Escherichia coli chaperonins Cpn60 (groEL) and Cpn10 (groES) do not catalyse the refolding of mitochondrial malate dehydrogenase

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In vitro refolding of pig mitochondrial malate dehydrogenase is investigated in the presence and absence of *Escherichia coli* chaperonins cpn60 (groEL) and cpn10 (groES). The refolded yields of active malate dehydrogenase are increased almost 3-fold in the presence of groEL, groES, Mg²⁺/ATP and K⁺ ions. Chaperonin-assisted refolding of malate dehydrogenase does not have an absolute requirement for K⁺ ions but Mg²⁺/ATP is obligatory. When ATP is replaced by other nucleoside triphosphates, or by non-hydrolysable ATP analogues, assisted

refolding is prevented. Optimal chaperonin-assisted refolding requires both groEL and groES homo-oligomers in molar excess over malate dehydrogenase. Kinetic analysis shows that the chaperonins do not catalyse the refolding of malate dehydrogenase but increase the flux of unfolded enzyme through the productive refolding pathway without altering and/or accelerating that pathway. Although not acting as refolding catalysts, the chaperonins are able to assist at least six consecutive cycles of malate dehydrogenase refolding.

INTRODUCTION

Chaperonins are a subclass of the molecular chaperones which are a ubiquitous, abundant and highly conserved group of proteins which assist protein folding in cells (Ellis and van der Vies, 1991; Gething and Sambrook, 1992). Chaperonins first came to attention because of their specific induction during the cellular response of all organisms to heat shock (Morimoto et al., 1990; Schlesinger et al., 1990) but are now known to be constitutively and abundantly expressed in the absence of any stress. Of all the chaperonins currently characterized, the best-known are the *Escherichia coli* chaperonins cpn60 (groEL) and cpn10 (groES). Both are structurally quite well characterized. GroEL is a 14 subunit homo-oligomer composed of two stacked rings of seven subunits each (Hendrix, 1979), while groES most probably consists of seven subunits arranged in a single ring (Chandrasekhar et al., 1986). Subunit molecular masses derived from the gene-derived amino acid sequences of groEL and groES are 57259 Da and 10368 Da respectively (Hemmingsen et al., 1988).

Until recently, little was known of the way chaperonins assist protein folding but recent studies on chaperonins groEL and groES have begun to probe the mechanism of chaperonin intervention in protein folding. In particular, a number of well-defined *in vitro* systems have been devised to study chaperonin-assisted refolding of well-characterized enzymes using the *E. coli* chaperonins. These include studies on the refolding of chemically denatured ribulose-1,5-bisphosphate carboxylase (Rubisco) (Goloubinoff et al., 1989; Viitanen et al., 1990; van der Vies et al., 1992), pre- β -lactamase (Laminet et al., 1990; Zahn and Pluckthun, 1992), citrate synthase (Buchner et al., 1991), lactate dehydrogenase (LDH) (Badcoe et al., 1991), rhodanase (Martin et al., 1991; Mendoza et al., 1991) and mouse dihydrofolate reductase (DHFR) (Martin et al., 1991; Viitanen et al., 1991). However, in spite of these studies, much of the mechanistic detail of chaperonin involvement in protein folding remains elusive. In

particular, it has still not been established whether or not chaperonins are catalysts of protein folding.

In an effort to understand the underlying chemical mechanism of chaperonin-assisted refolding of proteins, we have also been developing a number of model *in vitro* refolding systems using the *E. coli* chaperonins. In this paper we report results obtained while studying the chaperonin-assisted refolding of pig mitochondrial malate dehydrogenase (mMDH). Pig mMDH was chosen as the substrate for an *in vitro* refolding model system because the enzyme is structurally characterized (Roderick and Banaszak, 1986), the refolding pathway for the enzyme has already been studied in detail (Jaenicke et al., 1979) and the enzyme activity is readily determined by a simple spectrophotometric assay.

Results described in this paper show that the chaperonins should not be regarded as refolding catalysts, but as passive binding proteins which inhibit non-productive folding and aggregation reactions without substantially altering or accelerating the productive folding pathway itself.

EXPERIMENTAL

Materials

Bactotryptone and Bactoyeast extract were from Difco Laboratories, East Molsey, Surrey, U.K. Purification media Sephacryl S-300 and Q-Sepharose were obtained from Pharmacia LKB Biotechnology, Milton Keynes, Bucks., U.K. mMDH from pig heart was purchased from Boehringer-Mannheim U.K., Lewes, East Sussex, U.K. All other chemicals were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Deionized distilled water was used throughout.

Enzyme and protein assays

mMDH homodimer concentrations were calculated from $A_{280}^{1\%} = 2.5$ (Gregory et al., 1971) and a subunit molecular mass of 35 kDa

Abbreviations used: groEL, chaperonin 60 (cpn60); groES, chaperonin 10 (cpn10); Rubisco, ribulose-1,5-bisphosphate carboxylase; DHFR, dihydrofolate reductase; LDH, lactate dehydrogenase; p[NH]ppA, adenosine 5'-[β , γ -imido]triphosphate; 2-ME, 2-mercaptoethanol; mMDH, mitochondrial malate dehydrogenase.

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(Thorne and Kaplan, 1963). Concentrations of *E. coli* groEL and groES were evaluated using absorbance coefficients at A_{280} of $2.38 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for groEL and $3.44 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for groES (Viitanen et al., 1990).

Buffer solutions

pH values for buffer solutions were adjusted at room temperature, irrespective of the temperature at which they were subsequently used.

Plasmids

pAM1 was constructed by inserting a 2.2 kb *EcoRI*–*HindIII* fragment of plasmid pOF39 (Fayet et al., 1986), containing the *GroE* operon under control of a heat-shock promoter, into pBS(Bluescribe) (C. Lichtenstein and A. Moghaddam, unpublished work). pAM1 contains both ampicillin- and tetracycline-resistance markers.

Organisms

E. coli strain TG2 [*supE*, *hsd* Δ 5, *thi* Δ (*lac-proAB*), Δ (*srl-recA*)-306::Tn10(*ter'*)] was transformed using standard protocols (Sambrook et al., 1989) with plasmid pAM1 to provide a recombinant strain TG2/pAM1 which overexpressed both groEL and groES.

Growth of *E. coli*

Strain TG2/pAM1 was grown at 37 °C in 2YT medium containing ampicillin (100 mg/l). A portion (10 ml) of an over-night shake culture was used to inoculate 5 l of growth medium in a fermentation tank and the cells were further incubated at 37 °C while being stirred at 350 rev./min. Once the attenuation (D_{600}) had reached 0.5, the growth temperature was raised to 42 °C and incubation continued until the cell suspension had reached saturation (D_{600} of approx. 3.0). Cells were collected by centrifugation (10800 g, 20 min) and, if not used immediately, were stored at –20 °C. A typical 5 l culture gave 28 g wet wt. of cells.

Purification of groEL and groES

All solutions used during the purification of the chaperonins contained the following additions, unless otherwise stated: 2 mM 2-mercaptoethanol (2-ME), 2 mM EDTA, 0.6 mM phenylmethanesulphonyl fluoride (previously dissolved in ethanol, 5 ml/l of final solution), 0.6 mM benzamidine, aprotinin (1 mg/l) and pepstatin (1 mg/l).

Operations were performed at 0–4 °C, except for the f.p.l.c. steps which were carried out at ambient temperature (approx. 19 °C), but fractions from these columns were collected on ice. Both the Sephacryl S-300 and Q-Sepharose purification steps described below were performed with columns attached to a Pharmacia f.p.l.c. system and absorbance of the eluate was continuously monitored at 280 nm.

The purity of groEL and groES at each stage of the purification process was assessed by SDS/PAGE using 15 % (w/v) acrylamide gels according to procedures described by Sambrook et al. (1989).

Washed cells (thawed if previously frozen) were resuspended in 50 mM Tris/HCl buffer, pH 7.5, containing 0.1 M NaCl

(approx. 1 ml/g wet wt. of cells). After the addition of DNAase (0.1 mg/ml) and 2 mM MgCl_2 , cells were lysed by a single passage through a French press [cell pressure 7570 kPa (1100 p.s.i.)]. Cell debris was removed by centrifugation (26000 g, 30 min).

A 0.2 vol. of 6.2 % (w/v) streptomycin sulphate solution was added dropwise to the supernatant with stirring. After stirring for a further 15 min, the precipitate was removed by centrifugation (26000 g, 30 min). The supernatant was then fractionated with $(\text{NH}_4)_2\text{SO}_4$. Three stages of saturation from 0–10 %, 10–30 % and 30–60 % were used. Protein precipitating in the final fraction was collected by centrifugation (26000 g, 30 min) and then dissolved in a minimum volume of 50 mM Tris/HCl buffer, pH 7.5/0.1 M NaCl.

The solution was fractionated on a column (5.0 cm $\times$ 60 cm) of Sephacryl S-300 equilibrated with the last-mentioned buffer. Fractions (20 ml) were collected at a flow rate of 10 ml/min. Fractions containing groEL and groES were identified by SDS/PAGE and then separately combined. Both groEL and groES were further purified by ion-exchange chromatography using a column of Q-Sepharose (5.0 cm $\times$ 15 cm) previously equilibrated with 50 mM Tris/HCl buffer, pH 7.5/0.1 M NaCl. Elution was performed with a linear gradient of 0.1–0.5 M NaCl in a gradient volume of 840 ml. Fractions (20 ml) were collected at a flow rate of 10 ml/min. Fractions containing groEL and groES were combined separately, concentrated to 10 ml using a Flowgen N_2 -pressure concentrator (3 K membrane), and then dialysed into 50 mM Tris/HCl, pH 7.5/2 mM 2-ME (without other additives described above) and supplemented with 20 % (v/v) glycerol. Chaperonin solutions were then stored at 4 °C or –20 °C prior to use.

mMDH renaturing experiments

GroEL and groES were prepared for use by dialysis against 150 mM sodium phosphate (pH 7.6) containing 2 mM 2-ME and 1 mM EDTA at 4 °C. A stock solution of mMDH (approx. 2.5 mg/ml) was prepared in this buffer in a similar way.

An aliquot of mMDH stock solution was diluted to an enzyme concentration of approx. 0.3 mg/ml (4.3 μM dimer concentration) in 150 mM sodium phosphate, pH 7.6, containing 20 mM 2-ME, 10 mM EDTA and 6 M guanidinium hydrochloride. This solution was then incubated at 20 °C for at least 2 h so as to fully denature the mMDH. Renaturation of mMDH was performed by diluting denatured mMDH to a concentration of 10 $\mu\text{g/ml}$ (143 nM dimer concentration, 30-fold dilution) in a variety of renaturing buffers and incubating the resulting solutions for several hours at 20 °C. Renaturing buffers (1 ml) were typically composed of 150 mM sodium phosphate, pH 7.6, containing 20 mM 2-ME, 10 mM MgCl_2 , 10 mM KCl, 2 mM ATP and various amounts of groEL and groES. Fixed aliquots (20 μl) of renaturing mixtures were removed at defined times and mixed with aliquots of an assay buffer (980 μl) pre-incubated at 30 °C. Assay buffer consisted of 150 mM sodium phosphate, pH 7.6, 2 mM 2-ME, 0.5 mM oxaloacetate and 0.2 mM NADH. The initial rate of conversion at 30 °C of NADH into NAD^+ [determined by the initial decrease (absorbance units/min) in the A_{360} of assay mixtures] was used as a measure of mMDH reactivation during refolding. The activity of the refolding mMDH was expressed as a percentage relative to the activity of a control sample of undenatured mMDH (143 nM dimer concentration) incubated at 20 °C in a buffer of 150 mM sodium phosphate, pH 7.6, containing 20 mM 2-ME, 10 mM MgCl_2 , 10 mM KCl, and 2 mM ATP.

RESULTS AND DISCUSSION

A necessary prerequisite of mechanistic studies on chaperonin-assisted refolding of proteins is a reliable supply of pure chaperonins. Both groEL and groES are coded for by the *groE* operon (Fayet et al., 1986) which had previously been subcloned into plasmid pAM1 (C. Lichtenstein and A. Moghaddam, unpublished work).

Figure 1 Time course of chaperonin-assisted and spontaneous refolding of mMDH

mMDH was denatured as described in the text. Refolding was initiated by diluting mMDH to a concentration of 143 nM (dimer concentration) in renaturing buffer which either contained chaperonins (□), or from which chaperonins were absent (▲). The composition of renaturing buffer is described in the text but where appropriate (□) contained in addition groEL (858 nM homo-oligomer concentration) and groES (1716 nM homo-oligomer concentration). After the addition of mMDH, the mixtures were incubated at 20 °C and, at the times indicated, aliquots were removed and assayed for mMDH activity. The mMDH activity was expressed as a percentage of the activity of native non-denatured enzyme (143 nM dimer concentration) incubated in renaturing buffer.

Figure 2 The mechanism of chaperonin-assisted refolding of mMDH

The experiments were performed in a similar way to experiments described in the legend for Figure 1 and in the text. The illustrated curves show the effect of diluting denatured mMDH, to a concentration of 143 nM (dimer concentration), in renaturing buffer containing either groEL and groES (●) (homo-oligomer concentrations of 858 nM and 1716 nM respectively), groEL (◇) (homo-oligomer concentration 858 nM), or groEL (homo-oligomer concentration 858 nM) but without ATP in the renaturing buffer (■). In one further experiment (□), denatured mMDH was diluted into renaturing buffer containing groEL (homo-oligomer concentration 858 nM) and after incubation for 45 min, groES was added (final homo-oligomer concentration 1716 nM) as indicated by the arrowed position. Spontaneous refolding of mMDH, in renaturing buffer, was also observed (▲).

published work). *E. coli* strain TG2 was transformed with pAM1 to give a recombinant strain *E. coli* TG2/pAM1. Optimal yields of chaperonin were obtained when cells were grown to mid-logarithmic phase (D_{600} approx. 0.5) at 37 °C and then further grown to saturation under heat-shock conditions (42 °C). Heat-shock growth conditions were used because plasmid pAM1 contains the *GroE* operon under control of the heat-shock promoter. Following cell growth, essentially homogeneous groEL and groES were purified (as determined by SDS/PAGE) with respective yields of approx. 76 mg and 15 mg per litre of cell culture. Having developed a reliable source of large amounts of chaperonins, studies on the chaperonin-assisted refolding of proteins were possible.

The experimental conditions, under which the refolding of mMDH was investigated, were devised as a compromise between the conditions used by Viitanen et al. (1990) in the studies on chaperonin-assisted refolding of Rubisco and those conditions used by Jaenicke et al. (1979) for studying mMDH refolding in the absence of chaperonins. In initial experiments, it was apparent that the refolded yields of active mMDH were substantially higher in the presence of groEL and groES than in their absence. Indeed, chaperonin-assisted refolding of mMDH resulted in at least 90 % recovery of active enzyme, as compared with approx. 30 % recovery from spontaneous refolding in the absence of chaperonins and ATP (Figure 1). The concentration of mMDH in the renaturing buffer during all these initial studies was 143 nM (dimer concentration). Either increasing or decreasing this concentration failed to improve the yield of refolded mMDH in the presence of the chaperonins. Successful refolding of mMDH also required that the renaturation buffers contain phosphate and not Tris/HCl [in contrast with Viitanen et al. (1990)] and that 2-ME be present throughout as reducing agent. A similar requirement for reducing agent was noted by Mendoza et al. (1991) in their studies on rhodanase refolding.

A general scheme for the action of groEL and groES in refolding proteins has been outlined by Georgopoulos and Ang (1990). In this scheme, the unfolded protein binds to the large chaperonin groEL and then a combination of groEL-catalysed ATP hydrolysis and groES binding to groEL serves to promote protein refolding and/or subsequent release of the correctly folded protein from groEL. These studies on chaperonin-assisted refolding of mMDH closely support this general scheme. In the presence of groEL alone, without ATP present, there was no measurable recovery in mMDH activity with time at all, presumably because mMDH has all become bound to groEL (Figure 2). With groEL and ATP together, a small recovery (< 5%) of mMDH activity was observed with time. However, when unfolded mMDH was combined with groEL and ATP followed by the subsequent addition of groES (after 45 min) a dramatic increase in the recovery of mMDH activity was observed. Indeed the recovery of mMDH activity paralleled, without loss in the yield of active mMDH, results obtained when unfolded mMDH was combined directly with groEL, groES and ATP together (Figure 2). Hence groEL, groES and ATP are all required for successful assisted refolding of mMDH. The reason that a modest amount of mMDH reactivation was observed with groEL and ATP alone may be because a small amount of the purified groEL is phosphorylated (Sherman and Goldberg, 1992) so removing the requirement for groES to bring about the release of groEL-bound protein. Alternatively groEL may contain a small amount of groES contamination. It has been shown that groES is not an absolute requirement for chaperonin-assisted refolding but apparently acts to improve the efficiency of the process (Lamiet et al., 1990; Buchner et al., 1991; Martin et al., 1991; Viitanen et al., 1991). However, in the instance of chaperonin-

Figure 3 The percentage of unfolded mMDH that refolds is dependent on the ratio of groES to groEL

Denatured mMDH was diluted to 143 nM (dimer concentration) in renaturing buffer containing groEL (858 nM homo-oligomer concentration) and various amounts of groES homo-oligomer as indicated. After 2 h incubation at 20 °C, the mixtures were assayed for mMDH activity.

Figure 4 The percentage of unfolded mMDH that refolds is dependent on the ratio of groEL to mMDH

Denatured mMDH was diluted to 143 nM (dimer concentration) in renaturing buffer containing various amounts of groEL homo-oligomer as indicated. GroES homo-oligomer was also present in each case at a concentration twice that of the respective groEL concentration. After 2 h incubation at 20 °C, the mixtures were assayed for mMDH activity.

assisted refolding of mMDH, groES must be considered an absolute requirement since the efficiency of assisted refolding is so low in the absence of groES (Figure 2).

Further studies with mMDH revealed that the chaperonin-assisted refolding was optimum when the groEL and groES homo-oligomers were used in at least a stoichiometric mole ratio with respect to each other (Figure 3). The preferred groEL/groES homo-oligomer mole ratio in all the experiments described in this paper was 1:2 which conferred a slight improvement over the 1:1 ratio. Following on from this experiment, mMDH refolding was observed in the presence of both excess and substoichiometric amounts of the chaperonins relative to the mMDH homodimer (Figure 4). Excess chaperonin was found to be essential for optimal yields of active mMDH, with the best yields of refolded, active mMDH obtained using a 6-fold molar excess of groEL homo-oligomer (together with a 12-fold molar excess of groES homo-oligomer) over mMDH homodimer. Further

Table 1 Influence of various factors on chaperonin-assisted refolding of mMDH

Denatured mMDH was diluted to a concentration of 143 nM (dimer concentration) in renaturing buffer containing groEL (858 nM homo-oligomer concentration) and groES (1716 nM homo-oligomer concentration). All other components, unless omitted, were as described in the text. After incubation for 2 h at 20 °C the percentage recovery of mMDH activity was recorded.

Missing component	Added component	Recovery of mMDH activity after incubation for 2 h at 20 °C (%)
None	None	90
K ⁺ ions	None	70
ATP	None	0
ATP	GTP*	0
ATP	CTP*	0
ATP	p[NH]ppA*	0
ATP and groES	p[NH]ppA*	0

* Nucleotides included a concentration of 2 mM.

increases in the relative amounts of the chaperonins produced little further improvement. Similar observations have been made by Mendoza et al. (1991) in their studies on rhodanase refolding. There is some debate over the appropriate value of the absorbance coefficient for groEL (Fisher, 1992) so it must be emphasized that calculations of groEL concentrations here are based upon the absorbance coefficient determined by quantitative amino acid analysis (Viitanen et al., 1990; Zahn and Pluckthun, 1992).

Investigations into the cofactor requirements of chaperonin-assisted refolding of mMDH revealed (Table 1) that K⁺ is not obligatory, as has been observed in other *in vitro* folding systems (Viitanen et al., 1990), but ATP is essential. When ATP was substituted for by GTP or CTP, no active mMDH was formed. A similar observation was made when ATP was substituted for by the non-hydrolysable ATP analogue adenosine 5'-[β,γ -imido]triphosphate (p[NH]ppA). Hence ATP hydrolysis is essential for chaperonin-assisted refolding of mMDH. This latter result differs from results obtained during studies on the refolding of LDH (Badcoe et al., 1991) and DHFR (Viitanen et al., 1991) which show that p[NH]ppA is able to promote release of groEL-bound protein in an active form. At this stage there is no clear reason for the difference between these published observations and the results obtained here with mMDH.

In order to determine whether the chaperonins were changing the rate of mMDH refolding or not, a kinetic analysis of representative time course refolding data (see Figure 1) for chaperonin-assisted and spontaneous refolding of mMDH was carried out. In this analysis, an attempt was made to fit the observed refolding data using a simple kinetic model developed to explain the spontaneous refolding of mMDH by Jaenicke et al. (1979). Jaenicke et al. (1979) have suggested that the spontaneous renaturation and reactivation of chemically denatured mMDH can be described via an irreversible consecutive transconformation-association process according to the minimum scheme in eqn. (1) (where N and N' are native enzyme and an inactive dimeric intermediate respectively; M and M' are two conformational states of catalytically inactive mMDH monomers).

Figure 5 Curve fitting of representative time-course results for chaperonin-assisted and spontaneous refolding of mMDH

Using the mathematical approximation of a uni-bimolecular reaction sequence (Chien, 1948), two curves were calculated in which the concentration of monomeric folding intermediate M [see eqn. (2)] was taken to be 260 nM (○) and 94 nM (▲) respectively. Rate constants of $k_1 = 6.5 \times 10^{-4} \text{ s}^{-1}$ and $k_2 = 3 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, obtained by Jaenicke et al. (1979) for spontaneous refolding of mMDH, were also used to derive both curves. Representative time-course results obtained, as described in the legend to Figure 1 and in the text, by observing the reactivation of denatured mMDH (143 nM dimer concentration) in renaturing buffer containing chaperonins (●, ■) or from which chaperonins were absent (△, □) were plotted alongside these theoretical curves for comparison.

Figure 6 The observation of several cycles of chaperonin-assisted refolding of mMDH

Denatured mMDH was diluted to 143 nM (dimer concentration) in renaturing buffer containing groEL (143 nM homo-oligomer concentration) and groES (286 nM homo-oligomer concentration). The mixture was then incubated at 20 °C. Every 30 min over a period of 3 h further aliquots of denatured mMDH (of equal quantity to the first addition) were added to the renaturing buffer as indicated by the arrowed positions. At the indicated times, aliquots were removed and assayed for mMDH activity. The mMDH activity was expressed as a percentage of the activity of native non-denatured enzyme (858 nM dimer concentration) incubated in renaturing buffer.

Jaenicke et al. (1979) also suggested that the sigmoidal character of the kinetic traces for the time-dependent spontaneous recovery of mMDH activity (see Figure 1) indicate that the reactivation reaction cannot be determined by only one single rate-limiting step. Hence two alternative irreversible, consecutive reaction sequences, involving two rate-limiting steps, were

proposed so as to describe the observed kinetic behaviour [eqns. (2) and (3)]. In this instance, irreversibility only implies that the reverse reactions have rate constants much below those for the forward reactions.

Although both alternative pathways are plausible, Jaenicke et al. (1979) found that the consecutive uni-bimolecular reaction process [eqn. (2)] was sufficient to describe both the sigmoidicity and the concentration-dependence of the spontaneous reactivation process. Using a mathematical approximation of the consecutive uni-bimolecular reaction determined by Chien (1948), Jaenicke et al. (1979) derived one set of kinetic constants, $k_1 = 6.5 \times 10^{-4} \text{ s}^{-1}$ and $k_2 = 3 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, which was sufficient to describe the reactivation behaviour of mMDH over the full range of concentrations used.

All our foregoing studies on chaperonin-assisted and spontaneous refolding of mMDH were performed under the same conditions of temperature and pH as the original refolding studies of Jaenicke et al. (1979). Unsurprisingly therefore, representative time-course results obtained for spontaneous refolding of mMDH agree closely (Figure 5) with results calculated using the mathematical approximation (Chien, 1948) for the consecutive uni-bimolecular reaction [eqn. (2)] and using the values of k_1 and k_2 mentioned above. The calculated curve also assumes an initial concentration of 94 nM for the putative folding intermediate M [see eqn. (2)] which, when carried through the irreversible folding pathway [eqn. (1)], would result in a final concentration of 47 nM for native dimeric enzyme (N). Starting from an initial concentration of 143 nM (dimer concentration) for the unfolded enzyme, 47 nM corresponds to a 33% recovered yield of active enzyme which agrees with the experimental results (Figure 5). However, of considerable surprise was the finding that representative time-course results obtained for chaperonin-assisted refolding of mMDH also agree closely (Figure 5) with results calculated using the same mathematical approximation and values of k_1 and k_2 as above. In order to obtain this close fit, the only variable altered was the initial concentration of M [see eqn. (2)] which was changed to 260 nM. Following the same argument as above, this would result in a final concentration of 130 nM for the native dimeric enzyme (N) which corresponds to a 90% recovered yield of active enzyme, in agreement with the experimental results for chaperonin-assisted refolding of mMDH (Figure 5). Clearly the simple consecutive uni-bimolecular reaction scheme, used to describe spontaneous mMDH reactivation, applies equally well for the chaperonin-assisted reactivation. Moreover, a simple conclusion may be drawn from this result. The concentration of M represents the quantity of mMDH committed to entering the productive folding pathway leading to active enzyme. This quantity is three times higher for chaperonin-assisted refolding of mMDH than for the spontaneous process. Hence it appears that the chaperonins, groEL and groES, are promoting mMDH refolding merely by increasing the flux of unfolded enzyme through the productive refolding pathway and not by altering and/or accelerating that pathway. How general is this conclusion?

Recent studies on the chaperonin-assisted refolding of citrate synthase (Buchner et al., 1991) and pre- β -lactamase (Lamiet et al., 1990) have implied that refolding kinetics are unaffected by chaperonins and hence support the above conclusion. Moreover, recent work on chaperonin-assisted refolding of pre- β -lactamase

(Zahn and Pluckthun, 1992) has provided some evidence that the folding pathway of pre- β -lactamase is unaltered by the chaperonins. However, by contrast, it has been reported that chaperonins cause a rate acceleration of Rubisco refolding (Viitanen et al., 1990) and in other cases they have reportedly decreased the rate of protein refolding (Martin et al., 1991; Mendoza et al., 1991). Hence it is not possible to completely generalize the above conclusion to the chaperonin-assisted refolding of all other proteins.

The characteristics of the chaperonins thus far described are not those of folding catalysts. However, chaperonins were found to be able to turnover at least six times in the following experiment. Denatured mMDH was diluted to a final concentration of 143 nM (dimer concentration) in a renaturing buffer containing groEL homo-oligomer (143 nM) and groES homo-oligomer (286 nM). After 30 min, another mole equivalent of denatured mMDH was added to the renaturing buffer. Subsequently, four further mole equivalents of denatured mMDH were added to the renaturing buffer at 30 min intervals. Figure 6 shows the stepwise increase of mMDH activity resulting from the first three additions of denatured mMDH. The final concentration of mMDH, following these six sequential additions, was 858 nM (dimer concentration). Final mMDH activity was approx. 40% of that measured for a control sample of native mMDH at 858 nM (dimer concentration). Results with pre- β -lactamase (Zahn and Pluckthun, 1992) and homodimeric LDH (Badcoe et al., 1991) have shown that two polypeptide chains bind to the groEL homo-oligomer. Assuming the same is true of mMDH as well, then the refolding of mMDH observed in this experiment must arise from six sequential cycles of mMDH binding to groEL, refolding and release. In effect, each turnover of the chaperonin-assisted refolding process is about 85% efficient, a value which is in close agreement with the refolded yields of active mMDH found in the single turnover experiments (Figure 4).

Langer et al. (1992) have also demonstrated that groEL and groES may bring about successive rounds of protein refolding. In addition, they observed that groEL may act as a cellular catalyst of protein folding when working in collaboration with the *E. coli* chaperone DnaK and heat-shock proteins DnaJ and GrpE. The results of Langer et al. (1992) raise the possibility that groEL and groES may also increase the rate of mMDH refolding in the presence of DnaK, DnaJ and GrpE. With such a well-characterized model refolding system as mMDH refolding, it should be relatively easy to put this possibility to the test.

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