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The stability and hydrophobicity of cytosolic and mitochondrial malate dehydrogenases and their relation to chaperonin-assisted folding

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Abstract

mMDH and cMDH are structurally homologous enzymes which show very different responses to chaperonins during folding. The hydrophilic and stable cMDH is bound by cpn60 but released by Mg-ATP alone, while the hydrophobic and unstable mMDH requires both Mg-ATP and cpn10. Citrate equalises the stability of the native state of the two proteins but has no effect on the co-chaperonin requirement, implying that hydrophobicity, and not stability, is the determining factor. The yield and rate of folding of cMDH is unaffected while that of mMDH is markedly increased by the presence of cpn60, cpn10 and Mg-ATP. In 200 mM orthophosphate, chaperonins do not enhance the rate of folding of mMDH, but in low phosphate concentrations chaperonin-assisted folding is 3–4 times faster.

Key words: Chaperonin; Protein folding; Malate dehydrogenase

1. Introduction

The chaperonins, cpn60 and cpn10, have been demonstrated to aid the correct in vitro folding of denatured protein molecules [1–3] by increasing the yield of native protein over that of 'misfolded' or aggregated forms. This activity reflects their cellular function as constitutive molecular chaperones required for the folding and assembly of newly translated proteins and as heat-shock proteins which enable the cell to recover from conditions which may induce denaturation of existing protein components (for reviews, see [4–6]).

Chaperonin-60 is a large tetradecameric protein arranged in two, heptameric rings [7–9], each subunit having a molecular mass of 60 kDa and an ATPase activity. Chaperonin-10 is a co-protein which consists of a single, heptameric ring, each subunit having a molecular weight of 10 kDa [10]. Unfolded states of substrate proteins interact tightly with apo-cpn60, probably within the central cavity of the ring, with dissociation constants in the nanomolar range [11,12]. The binding of Mg-ATP weakens this interaction whilst hydrolysis and product release re-establishes it. Cpn10 can bind either to the Mg-ADP or Mg-ATP state but not to apo-cpn60 [10,13]. In most conditions it binds to just one side of the cpn60 oligomer to form an asymmetric complex [11], although during ATP turnover a proportion of the structures have cpn10 bound to both sides (M. Schmidt, personal communication). The presence of cpn10 in chaperonin-dependent refolding reactions facilitates the dissociation of bound protein substrates by potentiating the releasing effect of Mg-ATP [14] or by direct, competitive displacement [15] or both.

Substrate proteins have differing affinities for chaperonins and their nucleotide complexes. Some proteins dissociate quickly and spontaneously from apo-cpn60 [16], some require just Mg-ADP [17], others Mg-ATP or analogues [3,18] and some need both Mg-ATP and cpn10 [19,20]. The simplest explanation for this diversity of behaviour is that release depends on two factors; (i) the binding affinity between the unfolded protein and the chaperonin surface, and (ii) the free energy of folding (i.e. the stability) of the substrate protein. If the unfolded protein binds weakly and the folded state is very stable, then the substrate will be released easily; i.e. the energy of folding readily overcomes that of binding. If the unfolded protein binds tightly and the folded state is unstable, then displacement is more difficult.

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Abbreviations: mMDH, mitochondrial malate dehydrogenase; cMDH, cytosolic malate dehydrogenase; GdmCl, guanidinium chloride; cpn60, chaperonin 60; cpn10, chaperonin 10; P_i, orthophosphate.

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In this paper, we compare the folding requirements of two porcine malate dehydrogenase isoenzymes, one located in the cytosol of heart cells (cMDH), the other a mitochondrial enzyme (mMDH). The three-dimensional structure of both proteins has been determined [21–23] and, despite considerable sequence divergence, the subunit fold is the same. They also form dimers with homologous subunit interfaces.

Because of their similarity, mMDH and cMDH allow us to determine whether the secondary, tertiary and quaternary structural features of proteins are related to their affinity for the chaperonins and whether the free energy of folding influences this interaction. In addition, chaperonins are present at a high concentration in the mitochondrion where they assist in the folding of many proteins, but it is still somewhat unclear whether they have a general role in the cytosol: TCP-1, the cytosolic member of the chaperonin family, has as yet only been isolated in complexes with filamentous proteins such as tubulin and actin [24]. It is possible that these different environments should be reflected in the folding requirements of the two isoenzymes. The refolding reactions were monitored in the absence and presence of cpn60:Mg-ATP, cpn60:cpn10:Mg-ATP under a 'permissive' high concentration of inorganic phosphate (optimal folding conditions) and a more physiological but 'non-permissive' concentration of inorganic phosphate (poor folding conditions).

2. Materials and methods

2.1. Protein preparation

mMDH and cMDH from pig heart were obtained from Sigma. The chaperonins, cpn60 and cpn10, from *E. coli* were co-expressed in *E. coli* MC1061 cells from the pND5 plasmid [25]. Cells were cultured overnight at 37°C, exposed to 'heat shock' at 42°C for 1 h and grown for a further hour at 37°C before harvesting. The cells were resuspended in 50 mM triethanolamine hydrochloride (TEA) buffer at pH 7.5 containing 2 mM EDTA, and sonicated in order to obtain the cell extract. This was then treated with DNase (0.1%) in 5 mM $MgCl_2$ for 30 min before subsequently adding 7 mM EDTA, adjusting the pH to 8.5 and loading onto a Q-Sepharose ion-exchange chromatography column pre-equilibrated in 50 mM TEA, pH 8.5, and 2 mM EDTA. A 0–0.75 M NaCl 700 ml gradient was used to elute the chaperonins, cpn60 eluting at 0.6 M and cpn10 eluting at 0.4 M. Although at this point the cpn60 is 95% pure, greatly improved purity was obtained by adding 5 mM $MgCl_2$ and 2 mM ATP to the solution before re-loading on the Q-Sepharose column and re-purifying as above but in the presence of Mg-ATP. This removed any protein contaminants bound to the cpn60. The Mg-ATP was then removed by gel filtration (Sepharose cross-linked 6B). To obtain pure cpn10, the protein was equilibrated in 50 mM TEA, pH 8.5, and 2 mM EDTA buffer containing 1.5 M ammonium sulphate and loaded onto a phenyl-Sepharose column. The protein was eluted using a reverse-gradient from 1.5 to 0 M ammonium sulphate at a concentration of 0.5 M. Both chaperonins were stored in 70% ammonium sulphate at 4°C. Their concentrations were established as in [13].

2.2. Standard buffer conditions

The standard buffer conditions for all the experiments described in this paper except where stated is triethanolamine hydrochloride (TEA) 50 mM, pH 7.5, 20 mM $MgCl_2$, 50 mM KCl, 2 mM dithiothreitol (DTT). All experiments were performed at 25°C.

2.3. Denaturation experiments

The fluorescence intensities of solutions of 0.5 μM mMDH in different concentrations of the denaturant, guanidinium chloride (GdmCl), in the range 0–2 M, were recorded using an SLM spectrofluorometer (excitation λ = 278 nm and emission λ = 303 nm). The experiment was repeated in the presence of 2 mM citrate. The unfolding of cMDH was monitored in the same way using tryptophan fluorescence (excitation λ = 280 nm and emission λ = 340 nm).

The fluorescence data were fitted to the following relationship:

$$-RT \cdot \ln [(l_o - l_u)/(l_f - l_o)] = \Delta G_w + n \cdot \Delta G_{s,m} \cdot [D]/(K_{den} + [D])$$

where l_f and l_u are the fluorescence intensities of the folded and unfolded states, respectively, l_o is the observed intensity, ΔG_w is the free energy of folding in the absence of denaturant, n is the number of amino acid side chains becoming exposed to the solvent, $\Delta G_{s,m}$ (0.775 kcal/mol) and K_{den} (5.4 M) are denaturation constants for an average protein, and $[D]$ is the concentration of GdmCl [27]

2.4. Renaturation experiments

20 μM mMDH and cMDH were first denatured in a solution of standard buffer containing 4 M GdmCl and 10 mM DTT. After a 30 min incubation they were diluted 200-fold (to a subunit concentration of 100 nM) in standard buffer and in the absence and presence of the various chaperonin complexes. At different time intervals aliquots were taken and assayed for MDH activity by diluting 10-fold into cuvettes containing the two substrates 0.2 mM NADH and 0.6 mM oxaloacetate (OAA). The conversion of NADH to NAD^+ and, therefore, the enzyme's activity, was monitored by measuring the optical density (OD) at 340 nm with time in a Perkin Elmer spectrophotometer. The concentrations of chaperonin in the reactions were 500 nM cpn60, 1 μM cpn10 and, where present, 10 mM Mg-ATP. Extra aliquots of Mg-ATP were added every 2 h in experiments with mMDH in order not to limit the ATPase activity of cpn60.

3. Results

Figure 1 shows the equilibrium unfolding curves of mMDH in GdmCl in the absence and presence of its allosteric regulator citrate [26]. Both curves are of a similar shape with one major transition. The midpoint of

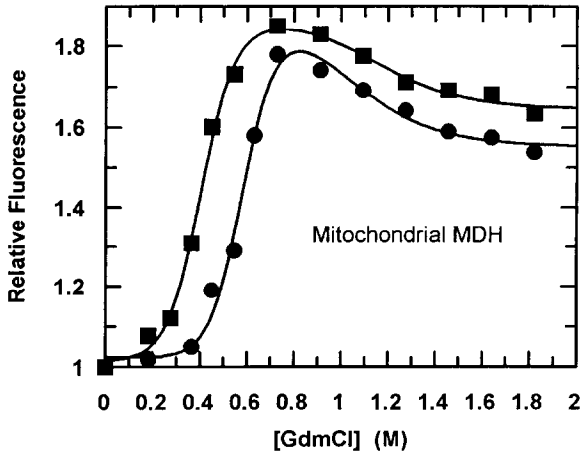


Fig. 1. The unfolding profile of mMDH in guanidinium chloride (GdmCl). The equilibrium unfolding of mMDH in GdmCl as measured by changes in tyrosine fluorescence intensity, in the absence (■) and presence (●) of 2 mM citrate. A fit of the data as described in section 2 to an equation based on the solvation energies of model compounds [27] is shown as a solid line. Values for the free energy of folding and the number of buried amino acids which become exposed to solvent for each transition were calculated and are listed in Table 1.

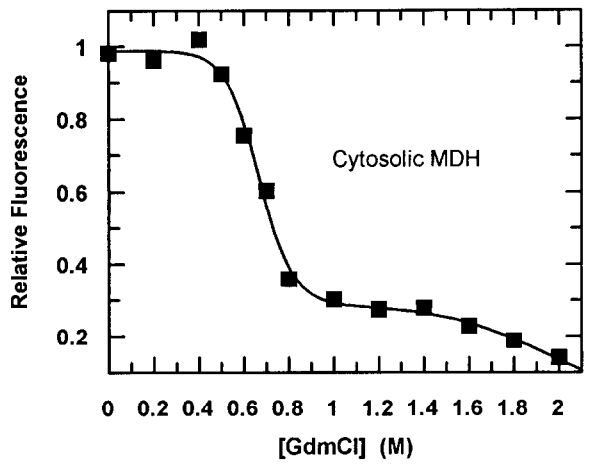


Fig. 2. The unfolding profile of cMDH in GdmCl. The unfolding of cytosolic MDH in GdmCl as measured by changes in tryptophan fluorescence intensity. As for mMDH (Fig. 1), the data were fitted to an equation based on the solvation energies of model compounds [27] (solid line).

this cooperative transition between the native state and the denatured state is 0.42 M in the absence of effector and 0.58 M in its presence. The effect of citrate is to shift the transition to a higher concentration of GdmCl and, therefore, to increase the stability of the native state. Fig. 2 shows the unfolding profile of cMDH. This protein also shows one major transition but the midpoint for unfolding is at a higher GdmCl concentration (0.67 M). In both cases there is a further, less cooperative change in fluorescence at higher concentrations of denaturant. This represents a further step in denaturation but, for the purposes of this present study, we concentrate only on the first transition from which we can extract the stability of the fully native state.

The stability of proteins is measured by the change in free energy of folding in water (ΔG_w) which can be calculated from the unfolding curves in denaturants. Here we used a method based on the solvation energies of model compounds [27]. The results, shown in Table 1, demonstrate that the native state of mMDH is less stable than its cytosolic counterpart having a ΔG_{w1} of −3.6 kcal/mol whereas that for cMDH is −5.9 kcal/mol. The effect of citrate on mMDH is to increase the value of ΔG_{w1} to −5.4 kcal/mol. In all cases the value for n_1, the number of internal residues becoming exposed to the solvent in the transition from the native state, is approximately 70. This similarity would be expected for proteins of equal molecular sizes unfolding through the same pathway. Table 1 also reports the free energy changes and n values for the second transition which represents complete unfolding. These were included in the fit to the data shown in Fig. 1 in order to deconvolve the correct values for the transition in which the native state is lost.

Fig. 3 shows the rate of regain of enzyme activity as mMDH refolds either spontaneously or in the presence of cpn60:cpn10:Mg-ATP. The experiment was repeated with 200 mM inorganic phosphate (P_i) in the refolding buffer. The inclusion of chaperonins was found to increase the efficiency of mMDH folding in both experiments. The yield of spontaneous folding is 25% in the absence of 200 mM P_i and increases to 75% in the presence of chaperonins. The inclusion of phosphate has the effect of increasing the yield of spontaneous folding to 45% and that in the presence of chaperonins to 90%.

The kinetics of the spontaneous mMDH folding reaction have been described previously and can be represented as follows [28]:

$$2U \xrightarrow{\text{fast}} 2I_1 \xrightarrow{k_1} 2I_2 \xrightarrow{k_{ass.}} D$$

where U_1 is the unfolded protein, I_1 and I_2 are intermediates and D is the native dimer. The rate-limiting unimolecular step, which causes the characteristic lag phase in the refolding curves of mMDH is described by k_1. This step is either preceded or followed by a unimolecular rearrangement which occurs with a rate too fast to evaluate in these experiments. The folded monomer (I_2) is competent for association which occurs with a bimolecular rate constant, $k_{ass.}$. The curves were fitted to two steps: one unimolecular with a rate constant, k_1, one bimolecular with a rate constant, $k_{ass.}$ [28,29]. The effect of the chaperonins on the rates of folding can then be quantified.

Table 2 shows the apparent values of k_1 and $k_{ass.}$ for the different folding conditions used. $k_{ass.}$ remains constant independent of the folding conditions at 3×10^4 $M^{-1} \cdot s^{-1}$. This is expected since it depends only on the rate of collision of competent monomers. At this stage in the folding reaction, the protein presumably has near-native secondary and tertiary structure and any intermolecular interactions are very specific [30]. k_1 is 2×10^{-4} s^{-1} for spontaneous folding in the absence of or at a low concentration (10 mM) of P_i. A high concentration of phosphate or the presence of chaperonins have the effect of increasing this rate 3-fold to produce a value of 6.5×10^{-4} s^{-1}. This value is identical to those previously reported in high concentrations of P_i with or without chaperonins

Table 1
The stability of folding of the malate dehydrogenases

	ΔG_{w1} (kcal/mol)	n_1	ΔG_{w2} (kcal/mol)	n_2
mMDH	−3.6	66	−4.6	34
mMDH + citrate	−5.4	72	−3.8	31
cMDH	−5.9	69	−6.4	31

Both the unfolding profiles of mMDH and cMDH contain two unfolding transitions. The change in energy (kcal/mol) which occurs at each transition (respectively, ΔG_{w1} and ΔG_{w2}) was calculated as in [27]. n_1 residues become exposed to solvent during the first transition and n_2 residues during the second transition.

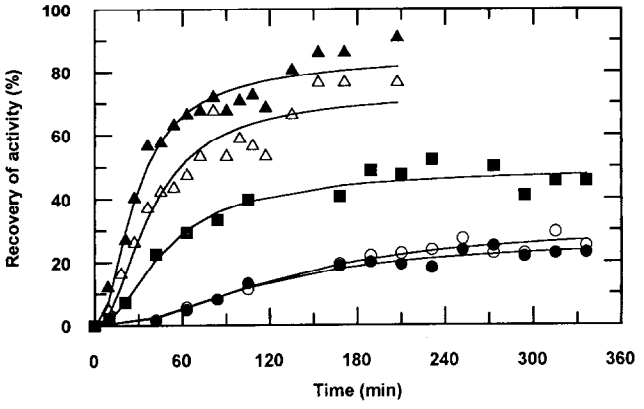


Fig. 3. The recovery of mMDH activity after denaturation. mMDH activity was measured at different time intervals after dilution into refolding buffer (see section 2) alone (○), containing 10 mM P_i (●), 200 mM P_i (■), cpn60:cpn10:Mg-ATP (△) or cpn60:cpn10:Mg-ATP and 200 mM P_i (▲). The data were fitted to two steps (see section 3): one with a first order rate constant, k_1; one with a second order rate constant, $k_{ass.}$ (dimerisation step).

[28,29]. In terms of the rates of folding, chaperonins have a similar effect on phosphate ions but are more potent in increasing the yield of active enzyme.

Cpn10 is essential for the folding of mMDH as demonstrated by the poor folding efficiency in its absence (Fig. 4). In addition, the rate of folding is dramatically retarded when cpn10 is omited, giving k_1 values of 1.2×10^{-4} s^{-1} without P_i and 1.5×10^{-4} s^{-1} in 200 mM P_i. The yields are reduced to 12 and 15%, respectively.

Table 2
Rates of refolding of mitochondrial and cytosolic malate dehydrogenase

Conditions	k_1 (s^{-1})	$k_{ass.}$ ($M^{-1} \cdot s^{-1}$)	Yields (%)
mMDH			
Spontaneous	1.89×10^{-4}	3.13×10^{4}	25
Spontaneous in low P_i	2.13×10^{-4}	3.05×10^{4}	25
Spontaneous in P_i	6.35×10^{-4}	3×10^{4}	45
+ cpn60:cpn10:ATP	5.95×10^{-4}	3.3×10^{4}	75
+ cpn60:cpn10:ATP + P_i	7.2×10^{-4}	2.9×10^{4}	90
+ cpn60:ATP	0.87×10^{-4}	3.03×10^{4}	12
+ cpn60:ATP + P_i	1.09×10^{-4}	3.11×10^{4}	15
cMDH			
Spontaneous	13.6×10^{-4}	$> 10^{6}$	64.2
+ cpn60	6.5×10^{-4}	$> 10^{6}$	28
+ cpn60:ATP	12.8×10^{-4}	$> 10^{6}$	60.3
+ cpn60:cpn10:ATP	13.6×10^{-4}	$> 10^{6}$	62.6

Two rate constants for the folding of mMDH can be extracted from the time-courses shown in Figs. 4 and 5 [28,29]: a unimolecular rate constant, k_1, and a bimolecular rate constant, $k_{ass.}$, for the dimerisation step. The folding of cMDH can be fitted to a similar model, although, under the conditions of the experiment, the dimerisation reaction is much faster than that of mMDH [31]. The yield of active protein obtained when MDH was renatured in the various folding conditions is also shown. Low P_i refers to 10 mM P_i whereas P_i refers to 200 mM P_i.

Fig. 5 shows the reactivation of cMDH spontaneously and in the presence of cpn60, cpn60:Mg-ATP and cpn60:cpn10:Mg-ATP. The curves were fitted to a single first order folding step since at this concentration of protein, the association step has a rate constant $> 10^6$. Our results are in agreement with previously reported data for the spontaneous folding of the protein [31]. cMDH refolds spontaneously to an efficiency of 60%; apo-cpn60 lowers the yield of active protein by binding to the unfolded protein, and retards the folding of that proportion of protein that reaches the native state (Table 2). This suggests that unfolded, bound cMDH exists in at least two populations, one having a much higher affinity for apo-cpn60 than the other. In the presence of cpn60:Mg-ATP or cpn60:cpn10:Mg-ATP, the folding of cMDH is practically indistinguishable from the spontaneous process.

4. Discussion

Mitochondrial MDH is synthesized as a pre-protein in the cytosol and transported into the mitochondrion in an unstable, non-native form. On migration across the inner mitochondrial membrane an N-terminal pre-sequence is proteolytically removed to allow folding to the native state in a chaperonin-rich medium. By contrast, the structurally homologous cytosolic enzyme has no pre-sequence and folds without modification in a compartment where the only chaperonin identified thus far is TCP1. This is present at relatively low concentrations and appears to be specific for the assembly of filamentous proteins [24]. It may, therefore, be expected that the formation of native mMDH would be assisted by chaperonins whereas the folding of cMDH would not.

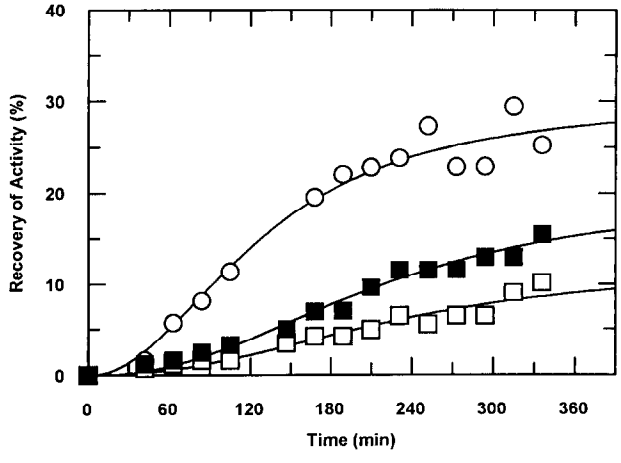


Fig. 4. Retardation of the recovery of mMDH activity after denaturation by cpn60:Mg-ATP. mMDH activity was measured at different time intervals after dilution into refolding buffer alone (○), containing cpn60:Mg-ATP (□) or cpn60:Mg-ATP and 200 mM P_i (■). The data were fitted to two steps as in Fig. 3 (see section 3).

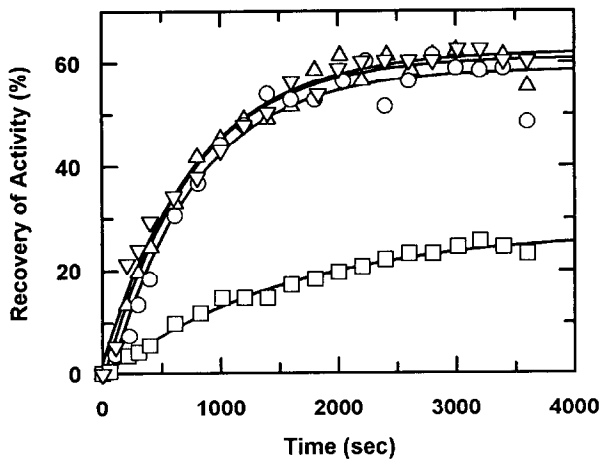


Fig. 5. The recovery of cMDH activity after denaturation. cMDH activity was measured at different time intervals after dilution into refolding buffer alone (○), or containing cpn60:Mg-ATP (△), cpn60:cpn10:Mg-ATP (▽) or cpn60 alone (□). The data were fitted to one first order folding rearrangement since, under these conditions, the dimerisation step in the refolding of cMDH is very fast (diffusion limited).

The results of these investigations demonstrate this expectation to be correct and prompt the more interesting enquiry into the structural or physical characteristics of these proteins which confer this behaviour.

Both proteins interact tightly with apo-cpn60, as evidenced by the blocking of folding. In the case of cMDH, Mg-ATP alone is able to release the protein from the chaperonin complex, whereas for mMDH the cpn10 co-protein is also required.

It may be said, a priori, that this difference in behaviour is a function neither of the folded conformation of the subunits nor of their quaternary arrangement. These features are near-identical in the two proteins [23]; a result which underlines previous conclusions that chaperonin dependence in folding is unrelated to the fold of the substrate [32].

A second factor may be the folding energy of the two proteins. Figs. 1 and 2 show the unfolding curves of mMDH and cMDH, respectively, which demonstrate that the two proteins unfold in a similar two-step process. For both proteins, the first step shows a dependence on the protein concentration (data not shown), indicating that this step includes the dissociation of subunits. The stability of the native state is measured by this first transition. The second and less well-characterised transition represents the unfolding of the monomers. The main difference between the two isoenzymes is the greater stability of the native state of cMDH (ΔG_w (cMDH) = −5.9 kcal/mol) compared with that of mDH (ΔG_w(mMDH) = −3.6 kcal/mol)) (Table 2). The stability of the folded state must have an influence on the amount of protein which can attain it, at equilibrium, in a chaperonin-assisted reaction. This is illustrated by the following scheme:

$$\begin{array}{ccc} & & K_f \\ & U & \longrightarrow F \\ C & \Big\{ & K_d \\ & C{:}U & \end{array}$$

where C is the chaperonin, U the unfolded protein, C:U the complex and F the folded state. Given that there is negligible free unfolded protein and the chaperonin is in large excess over the protein substrate, the proportion of folded protein, α, at equilibrium is given by:

$$\alpha = \frac{[F]}{[C{:}U] + [F]} = \frac{K_f \cdot K_d}{[C] + K_f \cdot K_d}$$

where K_f = [F]/[U], K_d = [C]·[U]/[C:U] and [C] is the free chaperonin concentration. When cpn60 is in excess, [C] ≈ [C_o], where [C_o] is the total concentration. It is evident that the amount of protein which can fold increases with K_f, which reflects the stability of the folded state. By adding nucleotide and then cpn10 the affinity of the unfolded state(s) for cpn60 is incrementally decreased and this is reflected in higher K_d values. It is thus possible that mMDH and cMDH may bind with the same affinity (K_d) to cpn60 but the yield of the former is limited by its instability, i.e. low K_f. To test this, the mMDH stability was enhanced by the addition of citrate to give a free energy of folding to the native state (ΔG_{wl}) of −5.4 kcal/mol, close to that of cMDH, ΔG_{wl} = −5.9 kcal/mol (see Table 2). Folding time-courses in all conditions shown in Table 1 for mMDH were then re-determined in the presence of citrate and were unchanged (results not shown), implying that the stability of the folded state is not a significant factor.

It appears, then, most likely that the extra requirement for cpn10 in the case of mMDH is due, at least partly, to its tighter binding to cpn60. When the protein folds in the presence of cpn60:Mg-ATP, the rate of folding is approximately two-fold slower than for the spontaneous process and the yield is lower. The additional presence of cpn10 allows more rapid folding (6-fold) and a greater yield is obtained (also 6-fold).

This cpn10 dependence may reflect either the presence of preferred binding sites on mMDH or some general physico-chemical feature of the protein which confers tight binding. One such property may be the hydrophobicity of the protein chain, since evidence suggests that protein:cpn60 interactions depend, at least in part, on hydrophobic interactions [33,34]. In this respect there is a clear difference between mMDH and cMDH. Using the hydrophobicity scales developed by Nozaki and Tanford [35] the value calculated for mMDH is −329 kcal/mol and for cMDH −424 kcal/mol. Thus the mitochondrial enzyme has a higher hydrophobic potential by almost 100 kcal/mol. Another aspect of the cpn10-depend-

cncc which may relate to the hydrophobicity of the chain is the precipitability of the substrate protein (i.e. are the conditions permissive or non-permissive [36] to folding?). If cpn10 can release the substrate into a chaperonin cavity to allow further folding, this would prevent aggregation.

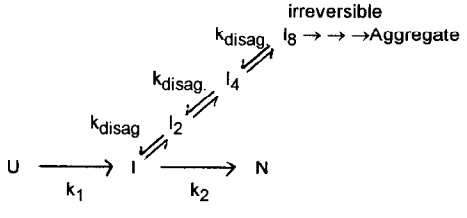
Further to this argument, the homologous, dimeric lactate dehydrogenase from *Bacillus stearothermophilus* (bLDH) has a chaperonin dependency intermediate between mMDH and cMDH. That is, in the presence of cpn60 and ATP there is an enhancement of the folding yield of this enzyme but there is no requirement for cpn10 [13]. This protein has a hydrophobic potential of −404 kcal/mol, intermediate between mMDH and cMDH. Thus the chaperonin dependency of these dehydrogenases is mMDH > bLDH > cMDH which is commensurate with their hydrophobicities. From this we can only suggest that overall hydrophobicity is a factor in binding affinity but cannot exclude the existence of preferred sequences. This latter possibility is now being addressed by examining the relative affinity for cpn60 of polypeptide fragments derived from the two proteins.

Figs. 3 and 4 show the kinetic time–courses of active mMDH recovery. These progress curves are 'sigmoidal' and show that the protein folds through the schematic pathway defined by Jaenicke et al. [28] (see section 3). Irrespective of folding conditions, the data can be fitted to a slow unimolecular step, representing subunit folding, and a bimolecular step representing subunit association. In 'non-permissive' conditions [36], where the orthophosphate concentration is in the physiological range, the unimolecular rate of folding is slow and the process inefficient. This rate is accelerated three-fold by the inclusion of either cpn60:cpn10:Mg-ATP or 200 mM orthophosphate, although with the chaperonins the yield is higher. Apart from the physiological point that chaperonins more than compensate for the absence of the artificial concentration of permissive ions, the important conclusion that can be drawn from the data is that in non-permissive conditions, chaperonins increase the measured rate of a folding step.

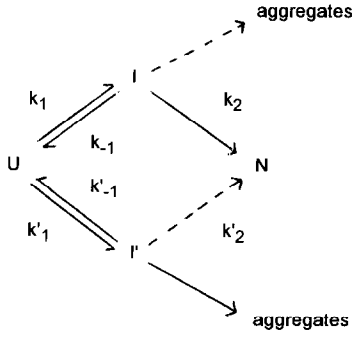
This enhancement of the rate appears to contradict previous proposed mechanisms of action for the chaperonins. However, it can be explained in at least two ways. First, consider a simple scheme for the folding pathway of a protein in which the unfolded (U) protein folds to the native form (N) via an intermediate state (I). If this intermediate becomes populated, it may be susceptible to aggregation and can dimerise to I_2 then I_4, I_8 ... until it reaches a state of aggregation which is irreversible. If, in the spontaneous folding reaction, material is partitioned between folding to N or forming irreversible aggregates, then the yield of native protein is reduced. Furthermore, if effectively all the protein forms slow but reversible aggregates (e.g. I_2 or I_4), then the rate-limiting step for those molecules which do attain the native state may be the dissociation of the aggregates (at a slow rate, $k_{disag.}$) rather than the conversion of I to N described by k_2. If the role of the chaperonin is to bind to I and prevent it from forming aggregates, the rate-limiting step would then become a function of I → N, as long as the release rate from cpn60 was rapid. In this case it is possible that the rate of folding can be accelerated over the spontaneous process as long as $k_2 > k_{disag.}$, but it must be remembered that the formation of a complex between the intermediate and the chaperonin will reduce the observed rate of this step. (Scheme 1).

A second explanation for the observed acceleration of folding in the presence of chaperonins becomes evident when one considers a more complex but perhaps more realistic folding pathway in which each state, as described by a common set of properties, is populated by a large number of conformers. In each state, therefore, there could be conformers which are competent for isomerisation into the next state and others which are less so [37,38]. Isomerisation out of these less 'competent' states can be slow and therefore these states are prone to aggregate. In scheme 2, U represents a population of unfolded states, I is a population of partially folded states which can fold relatively efficiently into the native state, N, and I′ is a population of partially folded states which are unproductive and highly prone to aggregation.

Effectors such as phosphate (mMDH) or chloride ions (rubisco) may stabilise intermediates competent for folding, thus increasing the flux through productive routes (increase in k_1) and minimising the need to isomerise in and out of less competent states. In a similar way, chaperonins, which are believed to stabilise more unfolded states of proteins [3,16,18,34] would facilitate the



Scheme 1.



Scheme 2.

isomerisation of proteins from incompetent, slowly folding intermediates (I′) into unfolded conformations by increasing k'_{-1}. This would allow progression along the more productive folding pathway and increase the observed rate of the process. The recent findings of Pluckthün and colleagues [34], that cpn60-bound cyclophilin has an amide exchange protection characteristic of the unfolded states, support the latter argument.

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