Refolding and recognition of mitochondrial malate dehydrogenase by *Escherichia coli* chaperonins cpn 60 (groEL) and cpn10 (groES)

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In vitro refolding of pig mitochondrial malate dehydrogenase is investigated in the presence of *Escherichia coli* chaperonins cpn60 (groEL) and cpn10 (groES). When the enzyme is initially denatured with 3 M guanidinium chloride, chaperonin-assisted refolding is 100 % efficient. C.d. spectroscopy reveals that malate dehydrogenase is almost unfolded in 3 M guanidinium chloride, suggesting that a state with little or no residual secondary structure is the optimal 'substrate' for chaperonin-assisted refolding. Malate dehydrogenase denatured to more highly structured states proves to refold less efficiently with chaperonin assistance. The enzyme is shown not to aggregate under the refolding conditions, so that losses in refolding efficiency result from irreversible misfolding. Evidence is advanced to suggest that the chaperonins are unable to rescue irreversibly misfolded malate dehydrogenase. A novel use is made of 100 K Centricon concentrators to study the binding of [^{14}C]acetyl-labelled malate dehydrogenase to groEL by an ultrafiltration binding assay. Analysis of the data by Scatchard plot shows that acetyl-malate dehydrogenase, which has previously been extensively unfolded with guanidinium chloride, binds to groEL at a specific binding site(s). At saturation, one acetyl-malate dehydrogenase homodimer (two polypeptides) is shown to bind to each groEL homo-oligomer with a binding constant of approx. 10 nM.

INTRODUCTION

Chaperonins are a subclass of the molecular chaperones which are a ubiquitous, abundant and highly conserved group of proteins which assist protein folding/refolding *in vitro* and *in vivo* (Gething and Sambrook, 1992; Hendrick and Hartl, 1993) as well as protecting proteins from stress-induced unfolding (Hendrick and Hartl, 1993). Of all the chaperonins currently characterized, the best known are the *Escherichia coli* chaperonins cpn60 (groEL) and cpn10 (groES). In an effort to understand the underlying chemical mechanism of chaperonin-assisted folding/refolding of proteins, we have been studying the chaperonin-assisted refolding of pig mitochondrial malate dehydrogenase (mMDH) *in vitro* using purified groEL and groES (Miller et al., 1993).

In the chaperonin-assisted refolding of mMDH by groEL and groES, unfolded mMDH binds to the large chaperonin groEL and then a combination of groEL-catalysed ATP hydrolysis and groES binding to groEL serves to promote release of correctly folded protein from groEL (Miller et al., 1993). While there has been intense recent interest in the interaction between groEL and either ATP or groES (Bochkareva et al., 1992; Jackson et al., 1993; Todd et al., 1993) there has been much less attention given to understanding the crucial interactions involved between groEL and the protein which is to be folded/refolded. The following paper describes our preliminary attempts to understand these interactions through studies on the chaperonin-assisted refolding of mMDH.

EXPERIMENTAL

Materials

Pig mMDH was purchased from Boehringer–Mannheim U.K., Lewes, East Sussex, U.K. [^{14}C]Acetic anhydride was purchased from Sigma Chemical Co., Poole, Dorset, U.K. All other chemicals were of the highest analytical grade available. Milli-Q water was used throughout.

Enzyme and protein assays

Pig mMDH homodimer concentrations were calculated from $A^{1\%}_{280} = 2.5$ (Gregory et al., 1971) and a subunit molecular mass of 35 kDa (Thorne and Kaplan, 1963). Concentrations of *E. coli* groEL and groES were evaluated by dry-weight calibration of the respective A_{280} coefficients using a modification of standard procedures (Kupke and Dorrier, 1978). In performing this calibration, preparations of groEL and groES were dialysed exhaustively against water, freeze-dried and then heated *in vacuo*, at 110 °C over phosphorus pentoxide, until constant weight was achieved. A_{280} coefficients of 2.92×10^4 $M^{-1} \cdot cm^{-1}$ for groEL and 4.72×10^3 $M^{-1} \cdot cm^{-1}$ for groES were obtained. These values are higher than those previously determined by quantitative amino acid analyses (Viitanen et al., 1990; Zahn and Pluckthun 1992) and other values reported (Hayer-Hartl and Hartl, 1993).

Buffer solutions

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

Purification of groEL and groES

GroEL and groES were prepared and purified as described previously (Miller et al., 1993). Protein stocks were stored in aliquots at −20 °C in 50 mM Tris/HCl, pH 7.5, 1 mM dithiothreitol, supplemented with 50 % (v/v) glycerol.

Abbreviations used: mMDH, mitochondrial malate dehydrogenase; cpn60, chaperonin 60 (groEL); cpn 10, chaperonin 10 (groES); 2-ME, 2-mercaptoethanol.

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Labelling of mMDH with [^{14}C]acetic anhydride

mMDH (approx. 5 mg) was prepared by extensive dialysis against vacuum-degassed 1.8 M sodium acetate at 4 °C under argon. The dialysate was concentrated to approx. 400 μl (in a 10 K Centricon concentrator), then treated every 15 min (while stirring at 4 °C) with four separate aliquots (5 μl) of a solution containing [^{14}C]acetic anhydride (100 μCi, specific radioactivity 14.3 mCi/mmol, 0.7 μl) in ethanol (20 μl). After 1 h, the acetylation mixture was fractionated on a Sephacryl S-100 HR column (1.5 cm × 15 cm) equilibrated with 150 mM sodium phosphate, pH 7.6, 2 mM 2-mercaptoethanol (2-ME), 1 mM EDTA at 4 °C. Elution was performed at a flow rate of 0.5 ml/min on a Pharmacia f.p.l.c. system with continuous monitoring of the eluate absorbance at 280 nm. Fractions corresponding to radiolabelled, enzymically active protein were pooled and concentrated to approx. 1 ml using 10 K Centricon concentrators. The concentrate was repeatedly dialysed against 150 mM sodium phosphate, pH 7.6, 2 mM 2-ME, 1 mM EDTA at 4 °C. Homogeneity of the labelled enzyme was confirmed by SDS/PAGE. Stocks of [^{14}C]acetyl-mMDH (typically 1.5 mg/ml) were stored at 4 °C and used within 2 weeks of preparation.

Specific radioactivity determination

Aliquots of [^{14}C]acetyl-mMDH solution (0.3 mg/ml, 4.3 μM dimer concentration) in 150 mM sodium phosphate, pH 7.6, 20 mM 2-ME, 10 mM $MgCl_2$, 10 mM KCl were diluted into the same buffer to give a range of final [^{14}C]acetyl-mMDH concentrations from 10 nM to 300 nM (dimer concentration). The radioactivity of 200 μl aliquots of each of these standard dilutions was determined by liquid-scintillation counting in Bray's fluid (5 ml). All samples were counted for 10 min and corrected for background. Duplicate determinations were used to construct a specific radioactivity curve.

C.d. measurements

mMDH (approx. 2.5 mg/ml) was dialysed against 150 mM sodium phosphate, pH 7.6, 2 mM 2-ME, 1 mM EDTA at 4 °C. Aliquots of the stock solution were then diluted to an enzyme concentration of 0.5 mg/ml in buffers composed of 150 mM sodium phosphate, pH 7.6, 20 mM 2-ME, 10 mM EDTA and various final concentrations of guanidinium chloride (0–6 M). Solutions were left to equilibrate for approx. 2 h at ambient temperature. C.d. spectra were recorded on a Jasco J-600 spectropolarimeter (25 °C, 0.2 mm path-length cell), and corrected for background absorbance. Percentage helical content of denatured mMDH was evaluated at 220 nm with reference to the c.d. spectrum of 6 M guanidinium chloride-denatured mMDH.

mMDH aggregation studies

A stock solution of mMDH was prepared by dialysis against 150 mM sodium phosphate, pH 7.6, 2 mM 2-ME, 1 mM EDTA at 4 °C and subsequently concentrated using 10 K Centricon concentrators to approx. 7 mg/ml. An aliquot of mMDH was diluted to an enzyme concentration of 2.5 mg/ml in 150 mM sodium phosphate, pH 7.6, 20 mM 2-ME, and 10 mM EDTA containing 3 M guanidinium chloride. This solution was incubated for 2 h at 20 °C so as to fully denature the mMDH.

Light-scattering effects were monitored at 20 °C using a Perkin–Elmer LS50 spectrofluorimeter, with excitation and emission wavelengths of 340 nm. The slit width was 2.5 nm for both excitation and emission. Denatured mMDH was diluted to enzyme concentrations ranging from 143 nM to 1000 nM in 150 mM sodium phosphate, pH 7.6, 20 mM 2-ME, 10 mM $MgCl_2$, 10 mM KCl at 20 °C in a fluorescence cuvette. In each case guanidinium chloride was adjusted to a final concentration of 0.1 M. After rapid mixing, light-scattering effects were monitored for 10 min.

mMDH refolding 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.

Aliquots of mMDH stock solution were diluted to a concentration of 0.3 mg/ml (4.3 μM dimer concentration) in buffers composed of 150 mM sodium phosphate, pH 7.6, 20 mM 2-ME, 10 mM EDTA and various final concentrations (0–6 M) of guanidinium chloride. These solutions were then incubated at 20 °C for 2 h to equilibrate. Renaturation of mMDH was initiated by diluting denatured protein to a concentration of 10 μg/ml (143 nM dimer concentration, 30-fold dilution) in renaturing buffers and incubating the resulting solutions for 3 h at 20 °C. Renaturing buffers consisted of 150 mM sodium phosphate, pH 7.6, 20 mM 2-ME, 10 mM $MgCl_2$ and 10 mM KCl, with ATP and a homo-oligomeric excess of groEL and groES. Fixed aliquots (20 μl) of renaturing mixtures were removed at recorded times and mixed with aliquots of an assay buffer (980 μl) pre-incubated at 30 °C. Assay buffer was composed 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 the assay mixtures, was used as a measure of mMDH reactivation during the refolding process. Recorded activities were expressed as a percentage relative to the activity of a control sample of native mMDH (143 nM dimer concentration) incubated at 20 °C in a buffer of 150 mM sodium phosphate, pH 7.6, 20 mM 2-ME, 10 mM $MgCl_2$, 10 mM KCl and 2 mM ATP.

Refolding experiments involving acetyl-mMDH were performed in an identical manner.

mMDH/groEL ultrafiltration binding assays

GroEL was prepared for use by dialysis against 150 mM sodium phosphate, pH 7.6, 2 mM 2-ME, 1 mM EDTA at 4 °C. 100 K Centricon concentrators were prepared by pre-centrifuging (1000 *g*) with a solution (1 ml) of mMDH (10 μg/ml) in the above buffer. Immediately before each binding assay, Centricon concentrators were washed thoroughly with water and then centrifuged (1000 *g*) with more water (1 ml). The Centricon concentrators were then centrifuged in an inverted position to remove excess liquid from the membrane and dried thoroughly.

Aliquots of [^{14}C]acetyl-mMDH stock solution were diluted to a concentration of 0.3 mg/ml (4.3 μM dimer concentration) in four different buffers composed of 150 mM sodium phosphate, pH 7.6, 20 mM 2-ME, 10 mM EDTA and guanidinium chloride of 0, 1, 2 and 3 M concentrations respectively. A separate set of groEL/[^{14}C]acetyl-mMDH ultrafiltration binding assays was then carried out with each different [^{14}C]acetyl-mMDH solution. Individual binding assays were performed by diluting an aliquot of a given [^{14}C]acetyl-mMDH solution into 150 mM sodium phosphate, pH 7.6, containing 20 mM 2-ME, 10 mM $MgCl_2$, 10 mM KCl and 40 nM groEL (homo-oligomer concentration).

Final mMDH concentrations ranged from 20 nM to 250 nM (dimer concentration). Special attention was made to adjust the guanidinium chloride concentrations in each binding assay to a final concentration of 0.2 M. Immediately after the addition of [^{14}C]acetyl-mMDH, binding assay mixtures were transferred to 100 K Centricon concentrators and centrifuged (1000 *g*, 1.5 min) at 4 °C (typically resulting in 300 μl of each mixture being transferred to the filtrate cup). [^{14}C]Acetyl-mMDH concentrations in the filtrate were then determined by scintillation counting of 200 μl aliquots in Bray's fluid (5 ml). These concentrations were then corrected using a correction plot specific to each separate set of binding assays. Correction plots were derived by performing control assays in the absence of groEL. From these control assays, the efficiency of [^{14}C]acetyl-mMDH partition through the Centricon membrane was determined as a function of enzyme concentration from 20 nM to 250 nM (dimer concentration) and then used to correct the filtrate concentrations for Centricon membrane binding. Corrected filtrate concentrations of [^{14}C]acetyl-mMDH now corresponded to the concentrations of unbound mMDH ([mMDH]$_{free}$) in each retentate. The amount of mMDH bound to groEL in the retentate (mMDH$_{bound}$) was then determined according to eqn. (1).

$$mMDH_{bound} = mMDH_{total} - (vol_{assay} \times [mMDH]_{free}) \quad (1)$$

For each assay, the assay volume (vol$_{assay}$) was 1 ml and mMDH$_{total}$ corresponded to the total amount of mMDH used in each binding assay. Scatchard plots were then made for each separate set of groEL/[^{14}C]acetyl-mMDH-binding assays.

RESULTS AND DISCUSSION

Chaperonin-assisted refolding of mMDH, previously denatured in 6 M guanidinium chloride, has been reported (Miller et al., 1993) to result in at least 90% recovery of active enzyme, whereas spontaneous refolding is about 30% efficient. Since then, we have found (results not shown) by the light-scattering methodology of Buchner et al. (1991) that mMDH, fully denatured with guanidinium chloride, does not aggregate under the refolding conditions reported. In fact even up to concentrations of 1000 nM no sign of aggregation was detected. Therefore the failure of the chaperonins to refold mMDH with 100% efficiency must arise from irreversible misfolding of mMDH.

In an effort to improve the efficiency of chaperonin-assisted refolding, mMDH was denatured using a range of lower guanidinium chloride concentrations from 1 M up to 3 M and then refolded using groEL and groES under the previously reported conditions (Miller et al., 1993). The results showed (Figure 1a) that chaperonin-assisted refolding resulted in 100% recovery of active enzyme when mMDH was denatured using 3 M guanidinium chloride. However, when 2.5 M, 2 M and 1.5 M guanidinium chloride solutions were used, the percentage recoveries of active enzyme were reduced to 90, 80 and 70% respectively [results for 2.5 M guanidinium chloride-denatured mMDH are not shown in Figure 1(a) for simplicity]. When 1 M guanidinium chloride was used, the mMDH enzyme activity was at least 50% recovered at the beginning of refolding, but was restored to 100% after about 2 h in the presence of the chaperonins. Spontaneous refolding of mMDH (Figure 1b) was investigated in parallel with the chaperonin-assisted refolding experiments described above. In all cases, spontaneous refolding was between 2- and 3-fold less efficient than the chaperonin-assisted process.

In order to provide some structural understanding for the different efficiencies of chaperonin-assisted refolding, c.d. spectroscopy was performed to determine the structural state of mMDH denatured with between 3 M and 1 M guanidinium chloride (Figure 2). The spectral line corresponding to native mMDH was typical of a protein with a substantial amount of α-helix. Surprisingly, mMDH lost secondary structure very rapidly as the concentration of guanidinium chloride was increased. Assuming 6 M guanidinium chloride-denatured mMDH to be a random coil, the helical content of mMDH in 3 M guanidinium chloride solution was estimated at 5% of the native protein (on the basis of the α-helix maximum at 220 nm). In 2 M guanidinium chloride solution, the helical content of the enzyme was approx. 22% of the native protein and in 1 M guanidinium chloride approx. 65%. However, we were unable to establish the oligomeric state of mMDH under these various denaturing conditions, even using cross-linking agents such as glutaraldehyde (Huang and Chang, 1992) and dimethylsuberimidate (Davies and Stark, 1970).

From the results shown in Figures 1(a) and 2, it appears that chaperonin-assisted refolding of mMDH is most efficient when the enzyme is initially denatured to a state with little or no residual secondary structure and that the efficiency of assisted refolding declines as the denatured state of the mMDH becomes

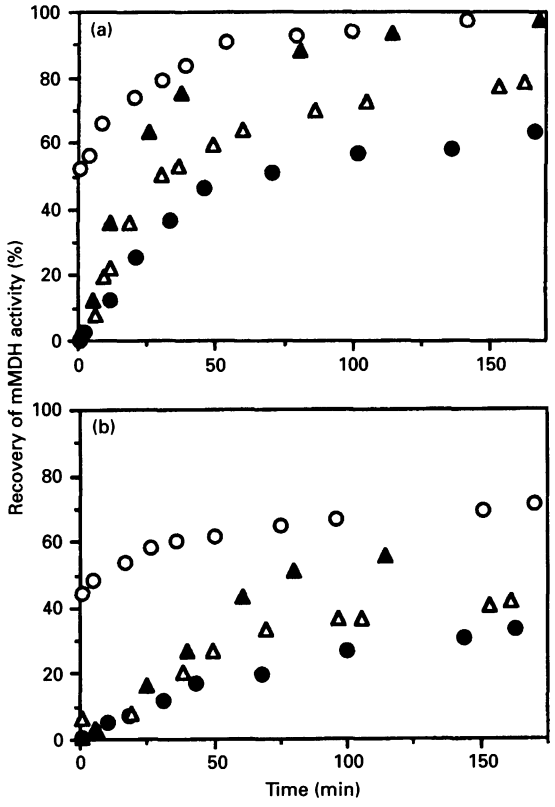

Figure 1 Time courses of chaperonin-assisted and spontaneous refolding of mMDH

(**a**) mMDH was denatured in 1 M (○), 1.5 M (●), 2 M (△) and 3 M (▲) guanidinium chloride as described in the text. Refolding was initiated by diluting mMDH to 143 nM (dimer concentration) in renaturing buffer containing groEL (858 nM homo-oligomer concentration), groES (1716 nM homo-oligomer concentration) and ATP (2 mM). 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 enzyme (143 nM dimer concentration) incubated in the renaturing buffer. (**b**) mMDH was denatured in 1 M (○), 1.5 M (●), 2 M (△) and 3 M (▲) guanidinium chloride as above. Spontaneous refolding was initiated by diluting mMDH to 143 nM (dimer concentration) in renaturing buffer containing ATP (2 mM).

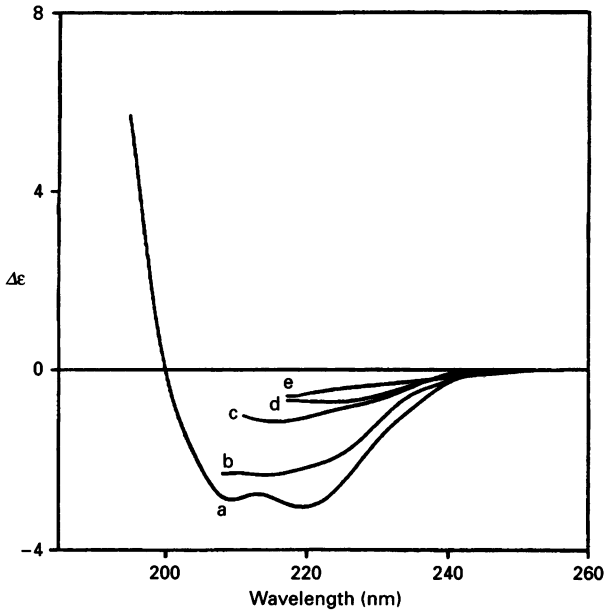

Figure 2 C.d. spectra of mMDH at increasing levels of denaturation

mMDH was denatured at the indicated concentration of guanidinium chloride as described in the text. C.d. spectra were recorded at 25 °C in a 0.2 mm path-length cell. (**a**) corresponds to native mMDH (0.5 mg/ml), (**b**) to 1 M guanidinium chloride-denatured mMDH (0.5 mg/ml), (**c**) to 2 M guanidinium chloride-denatured mMDH (0.5 mg/ml), (**d**) to 3 M guanidinium chloride-denatured mMDH (0.5 mg/ml) and (**e**) to 6 M guanidinium chloride-denatured mMDH (0.5 mg/ml).

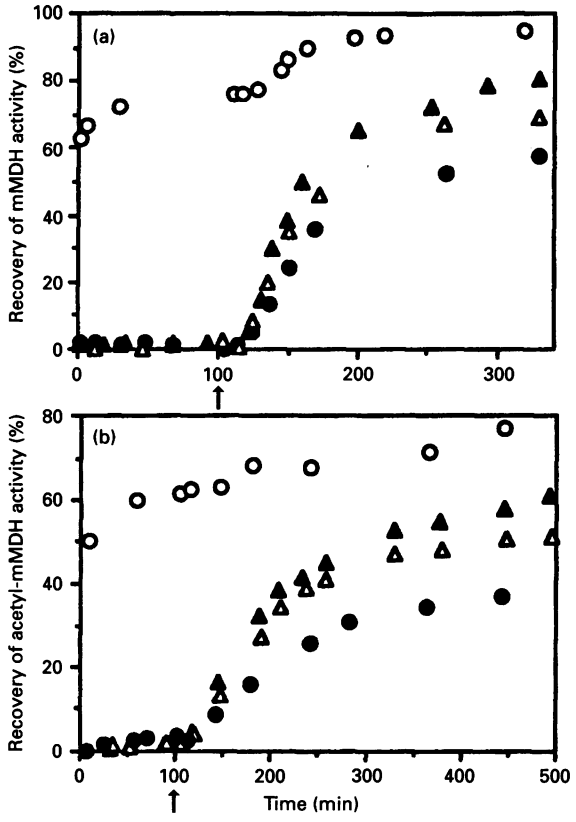

Figure 3 GroEL suppression of mMDH and acetyl-mMDH refolding

(**a**) Experiments were performed analogously to those described in the legend of Figure 1 and in the text. mMDH, denatured in 1 M (○), 1.5 M (●), 2 M (△) and 3 M (▲) guanidinium chloride, was diluted to 143 nM (dimer concentration) in renaturing buffer containing groEL only (858 nM homo-oligomer concentration). After incubation for 100 min, groES (final homo-oligomer concentration 1716 nM) and ATP (2 mM) were added at the arrowed position. (**b**) Acetyl-mMDH, denatured in 1 M (○), 1.5 M (●), 2 M (△) and 3 M (▲) guanidinium chloride, was diluted to 143 nM (dimer concentration) in renaturing buffer containing groEL only (858 nM homo-oligomer concentration). After incubation for 100 min, groES (final homo-oligomer concentration 1716 nM) and ATP (2 mM) were added at the arrowed position.

more structurally complex. However, if the denatured state of the mMDH is too highly structured (i.e., when the protein is denatured with 1 M guanidinium chloride) then a significant proportion of mMDH (approx. 50%) refolds to active enzyme immediately, presumably with little or no chaperonin participation.

In order to determine the extent of chaperonin participation in the assisted refolding experiments described in Figure 1(a), mMDH was once more denatured using a range of guanidinium chloride concentrations from 1 M up to 3 M and then refolded initially in the presence of groEL alone followed by the addition of groES (and ATP) after 100 min. When 1 M guanidinium chloride was used, mMDH enzyme activity was at least 60% recovered at the beginning of refolding, reaching a plateau at 75% recovery after 100 min. Addition of groES and ATP resulted in a further recovery of mMDH activity up to 95%. Thus up to three-quarters of the mMDH appeared to refold without chaperonin participation. By contrast, when mMDH was denatured with 1.5 M, 2 M and 3 M guanidinium chloride and then combined with groEL, mMDH refolding was completely inhibited until groES and ATP were added (Figure 3a). After the addition of groES and ATP the percentage recoveries of active enzyme paralleled the results shown in Figure 1(a). In these cases, the complete inhibition of spontaneous refolding by groEL, followed by the restoration of refolding (with the addition of groES and ATP), demonstrated that the chaperonins were participating completely in the refolding of mMDH. However, the percentage recoveries of active enzyme were not the same, in spite of the complete participation of the chaperonins in each case. As discussed previously, the failure of the chaperonins to refold mMDH with 100% efficiency must arise from irreversible misfolding of mMDH. Hence a crucial question arises. To what extent is irreversibly misfolded mMDH associated with the chaperonins during assisted refolding?

To investigate this question, quantitative binding studies were performed between mMDH and groEL. To do this mMDH was radioactively labelled by acetylation with [^{14}C]acetic anhydride (Riordan and Vallee, 1972) and purified by gel filtration. The [^{14}C]acetyl-mMDH (approx. 50 c.p.m./pmol) appeared homogeneous on overloaded, Coomassie Blue-stained, SDS/12% polyacrylamide gels and co-migrated with native mMDH. Enzyme activity of the labelled protein corresponded to that of native mMDH and was undiminished even after several weeks storage in solution at 4 °C. Furthermore, experiments on the chaperonin-assisted refolding of acetyl-mMDH revealed (Figure 3b) that acetyl-mMDH behaved in the same way as mMDH (Figure 3a). Hence, in every respect acetyl-mMDH was found to behave in a fashion almost identical with mMDH. Four sets of groEL ultrafiltration binding assays (Spector et al., 1972) were then carried out with native [^{14}C]acetyl-mMDH and enzyme which had been previously denatured with 1, 2 and 3 M guanidinium chloride respectively. Individual ultrafiltration assays were performed using 100 K Centricon membranes. Each assay

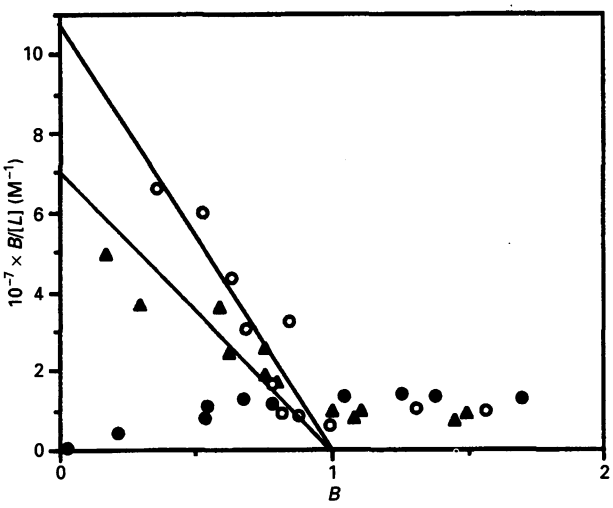

Figure 4 Scatchard plots for the binding of [^{14}C]acetyl-mMDH to groEL

[^{14}C]Acetyl-mMDH was denatured as described in the text and then diluted into binding assay buffer containing groEL (40 nM homo-oligomer concentration). Enzyme concentrations ranged from 20 nM to 250 nM (dimer concentration) and guanidinium chloride was always adjusted to a final concentration of 0.2 M. The amount of mMDH binding to groEL was determined in each case by ultrafiltration assay using 100 K Centricon concentrators. Scatchard plots are shown for the interaction with groEL of [^{14}C]acetyl-mMDH previously denatured in 1 M (●), 2 M (▲) and 3 M (○) guanidinium chloride respectively. *B* represents mol of [^{14}C]acetyl-mMDH homodimer bound per mol of groEL, [*L*] represents free [^{14}C]acetyl-mMDH homodimer concentration. Where appropriate, data were fitted by a least-squares analysis.

relied on the assumption that the concentration of acetyl-mMDH in the Centricon filtrate is equivalent to the concentration of acetyl-mMDH remaining unbound to groEL in the retentate. From the amount of unbound acetyl-mMDH, the amount bound to groEL is then determined according to eqn. (1). However, control experiments performed in the absence of groEL revealed that a proportion of acetyl-mMDH was always bound to the Centricon membrane after centrifugation, thereby reducing the concentration of acetyl-mMDH in the filtrate. Fortunately, this non-specific binding behaviour was found to increase linearly with respect to the initial concentration of acetyl-mMDH and could easily be corrected for. Hence, during the binding studies with groEL, filtrate concentrations of acetyl-mMDH were always corrected for membrane binding so as to provide an accurate determination of the concentration of unbound acetyl-mMDH in the retentate. With the exception of Centricon membrane binding, other losses in acetyl-mMDH concentration through non-specific binding were found to be insignificant. To the best of our knowledge, this represents the first application of an ultrafiltration binding assay to study protein–protein interaction.

The results of the different sets of binding assays were expressed as Scatchard plots (Figure 4). When acetyl-mMDH was unfolded in 1 M guanidinium chloride and then combined with groEL, data acquired over the full concentration range of [^{14}C]acetyl-mMDH fell on an almost horizontal line parallel to the *x*-axis. Native acetyl-mMDH was found to behave similarly with groEL (results not shown). In both cases, acetyl-mMDH appeared to be interacting with groEL by non-saturable, non-specific interactions. By contrast, when acetyl-mMDH was unfolded in either 3 M or 2 M guanidinium chloride and then combined with groEL, the binding behaviour was clearly biphasic. In both cases, data acquired using the lower [^{14}C]acetyl-mMDH concentrations could be fitted by least-squares analysis to straight lines which both intercepted at a *B* value of 1 (corresponding to 1 mol of acetyl-mMDH homodimer bound per mol of groEL homo-oligomer). Data acquired at the higher [^{14}C]acetyl-mMDH concentrations once more gave almost horizontal lines parallel to the *x*-axis. In these two cases, denatured acetyl-mMDH is binding to groEL specifically by a defined binding site(s), which saturate with two acetyl-mMDH polypeptides. Thereafter non-saturable, non-specific interactions with the chaperonin predominate. From the two straight lines, binding constants (K_d) of 9 nM and 14 nM were estimated from the Scatchard plots for the specific, saturable binding of 3 M and 2 M guanidinium chloride-denatured acetyl-mMDH respectively to groEL. Given the very close similarity between acetyl-mMDH and mMDH it seems reasonable to presume that mMDH would bind to groEL with similar binding constants and the same stoichiometry. These values agree closely with the value of 7 nM determined for the binding of unfolded LDH to groEL (A. R. Clarke, unpublished work).

The non-specific binding behaviour of the 1 M guanidinium chloride-denatured acetyl-mMDH neatly accounts for the lack of involvement of the chaperonins in the assisted refolding of 1 M guanidinium chloride-denatured acetyl-mMDH (Figure 3b) and mMDH [Figures 1(a) and 3(a)]. Similarly, the specific binding behaviour of the 2 M and 3 M guanidinium chloride-denatured acetyl-mMDH is very much in keeping with the complete participation of the chaperonins in assisted refolding of 2 M and 3 M guanidinium chloride-denatured acetyl-mMDH and mMDH [Figures 1(a), 3(a) and 3(b)]. In addition, the close similarity of the specific binding of 3 M and 2 M guanidinium chloride-denatured acetyl-mMDH makes it reasonable to suggest that irreversibly misfolded, as well as productively folding, acetyl-mMDH and mMDH polypeptides bind equally well to groEL but that irreversibly misfolded proteins are not being rescued by the chaperonins. In view of the previously reported observation that the chaperonins have little control over the kinetics of mMDH refolding (Miller et al., 1993), this suggestion appears all the more probable. The participation of other molecular chaperones when proteins fold/refold *in vivo* (Langer et al., 1992) would then be to prevent irreversible misfolding prior to chaperonin-assisted refolding.

Current models of the chaperonin mechanism (Hendrick and Hartl, 1993) described two polypeptides binding within the cavity of groEL, folding and then being released following ATP-driven conformational changes (Saibil and Wood, 1993; Saibil et al., 1993; Jackson et al., 1993). Our data are in no way contradictory to this currently accepted model. Given the numbers of acetyl-mMDH polypeptides binding to groEL and the known dimensions of the chaperonin inner cavity (6 nm, Braig et al., 1993) unfolded acetyl-mMDH or mMDH could certainly bind within the central cavity, although our results do not rule out the possibility that unfolded enzyme is alternatively binding to surface depressions on groEL. Two defined binding sites, located at either end of the groEL homo-oligomer, have already been demonstrated by electron microscopy (Braig et al., 1993). Bochkareva et al. (1992) have reported, on the basis of concave Scatchard plots, that co-operativity exists in the binding of rhodanese to groEL. By contrast, our data do not show co-operative behaviour in the binding of acetyl-mMDH to groEL.

Finally, the conclusion that mMDH with residual secondary structure refolds by chaperonin-assistance with the highest efficiency suggests that the chaperonin groEL recognizes first either the stereo-electronic properties of a linear chain of amino acids or those similar properties of secondary structures (α-helices, β-sheets or β-turns). Since groEL is known to bind peptides in an α-helical conformation (Landry et al., 1992) and secondary

structures form readily in peptides at equilibrium (Wright et al., 1988), then secondary structure recognition by groEL seems the more plausible. We are currently investigating this recognition process further.

We thank the SERC, The Royal Society and Roche Products Ltd. for financial support. We also thank Dr. Alex Drake for his assistance in performing the c.d. spectroscopy.

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Received 19 October 1993/11 March 1994; accepted 18 March 1994

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