was used to fit the enzyme-renaturation data to the minimal kinetic scheme, as described previously (Badcoe et al., 1991).

RESULTS

Figure 1(a) shows the folding time course of LDH alone and in the presence of cpn60 with different concentrations of the nucleotide ligand Mgp[NH]ppA. The fact that LDH folds through a series of slowly formed intermediates is reflected in the noticeable lag before the appearance of the active enzyme (Badcoe et al., 1991), and the sigmoidal shape of the progress curve is retained in the presence of chaperonins and nucleotide. The general observation is that interaction of folding LDH with the chaperonins reduces the rate of folding. The extent of retardation is reduced as the concentration of Mgp[NH]ppA is increased, underlining the ability of nucleoside triphosphate to weaken protein binding (Badcoe et al., 1991). The presence of cpn10 (Figure 1b) reduces the folding time still further showing that the association with cpn60–Mgp[NH]ppA lowers the affinity of the chaperonin for the unfolding protein. Interestingly, in this case, cpn10 appears to lower the amount of recovered protein, perhaps by lowering the number of available binding sites on the complex (conditions for increasing the yield are no longer optimal).

Figures 2(a) and 2(b) show the time courses for the refolding

Figure 1 Effect of Mg[ppA] on the refolding of LDH in the presence of (a) cpn60 and (b) cpn60-cpn10

The recovery of LDH activity was measured as a function of time in the absence of chaperonin (—), in the presence of cpn60-Mg[ppA] (a; open symbols) and cpn60-cpn10-Mg[ppA] (b; filled symbols). The concentration of Mg[ppA] was varied from 1 mM ($\square$, $\blacksquare$), 2 mM ($\triangle$, $\blacktriangle$), 4 mM (∇ , $\blacktriangledown$), 6 mM ($\circ$, $\bullet$) to 10 mM ($\diamond$, $\blacklozenge$). Protein concentrations in the assays were 75 nM (LDH), 200 nM (cpn60 oligomer) and 400 nM (cpn10 oligomer) and the assays were performed in standard buffer conditions (see the Materials and methods section). The activity of 75 nM native LDH is 1.35 $\Delta A/\text{min}$ in these conditions. The curves show the optimal fit of the data to a model in which cpn60 is able to bind to three folding intermediates in order to retard the appearance of native LDH.

of LDH in the presence of cpn60-MgADP and cpn10-MgADP-cpn60 respectively. The curves show that MgADP also has some ability to displace LDH from the chaperonin. When compared with the effect of the MgATP analogue, a much greater concentration of MgADP is required to weaken the interaction of cpn60 with the LDH substrate. This is consistent with the weaker interaction of MgADP with cpn60 (Jackson et al., 1993). The presence of the co-chaperonin has only a small effect on the retardation time, i.e. with MgADP as the nucleotide, the cpn10 interaction does little to displace bound protein. Inclusion of 10 mM phosphate in these experiments with MgADP had no measurable effect on the refolding time courses (results not shown).

When LDH is folded in the presence of cpn60-MgATP or cpn60-MgATP-cpn10 (Figure 3), the rate of refolding is retarded only slightly. As in the case of complexes with Mg[ppA], cpn10 causes a reduction in the folding time over that without

Figure 2 Effect of MgADP on the folding of LDH in the presence of (a) cpn60 and (b) cpn60-cpn10

The experiment described in Figure 1 was repeated using MgADP instead of Mg[ppA]. The nucleotide concentrations in the assays were 50 μM ($\square$, $\blacksquare$), 0.5 mM ($\triangle$, $\blacktriangle$), 4 mM (∇ , $\blacktriangledown$) and 20 mM ($\circ$, $\bullet$). The inclusion of 10 mM phosphate has no effect on these folding time courses.

the co-protein. It is apparent from these results that MgATP hydrolysis results in an increase in the efficiency of folding, i.e. 60% higher than in the absence of chaperonins. It was not possible in these experiments to look at the folding of LDH with chaperonin at low concentrations of MgATP because of the rapid hydrolysis of the nucleotide once a protein substrate is added (Martin et al., 1991; Jackson et al., 1993).

The degree to which these chaperonin complexes retard the refolding of LDH, compared with the spontaneous rate, can be taken as a measure of affinity of the protein-protein interaction. The simplest case:

where U is the unfolded state of the protein, N is the native state, k_f is the measured rate constant of folding and K_d is the dissociation constant describing the interaction of the unfolded protein with the chaperonin, C. If equilibration of the chaperonin-

Figure 3 Effect of MgATP on the folding of LDH in the presence of cpn60 and cpn60-cpn10

The experiment described in Figure 1 was repeated using MgATP instead of Mgpp[NH]ppA. Open symbols represent experiments performed using cpn60-MgATP and filled symbols are experiments performed with cpn60-cpn10-MgATP. The concentrations of nucleotides in the assays were 1 mM ($\square$, $\blacksquare$), 2 mM ($\triangle$, $\blacktriangle$), 4 mM (∇ , $\blacktriangledown$) and 8 mM ($\diamond$, $\blacklozenge$). The refolding of LDH was observed to be independent of the concentration of MgATP and so only one fit to the data was performed for each type of complex.

binding step is rapid compared with the rate of folding and the chaperonin concentration is high compared with that of the unfolded protein [as is usually the case (Martin et al., 1991; Badcoe et al., 1991; Jackson et al., 1993)] then:

$$K_d = C_o k_{obs} / (k_f - k_{obs})$$

where k_{obs} is the observed first-order rate constant for folding and C_o is the total chaperonin concentration. In this case, analysis of the effect of nucleotides and cpn10 on the affinity for unfolded protein can be determined by measuring k_{obs} for the refolding process. In the case of LDH folding, such a simple analytical solution is not possible because the protein refolds through several slow steps and cpn60 is able to bind tightly to two unfolded states. The folding pathway of *B. stearotherophilus* LDH is atypical and can be summarized:

Each state can be observed at equilibrium when the protein is unfolded in increasing concentrations of GdmCl (Smith et al., 1991; Badcoe et al., 1991) and the kinetics of folding from any state are consistent with the above sequential progression (Burstion et al., 1992). Three features of this kinetic pathway should be pointed out. First, when folding is initiated from the random-coil state at 4 M GdmCl, the coil will immediately collapse. Thus U_1 represents this initial collapsed state. Secondly, if the protein is unfolded only as far as the U_3 state (2–2.2 M GdmCl), its properties are characteristic of a 'molten globule' (Smith et al., 1991), yet when this species is refolded cpn60 is unable to bind it (Badcoe et al., 1991). Thirdly, the unimolecular step described by the rate constant, k_3 , is fast compared with k_1 , k_2 and the second-order rate of dimerization at the protein concentrations used in these experiments. We can therefore most simply describe the

Figure 4 Variation of the averaged dissociation constant, K_d , of LDH folding intermediates from cpn60 and cpn60-cpn10 as a function of nucleotide concentration

Variations in dissociation constants, K_d , of the three folding intermediates of LDH complexed with cpn60 (a) or cpn60-cpn10 (b) with increasing nucleotide concentration are plotted. The average of these was taken as a measure of the affinity of cpn60 (or cpn60-cpn10) for unfolded LDH. Dependence on the concentration of MgATP ($\circ$, $\bullet$), Mgpp[NH]ppA ($\square$, $\blacksquare$) and MgADP ($\triangle$, $\blacktriangle$) is shown and a standard spline fit is drawn through the data in order to emphasize, for example, the co-operativity of the effect of Mgpp[NH]ppA.

kinetics of refolding, when initiated from the random-coil state and in the presence of chaperonins, as:

Previous analyses (Badcoe et al., 1991; Burstion et al., 1992) have shown that cpn60 interacts tightly with U_1 and U_2 but weakly or not at all with U_3 and these interactions have been included in the above scheme. By applying numerical integration (see the Materials and methods section), the data shown in Figures 1, 2 and 3 were fitted to this model to estimate the effect of cofactors on the affinity of cpn60-LDH interaction. Parameters k_1 , k_2 and k_3 were determined independently for LDH refolding in the absence of chaperonins and fixed (Badcoe et al.,

Table 1 Dissociation constants, K_d , of LDH folding intermediates from complexes with cpn60 or cpn60–cpn10 with various concentrations of nucleotide

As explained in the text, the folding curves of LDH were fitted to a model in which the binding of cpn60 (or cpn60–cpn10) to three intermediates on the folding pathway of LDH causes the retardation of folding. The dissociation constants of the three intermediates K_{d1} , K_{d2} and K_{d3} (nM) are given for each complex of cpn60 with and without cpn10, in the presence of the various concentrations of nucleotides MgATP, Mgp[NH]ppA and MgADP (mM). All values contain errors of $\pm 6\%$. An average value of K_{d1} and K_{d2} , K_{av} (nM), as a representative dissociation constant of unfolded LDH from the chaperonin complexes is also given.

Nucleotide	– cpn10				+ cpn10			
	K_{d1}	K_{d2}	K_{d3}	K_{av}	K_{d1}	K_{d2}	K_{d3}	K_{av}
MgATP	115.5	125.5	556.9	118.5	453.6	424.1	1280	439
MgADP								
0.05 mM	8	7.58	44.8	7.8	17.22	17.94	74	17.6
0.5 mM	17.3	13.9	60.5	15.6	16.58	14.95	587	15.8
4 mM	22	14.25	137.7	18.12	25.85	23.45	90.8	24.6
20 mM	33.2	26.6	98.4	29.9	35.8	31.33	105.4	33.6
Mgp[NH]ppA								
1 mM	18.3	22.8	270.2	20.6	29.8	28.8	559.4	29.3
2 mM	28.2	25.3	101.1	26.7	51.3	50	136.3	50.6
4 mM	54.4	52.01	176.6	53.25	82	84.4	329.4	83.2
6 mM	66.2	65.3	157.7	65.75	130.2	172.4	11300	151.3
10 mM	70.1	72.5	192.0	71.8	149.1	159.3	1212	154.2

1991; Burston et al., 1992). Values for K_{d1} , K_{d2} and K_{d3} were then extracted from fits to refolding time courses in the presence of chaperonins. In all cases K_{d1} and K_{d2} were of the same magnitude and much tighter than K_{d3} (see Table 1). Presented in Figure 4 are averages of these values plotted as a function of the concentrations of Mgp[NH]ppA, MgADP and MgATP in the absence (Figure 4a) and presence (Figure 4b) of cpn10.

These plots are useful in two ways. First they provide estimates of the affinities of each of the chaperonin–nucleotide complexes for the unfolded LDH. These are given by the apparent dissociation constants at saturating nucleotide concentrations. Apo-cpn60 has the highest binding affinity (7 nM), followed by cpn60–MgADP (30 nM), cpn60–Mgp[NH]ppA (70 nM) and cpn60–MgATP (120 nM). The presence of the co-chaperonin increases the dissociation constant for the Mgp[NH]ppA (150 nM) and MgATP (440 nM) states still further while having little effect on the MgADP state (34 nM). Secondly, they give an estimate of the binding affinity of the nucleotides for the cpn60–LDH complex. These affinities follow the same order as those in the absence of a protein substrate, i.e. MgATP > Mgp[NH]ppA > MgADP and, in the case of the latter two nucleotides, the presence of bound protein is seen to weaken the interaction over that seen in its absence (Jackson et al., 1993), in line with their ability to ‘compete’ with protein binding. In the MgATP experiments, data at sufficiently low concentrations of nucleotide to determine its apparent affinity were unattainable since, at submillimolar concentrations, the MgATP would be exhausted within the time required to refold the substrate.

DISCUSSION

There are two major models to account for the ability of chaperonins to increase the yield of native protein during folding. Neither requires specificity in the interaction with the folding protein, thus either is able to account for the extraordinary diversity of substrates on which chaperonins act.

One model proposes that the role of chaperonins is to sequester ‘sticky’ protein-folding intermediates from the bulk phase, thereby preventing their irreversible aggregation (Buchner et al., 1991). An inevitable consequence of this mechanism is that the

interaction of chaperonins with folding intermediates will slow down the rate of folding in proportion to the decrease in free concentration of these intermediates (Jackson et al., 1993). As a result, a large increase in the yield of native material in the presence of chaperonins will be accompanied by a large reduction in the rate of folding. In several cases this reciprocal relationship between folding efficiency and folding rate is not observed. This is particularly well demonstrated by Buchner et al. (1991) who show that, in the case of citrate synthase, the kinetics of formation of the active enzyme are unaffected by the presence of chaperonins and MgATP in experiments where the improvement in yield is 10-fold. This has also been seen in experiments with malate dehydrogenase where the yield is improved 3-fold whereas the kinetics are unchanged (Miller et al., 1993). Therefore the action of the chaperonins cannot be solely due to passive binding of the unfolded protein states. A version of this model in which the folding protein is encapsulated in the central cavity would not necessarily result in slow folding, as the molecule could have little or no interaction with chaperonin surfaces. However, recent data (Horwich et al., 1993) suggest that protein substrates are too large for this to be possible.

A second model proposes that chaperonins have the ability to unfold misfolded states and thus allow another chance to refold to the native structure. Here the energy of MgATP hydrolysis is used to cycle the chaperonin between states that have alternatively high and low affinities for an unfolded substrate and so produce a ‘binding–unfolding–release’ mechanism (Hubbard and Sander, 1991; Jackson et al., 1993).

In either case, it is important to measure the affinity of interaction between the binding surface on cpn60 and the protein substrate. In the first mechanism, binding is responsible for the sequestration of the protein to prevent aggregation, and in the second it is used to stabilize more unfolded states. As it is known that nucleotide interactions with cpn60 modulate this interaction, it appears that a key to understanding the requirement for MgATP binding and hydrolysis in chaperonins is to define the protein–protein binding affinity at each stage of the hydrolytic cycle.

Table 1 shows the LDH–cpn60 dissociation constants extracted from numerical analysis of the refolding curves shown in Figures

1, 2 and 3. The most obvious feature is that in all cases the chaperonin binds tightly to the first two states in the LDH folding pathway and only very weakly to the third. This accords with previous conclusions that chaperonins bind tightest to the least folded states (Badcoe et al., 1991; Gray and Fersht, 1993). It also confirms the finding that when LDH is unfolded only as far as the productive 'molten globule' state and then allowed to refold, it does not interact detectably with cpn60 (Badcoe et al., 1991; Burston et al., 1992). A good discussion of the state of folding of cpn60 substrates is given by van der Vies et al. (1992); as all folding intermediates are in equilibrium and often rapidly interchangeable, it is difficult to establish whether at any one time cpn60 is binding to the major or the minor species. There are reports of proteins binding in 'molten globule' states (Goloubinoff et al., 1989; Martin et al., 1991) or from more folded states even (Lamiet et al., 1990; Martin et al., 1992), but the general trend is that cpn60 has greater affinity for the more unfolded states.

It is evident that different proteins show marked differences in their affinities for chaperonins [for examples, see Martin et al. (1991), Mizobata et al. (1992) and Kubo et al. (1993)]. One extreme would include proteins such as rhodanese and ribulose biphosphate carboxylase (rubisco) which can only be dissociated from cpn60 by the addition of both MgATP and cpn10 (Goloubinoff et al., 1989; Martin et al., 1991). The other extreme is tryptophanase from *E. coli* which dissociates readily from cpn60 by the addition of MgADP alone (Mizobata et al., 1992). These studies with LDH, in which we can observe and compare dissociation from any chaperonin state, suggest that a tightly bound protein may require the 63-fold weakening brought about by MgATP and cpn10 whereas a weakly bound one may only require the 4-fold weakening induced by MgADP.

In saturating the protein-displacement effect, the nucleotides follow the same pattern as their binding affinity, i.e. MgATP > MgppA > MgADP (Jackson et al., 1993), and in the case of MgADP complexes the inclusion of orthophosphate has no measurable effect on its properties. This picture, however, is slightly complicated by the asymmetry of the cpn60–cpn10 complex which produces two classes of nucleotide site. Both MgADP and MgATP can support the formation of the cpn60–cpn10 complex, but once MgADP is bound to the cpn10 side of the complex (either by direct association or *in situ* by MgATP hydrolysis) it is extremely stable (Jackson et al., 1993; Todd et al., 1993). From this we assume that, in the presence of cpn10, these seven sites are occupied with MgADP in the steady-state hydrolysis of MgATP and at all equilibrium MgADP concentrations used in the experiments reported here. The corollary of this argument is that these sites are 'silent' and the nucleotide-concentration-dependences we quote here are for binding events at the open end of the complex. The results shown in Table 1 demonstrate that MgADP binding at low concentrations, but sufficient to saturate the cpn10 side, does not measurably lower the protein-binding affinity in comparison with apo-cpn60. In this regard it is important to note that, for both MgATP and its analogue, the inclusion of cpn10 potentiates the action of the nucleotide. It is clear that, for some proteins (e.g. mitochondrial malate dehydrogenase, rhodanese), this extra stimulus is necessary to trigger release and folding. However, for other presumably more weakly bound proteins (e.g. tryptophanase, LDH), cpn10 is not required for release or to enhance folding yields. Given these observations, it cannot be said that cpn10 is an obligatory component of the chaperonin mechanism.

The major question is how do these modulated chaperonin–protein-binding affinities help us understand the mechanism of action and biological activity? In order to attempt to answer this,

Figure 5 Changes in the binding affinity for unfolded LDH during the MgATP-hydrolytic cycle of the cpn60–cpn10 complexes

The cpn60 oligomer is asymmetric when associated with cpn10 and the available evidence suggests that MgADP is so tightly bound at the cpn10 side of the complex that it will not exchange during the catalytic cycle (Jackson et al., 1993). The cycle thus shows hydrolysis occurring at only one side of the oligomer and it is with this side that substrate proteins associate. The affinity of each complex in the ATPase cycle for the unfolded LDH molecules is given in parentheses and represented by the vertical lines between the complex and its substrate.

the results should be viewed in relation to the conditions within the cell.

The initial conclusion is that, in the cell with a MgATP concentration of about 8 mM (Lehninger, 1982), the cpn60–cpn10 complex will be in the lowest-affinity MgATP-saturated form [K_i for MgATP is 8 μ M and hydrolysis is the rate-determining step (Jackson et al., 1993)]. This refers to the 'open' end of the complex since, as stated above, the end to which cpn10 is bound is 'silent'. The apparent K_d for unfolded LDH in this state is 0.44 μ M. On association with an unfolded substrate protein, MgATP hydrolysis is stimulated (Martin et al., 1991; Jackson et al., 1993) to generate the MgADP form. In the case of LDH this tightens the interaction 13-fold (or 6.7 kJ/mol). As the cellular concentration of MgADP is about 1 mM (Lehninger, 1982) and the K_i describing the MgADP interaction is 2.5 mM (Jackson et al., 1993), the MgADP will be readily exchanged with MgATP. During this exchange there is a transient 'apo' state with no nucleotide bound at the open end which has an even higher protein affinity; $K_d = 7$ nM (or 10.5 kJ/mol more stable than in the MgATP state). This cycle is summarized in Figure 5.

The observations show that, at least in part, the energy derived from MgATP hydrolysis is used to alter substrate-binding affinity through the stabilization of different chaperonin conformers. The question remains as to whether these observations help discriminate between a 'suppression-of-aggregation' or a 'recycling' model. We suggest that the properties of chaperonins make it inevitable that both of these activities are integral parts of the mechanism of enhanced folding. Unfortunately, the affinities reported here do not contain any information as to the physical properties of protein substrate bound to cpn60. 'On-chaperonin' folding has been measured in the case of barnase by Gray and Fersht (1993) but the rate is so small that its mechanistic significance is as yet unclear. An interesting argument in its favour is that misfolding/aggregation of proteins such as rhodanese can be almost totally prevented in the presence of chaperonins, suggesting 'on-chaperonin' folding to at least a state that does not aggregate (folding efficiency can approach 100% in the presence of chaperonins when in its absence there is a strong tendency to misfold or aggregate). However, the assisted

Figure 6 Schematic free-energy profiles for spontaneous and chaperonin-assisted protein folding

Each profile shows a hypothetical protein-folding pathway in free solution (A), on the surface of a weak-binding complex (e.g. cpn60–cpn10–MgATP) (B) and on the surface of a tight-binding complex [e.g. apo-cpn60 or cpn60–MgADP(–cpn10)] (C). State 3 is a fully unfolded protein which, when placed in native conditions, can either partially fold to form a population of productive intermediates (state 4) which are then able to fold further to the correct native state of the protein (state 5) or spontaneously misfold to an unproductive structure (state 2) which usually coalesces to form insoluble aggregates (state 1). In each of the cases presented in profiles A, B and C, the rate-determining step of folding is from state 4 to state 5. As aggregation does not occur on the surface of the chaperonin (profiles B and C), the chaperonin functions by stabilizing the unfolded protein (state 3) in order to reduce the energy barrier between the misfolded state 2 and the productive intermediate (state 4). This mechanism is discussed fully in the text.

folding of LDH is less successful (when the conditions are optimized, a maximum efficiency of 50% is obtained) and may require other factors suggesting that the released protein may still be in a state prone to aggregation. The degree of 'on-chaperonin' folding may therefore vary with protein substrate. We suggest that the properties of chaperonins make it inevitable that both the proposed activities are integral parts of the mechanism of enhanced folding.

The argument is illustrated in Figure 6 which shows three schematic profiles of the energetics of protein folding in free solution (profile A), on the surface of the weak-binding chaperonin–MgATP complex (profile B) and on the surface of the tight-binding apo-chaperonin or MgADP complex (profile C). On each profile are represented five states of the substrate protein. The unfolded protein (state 3) can fold rapidly to a productive intermediate (state 4) or can misfold (state 2). Both these states may have the general properties of the 'molten globule' but only the misfolded state will have high affinity for cpn60 (Badcoe et al., 1991). Proteins that fold efficiently will preferentially populate productive states, and those that do not will populate misfolded states. From the misfolded state(s) the protein can either aggregate (state 1) or isomerize to a productive intermediate (state 4) via a more unfolded conformation. The energetic barrier to this latter process may be high thus predisposing the protein to aggregation. This behaviour is exemplified by LDH which, when unfolded to a 'molten globule'-like intermediate state, refolds efficiently but when fully denatured refolds poorly (Badcoe et al., 1991).

In the presence of chaperonin–MgATP, a spontaneously misfolded protein (2,A) (i.e. Figure 6, state 2, profile A) can associate to form (2,B) so preventing the formation of aggregates (1,A). Hydrolysis of MgATP then produces state (2,C). The

increased affinity will then allow spontaneous unfolding to state (3,C). The problem with this state is that it may be so tightly bound as to produce a large barrier to folding. However, reassociation of MgATP (to generate 3,B) will weaken the interaction to allow spontaneous folding to a productive intermediate 'on-chaperonin' (4,B) or 'off-chaperonin' (4,A). From the present results, we cannot distinguish between these two possibilities. In either case the chaperonin has used the energy of MgATP hydrolysis to drive the formation of productive intermediates and has sequestered the reaction to prevent aggregation of the folding protein molecules; such a combination of effects, which are intrinsic to chaperonin behaviour, may provide a general explanation for the coupling of MgATP hydrolysis to improved protein folding in the cell.

R.A.S. acknowledges personal support from Porton Industries. A.R.C. is the recipient of a fellowship from the Lister Institute, London, U.K. This work was supported by a grant from the Wellcome Trust.

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Received 10 November 1993/13 January 1994; accepted 20 January 1994