Characterisation of Cpn60 (GroEL) bound cytochrome c: the passive role of molecular chaperones in assisted folding/refolding of proteins

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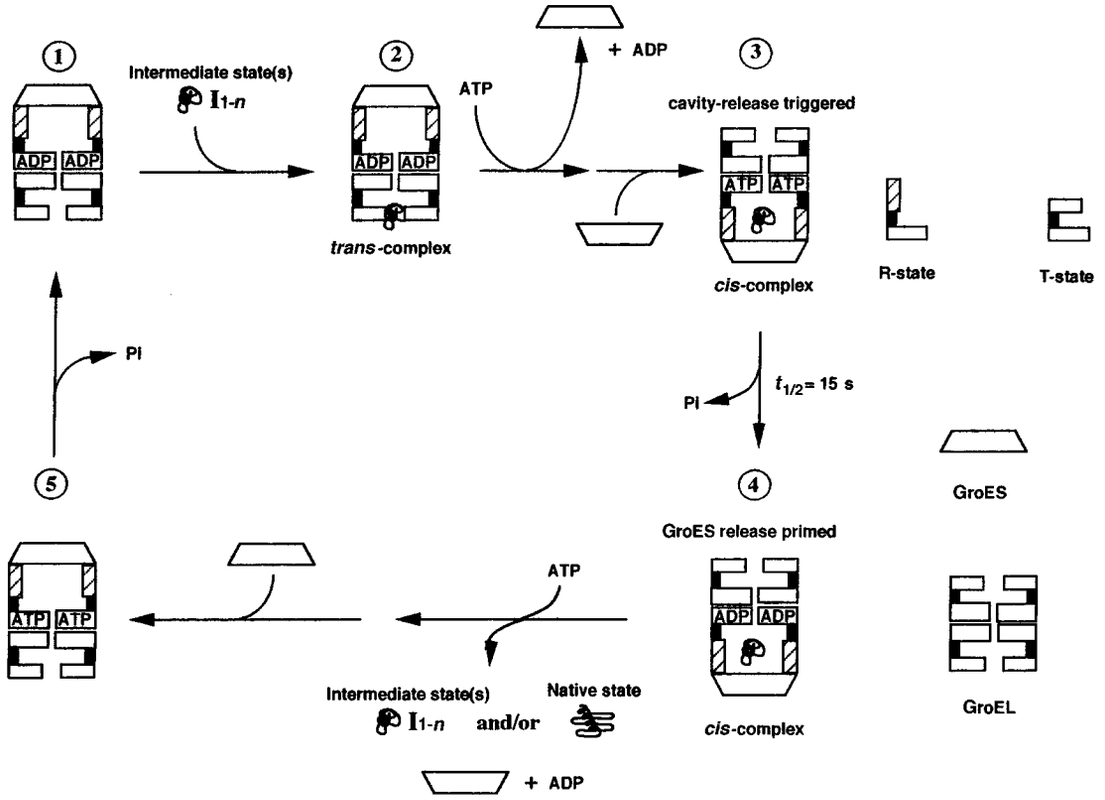
Molecular chaperone GroEL and cytochrome c were shown to form a stable complex at low ionic strength which was structurally characterised by means of UV-visible, CD and fluorescence spectroscopy. GroEL-bound cytochrome c was demonstrated to be in a compact, non-native state which could correspond to GroEL-bound forms of two well known cytochrome c folding intermediates I^{H}_{NC} and I*. These were selectively released from GroEL using adenosine 5′-triphosphate and co-chaperone GroES. Drawing from these results and our previous data, a simple passive kinetic partitioning mechanism is proposed for molecular chaperone assisted folding/refolding of substrate proteins in which molecular chaperone GroEL binds to substrate proteins in order to control the steady state concentration of substrate protein folding intermediates below the critical threshold for aggregation, thereby encouraging substrate protein folding intermediates to partition kinetically along routes to correctly folded protein in preference to aggregated states. In this way the yield of correctly folded protein may be maximised, unless the extrinsic folding conditions themselves otherwise prevent this happening by promoting protein misfolding through the formation of incorrect intramolecular interactions.

Introduction

Molecular chaperones assist the folding/refolding of other proteins without becoming part of the final folded structure.[1] Perhaps the best known and characterised molecular chaperones are the *Escherichia coli* (*E. coli*) molecular chaperone Cpn60 (GroEL) and the co-molecular chaperone Cpn10 (GroES). As a result, it is now well established that GroEL is a homo-oligomer comprising 14 subunits (each 57 259 Da) arranged in two stacked rings of 7 subunits each, whilst GroES consists of 7 subunits (each 10 368 Da) arranged in a single ring. Both GroEL and GroES have been extensively characterised by atomic force microscopy,[2] electron microscopy,[3,4] and the X-ray crystal structures of both GroEL and GroES are now available.[5–7] Schematic diagrams of GroEL and GroES are shown in Scheme 1 together with a schematic representation of the GroEL-GroES-$(ADP)_7$ "resting complex" **1**, which has been most recently characterised by X-ray crystallography.[6]

The mechanism by which GroEL, assisted by GroES and adenosine 5′-triphosphate (ATP), promotes substrate protein folding/refolding is still the subject of some debate. What is beyond doubt is the fact that GroEL will bind to a very wide range of unfolded/non-native protein substrates,[8] *via* hydrophobic interactions supplemented by short-range electrostatic interactions.[9–11] ATP and GroES binding followed by ATP hydrolysis then promote extensive conformational changes in the structure of GroEL which have an effect upon the affinity of GroEL for bound substrate protein.[3,6,12,13] Recently, Horwich and co-workers have reported some incisive new data, derived from experiments with GroEL mutants and X-ray crystal data, which has enabled them to knit this tapestry of information together and come up with a credible model for the interplay between GroEL, GroES, ATP and unfolded substrate protein.[6,14] This has been described as a "two stroke motor" mechanism.[15] The main features of this mechanism are illustrated (Scheme 1), borrowing from the notation of Sparrer and Buchner.[16] *trans*-Complex **2** probably forms from resting complex **1** at a very rapid rate ($>1 \times 10^8$ M^{-1} s^{-1}).[17] Thereafter, the rate of formation of *cis*-complex **3** is likely to be just a little slower,[14,16] but involves substantial conformational changes in GroEL itself accompanied by a volumetric expansion and a loss of hydrophobic binding regions which leads to a release of bound protein substrate into the cavity of *cis*-complex **3**. Rebinding of substrate protein to GroEL is not possible at this stage owing to the hydrophilic character of the cavity in *cis*-complex **3** following the conformational changes, and also the presence of the GroES "cap". A key feature of the two stroke motor mechanism is that *cis*-complex **3** must be converted to complex **4** before substrate protein folding intermediates may be released from the cavity, alongside adenosine 5′-diphosphate (ADP) and GroES, by a further step of ATP-binding in the *trans*-ring.[6,14] Moreover, the conversion of **3** to **4** appears to be on a regular clock ($t_{1/2} = 15$ s) and is a fixed slow step in the cycle.

However, the effect of this cyclical process of sequestration and release upon the folding of the substrate protein itself is still very much an open question. On the one hand, it has been suggested that GroEL/GroES assist substrate protein folding through the active unfolding (possibly catalytically) of misfolded substrate protein.[18–21] On the other hand, it has been suggested that GroEL/GroES may operate by actively promoting folding (possibly catalytically) of the substrate protein itself.[21,22] In the following, we shall describe results obtained whilst studying a stable, functional complex formed between GroEL and cytochrome c, a well characterised, small substrate protein (Fig. 1). Drawing from these results and our previous data, we come to an alternative conclusion that a passive kinetic partitioning mechanism is probably the most appropriate mechanism to account for GroEL/GroES-assisted folding of substrate proteins.



Scheme 1 Depiction of GroEL/GroES assisted folding/refolding of proteins, based upon observations of Rye *et al.*,[14] using a diagrammatic representation derived from Sparrer and Buchner.[16] Abbreviations are: ATP, adenosine 5′-triphosphate; Pi, inorganic phosphate; ADP, adenosine 5′-diphosphate. The T-state and R-state nomenclature,[12] refer to the conformations of individual GroEL subunits in the homo-oligomeric structure. The T-state has a high affinity for unfolded substrate protein and the R-state a low affinity (the affinity for ATP is reversed; *i.e.*, the T-state has a low ATP binding affinity and the R-state a high affinity). The term $\mathbf{I}_{1-n}$ refers to discrete substrate protein folding intermediates, **I**, of between 1 and *n* in number, all of which may interact with GroEL. See text for further details.

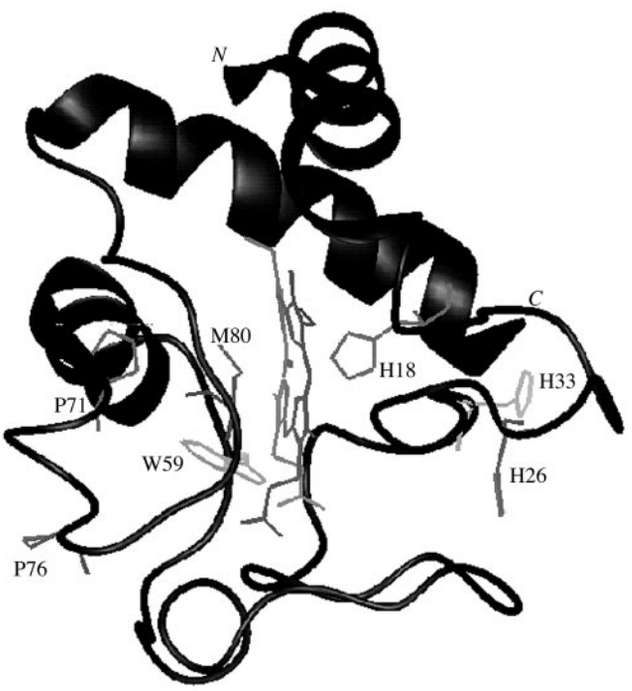


Fig. 1 Side view of the X-ray crystal structure of horse heart cytochrome c (Brookhaven Protein Data Bank: 1hrc).[60] The ribbon represents the α-carbon backbone. Positions of N and C termini of the polypeptide are indicated (N and C respectively), as are the positions of key amino acid residues mentioned in the text. The amino acid residue abbreviations are: H; histidine (H18, H26 and H33): M; methionine (M80): W; tryptophan (W59): P; proline (P71 and P76). The covalently attached heme group of cytochrome c may be seen at the centre of the structure, sideways on. The image was constructed using Quanta 97 (Molecular Simulations, CA, USA).

Experimental

Materials

Sephacryl S300 was purchased from Amersham Pharmacia Biotech, Bucks, UK. Horse heart cytochrome c was purchased from Sigma Chemical Co., Poole, Dorset, UK. All other chemicals used were of analytical grade or better and were also obtained from Sigma Chemical Co., Dorset, UK. Cytochrome c oxidase from beef heart was a gift from Professor M. T. Wilson, Department of Chemistry, University of Essex, Colchester, UK. Deionised distilled MilliQ water was used throughout. pH values of buffer solutions were adjusted at room temperature, irrespective of the temperature at which they were subsequently used. GroEL and GroES were purified from a recombinant strain of *Escherichia coli* according to methods described previously.[9] GroEL stock solutions (approx. 150 μM homo-oligomer concentration) were dialysed against a standard folding buffer A (50 mM Tris-Cl [tris(hydroxymethyl)-methylammonium chloride] buffer, pH 7.5, containing 2 mM ascorbate) ready for complex formation. GroES was prepared for use in a similar way. Concentrations of *E. coli* GroEL and GroES were evaluated as described previously,[9] and by Biorad protein assay, using bovine serum albumin as a standard, when ascorbate was present in the solutions. Cytochrome c concentration was calculated using the absorbance coefficient at A_{410} of 106 100 M^{-1} cm^{-1} and a molecular weight of 12 300 Da.[23] UV-visible spectroscopy was performed on a Amersham Pharmacia Biotech Ultrospec III with spectra recorded at 25 °C. CD spectra were obtained using a Jasco J-620 spectropolarimeter at 25 °C (1 cm pathlength cell) and where appropriate, corrected for background absorbance of GroEL. Fluorescence measurements were carried out at 25 °C using a Shimadzu RF5001PC spectrofluorophotometer with slit widths of 10 nm. All spectra were corrected for background fluorescence.

Complex formation

Unless otherwise stated, all steps in this section were performed at 4 °C. Cytochrome c (1.6 mM) was unfolded in buffer B (buffer A containing 6 M guanidinium chloride [Gu-HCl]). After 2 h at 20 °C, an aliquot (50 μl) of this solution was rapidly mixed at 4 °C with a 0.5 mol equivalent of GroEL (40 μM, homo-oligomer concentration) in buffer A (1 ml). This solution was then immediately applied to a column of Sephacryl S300 (1.6 × 78 cm) attached to an Amersham Pharmacia Biotech fast protein liquid chromatography (fplc) system, pre-equilibrated with the same buffer A. The column was eluted with buffer A, at a flow rate of 1.5 ml min^{-1}, and fractions (2 ml) were collected. Elution of GroEL-bound and unbound cytochrome c was monitored, fraction by fraction, at A_{410} and where appropriate, fractions containing GroEL-bound cytochrome c were combined and concentrated (Amicon, MA, USA, stirred cells) over 100 kDa cut-off membranes (Omega membranes, Flowgen Instruments, Kent, UK) until the GroEL-bound cytochrome c concentration in the retentate reached 10–20 μM. At this point, GroEL-bound cytochrome c was stored at 4 °C for up to 20 h before use.

Complex stability

Samples of GroEL-bound cytochrome c solution (20 μM cytochrome c concentration) were incubated at either 4 °C, 25 °C or 37 °C and at appropriate time intervals aliquots (150 μl) were removed and applied to a Superose-12 HR10/30 column (1 × 30 cm) attached to an Amersham Pharmacia Biotech fplc system, pre-equilibrated with buffer A. The column was eluted with the same buffer, at a flow rate of 0.8 ml min^{-1}, and fractions (1 ml) were collected and monitored for cytochrome c content by their absorbance at A_{410}.

Cytochrome c release

GroEL-bound cytochrome c (4 μM cytochrome c concentration) in buffer A, was divided into three equal aliquots (each 10 ml). The first aliquot was supplemented with $MgCl_2$ and KCl (final concentrations each 10 mM), the second with $MgCl_2$, KCl and ATP (final concentrations 10 mM, 10 mM and 2 mM respectively), and the third with $MgCl_2$, KCl, ATP, and GroES (final concentrations 10 mM, 10 mM, 2 mM and 8 μM respectively). After 1 h stirring at 20 °C, each aliquot was separately transferred to a 100 kDa centricon concentrator and centrifuged (1000 g, 1.5 h) at 4 °C to separate released cytochrome c from GroEL. For each aliquot, the amount of cytochrome c released from GroEL, cyt $c_{released}$, was estimated as a percentage of the total amount of cytochrome c present, cyt c_{total}, by means of eqn. (1), where $[cyt\ c]_{released}$ was the concentration of cytochrome c in the centricon filtrate, as determined by absorbance at A_{410}, and vol_{assay} the total aliquot volume (approx. 10 ml). Eqn. (1) was derived from a similar equation used elsewhere for a similar purpose.[24] Cytochrome c in the retentate was usually transferred to a 3 kDa centricon concentrator and repeatedly washed by dilution with buffer A, and centrifugation (1000 g, approx. 1 h). After this, cytochrome c in the retentate was evaluated as a substrate for cytochrome c oxidase in a redox-coupled assay.

$$cyt\ c_{released} = 100 \times [(vol_{assay} \times [cyt\ c]_{released})/cyt\ c_{total}] \quad (1)$$

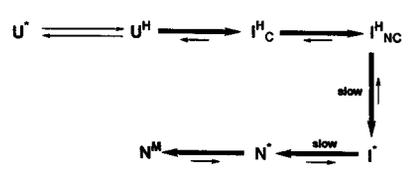
Redox-coupled assay

Assays were performed at room temperature in a specially designed sealed reaction vessel attached to the oxygen electrode. Cytochrome c oxidase (stored frozen at −20 °C) was diluted to a concentration of 59 nM in an assay buffer C (Tris-acetate 25 mM, pH 7.4, containing Tween-80 [0.1%, v/v]) and then ascorbate and *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) were added (final concentrations 7 mM and 0.7 mM respectively). The electrode reading (read as percentage oxygen saturation) was allowed to stabilise and then cytochrome c (0.4–5 μM final concentration) was carefully introduced by microsyringe. The change in percentage oxygen saturation was observed over 3 min and then converted into a rate of cytochrome c oxidation in units of μM s^{-1}. This conversion was made on the basis that cytochrome c is a one electron donor whilst oxygen is a four electron acceptor so that four molecules of cytochrome c are needed for cytochrome c oxidase to reduce one molecule of oxygen. Also, each one percent fall of oxygen saturation corresponds to a decline of 2.5 μM in oxygen concentration.[25] All assays were carried out using a Jenway 9010 polarographic oxygen electrode connected to a Corning 240 pH meter. Before use, the electrode was always calibrated in air (100% oxygen) followed by immersion in 2% w/v sodium sulfite solution (0% oxygen).

Results and discussion

Without doubt, cytochrome c is one of the best characterised small proteins (Fig. 1). The spontaneous folding pathway and structures of various folding intermediates have been extensively characterised over the last few years.[26–31] In addition, spectroscopic analysis of the covalently bound heme group has enabled the conformational state of the protein to be analysed in a wide range of circumstances and under a wide range of conditions.[32] For these reasons, we considered that cytochrome c would be an ideal small protein substrate to investigate the conformational behaviour of a substrate protein bound to GroEL. Our basic strategy was to combine unfolded cytochrome c with GroEL by manual-mixing and isolate by gel filtration a GroEL–cytochome c complex which could then be studied spectroscopically.

Small single domain proteins (<20 kDa) usually fold spontaneously in a matter of a few milliseconds. However at neutral pH, cytochrome c folds rather more slowly than might be anticipated taking anything up to 10 s to reach native state.[28,29] The presence of the heme group is the main reason for this anomalous behaviour. In the native state of cytochrome c, the heme iron atom is axially coordinated by H18 and M80 (Fig. 1). At neutral pH, the predominant unfolded state of cytochrome c is known as U^H. In this state, the heme iron remains coordinated by His18 but the Met80 ligand found in the native state is replaced either by H26 or H33 (Fig. 1).[29] Of these latter two residues, H33 is used most frequently.[30] The first event in folding at low denaturant concentrations (<1.5 M guanidinium chloride [Gu-HCl]), is the rapid formation (≈1 ms) of a compact intermediate, I^H_C (Scheme 2). Folding then continues



Scheme 2 Cytochrome c folding pathway adapted from Colón *et al.*[29] **U** correspond to unfolded states and **I** to intermediate states. **N*** is a native-like state of cytochrome c and $\mathbf{N^M}$ the final biologically active native state of cytochrome c. See text for details. Reprinted with permission from *Biochemistry*, 1996, **35**, 5538. Copyright 1996, American Chemical Society.

through a partially folded intermediate, I^H_{NC}, which is stabilised through the interaction of the main *N*- and *C*-terminal α-helices.[31] In this intermediate, the heme iron still remains coordinated by either H26 or H33, thereby preventing the formation of stable secondary and tertiary structure in other regions of the protein. In order for the native state to form, this non-native histidine residue must dissociate to form a five-coordinate intermediate, I*, with similar structural properties. This dissociation step is one of the key rate limiting steps in

cytochrome c folding at neutral pH. The folding process is then completed by slow *cis* to *trans* isomerisation of proline residues P71 and P76 to give a five-coordinate intermediate N*. The native state, N^M, is then rapidly assembled by the coordination of M80 to the heme iron atom (Scheme 2).[28,29]

GroEL is well known to recognise and interact with many different unfolded states of protein substrates ranging from "early folding intermediates" and "molten-globules", to "late folding intermediates", not to mention misfolded states, but not to native states of proteins.[24,33–35] Given the anomalously slow folding of cytochrome c at neutral pH, we anticipated that there would be sufficient time for some intermediate folded states of cytochrome c to interact with GroEL if unfolded cytochrome c were diluted into a stirred, concentrated solution of the molecular chaperone. We were supported in this supposition by Hoshino *et al.*,[36] who estimated that the rate constant for apocytochrome c binding to GroEL is 7.8×10^7 M^{-1} s^{-1} implying that substantial GroEL-binding of unfolded states of cytochrome c could take place within 100 ms to 1 s at high GroEL concentrations (>1.6 μM homo-oligomer concentration). In the event, our best binding results were obtained when an aliquot of 6 M Gu-HCl unfolded cytochrome c (80 μM, final concentration) was mixed with 0.5 mol equivalent of GroEL (40 μM homo-oligomer concentration) at 4 °C in low ionic strength buffer (standard folding buffer A). Approximately 30–40% of the cytochrome c was found routinely to co-elute with GroEL when the mixture was resolved by gel filtration on S300-medium (size separating range 10 kDa to 1500 kDa) suggesting that a stable 1 : 1 complex had been formed between cytochrome c and GroEL (Fig. 2a). This complex was still in evidence after several hours concentrating (to a cytochrome c concentration of approximately 10–20 μM) at 4 °C (over a 100 kDa membrane) and proved reasonably stable to overnight storage at 4 °C (Fig. 3). There is a substantial size difference between cytochrome c (approx. 12 kDa) and GroEL (approx. 850 kDa). Therefore a clean peak separation should reasonably have been expected between GroEL-bound cytochrome c and unbound cytochrome c using S300-medium (see Fig. 2b). However, no such clean separation was found owing to the tendency of a proportion of the cytochrome c to elute as a broad band covering the molecular weight range from GroEL-bound to monomeric cytochrome c. This same broad band was also observed when spontaneously refolded cytochrome c was eluted through the S300 column (Fig. 2c). Therefore, we concluded that a proportion of Gu-HCl unfolded cytochrome c was probably forming high molecular weight cytochrome c aggregates following dilution into the standard folding buffer. Indeed, cytochrome c is well known to form such high molecular weight aggregates.[32] Fortunately, these high molecular weight cytochrome c species only very slightly co-eluted with the tail-end of the GroEL-bound cytochrome c peak (Fig. 2). Hence, with careful selection of gel filtration fractions, we were able to isolate what appeared to be homogeneous GroEL-bound cytochrome c, as judged by further gel filtration experiments (Fig. 3).

Spectroscopic characterisation of this complex was performed to determine the conformational state of the GroEL-bound substrate protein. However prior to this, stability studies were conducted to ensure that GroEL-bound cytochrome c would be sufficiently stable to obtain meaningful spectroscopic data at the concentrations and temperatures required for spectroscopic analysis. According to Superose-12 analytical gel filtration experiments, GroEL-bound cytochrome c appeared to be stable over a period of 20 h at 4 °C, partially so at 25 °C, and unstable at 37 °C (Fig. 3). These results suggested that reliable spectroscopic data could be acquired on GroEL-bound cytochrome c provided that the complex was prepared and stored at 4 °C and provided that spectroscopic analyses were then performed either at 4 °C or within a few hours at 25 °C. Consequently, the latter protocol was followed for all subsequent spectroscopic analyses of GroEL-bound cytochrome c. The

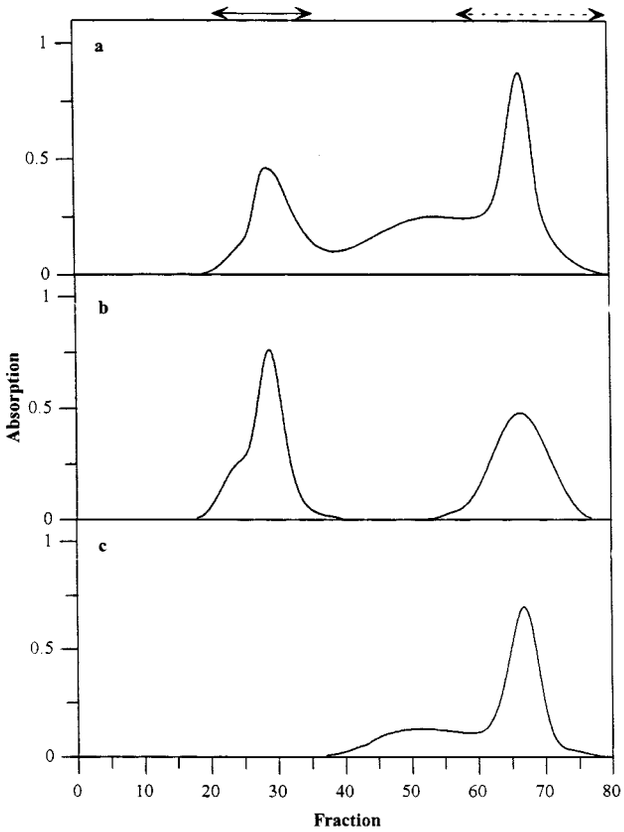


Fig. 2 Gel filtration elution profiles. (a) An aliquot of guanidinium chloride (Gu-HCl) unfolded cytochrome c (80 μM final concentration) was combined with GroEL (40 μM homo-oligomer concentration) in standard folding buffer A (1 ml) at 4 °C. After mixing, the mixture was eluted through a Sephacryl S-300 gel-filtration column. Fractions (2 ml) were analysed for the presence of cytochome c by their absorption at 410 nm. GroEL-bound cytochrome c elutes first (fractions 25–35 approx.). (b) Native cytochrome c (80 μM final concentration) and GroEL (40 μM homo-oligomer concentration) in standard folding buffer A (1 ml) were eluted through the S-300 column in the same way as above. GroEL, eluting first (fractions 25–35 approx.), was detected by absorption at 280 nm; native cytochrome c, eluting second (fractions 65–75 approx.) was detected by absorption at 410 nm. (c) Spontaneously refolded cytochrome c, formed by diluting an aliquot of Gu-HCl unfolded cytochrome c (40 μM final concentration) into standard folding buffer A (1 ml), was eluted through the S-300 column as above and the presence of cytochrome c detected by absorbance at 410 nm.

instability of the complex at 25 and 37 °C as compared to 4 °C is probably due to two main reasons. Firstly, GroEL-bound cytochrome c must necessarily be in equilibrium with unbound cytochrome c even though the interaction is probably characterised by a low dissociation constant of about 10 nM or so.[13,24] Clearly unbound cytochrome c would have the potential to fold to the native state as an alternative to binding to GroEL and this would be more likely at higher temperatures (25 or 37 °C) when cytochrome c folding is faster. Therefore, lower temperatures would favour complex stability and persistence. Secondly, it is now well established that the interaction between GroEL and bound substrate proteins results from a combination of hydrophobic and electrostatic interactions,[9–11] of which electrostatic forces are frequently stronger at lower rather than higher temperatures. Given the fact that GroEL has a net negative charge at neutral pH (pI 4.5)[37] and cytochrome c a net positive charge (pI > 9), the interaction between GroEL and cytochrome c at neutral pH is likely to involve significant electrostatic interactions which would of course be enhanced at lower, rather than higher temperatures.

Initially, the UV-visible absorption spectrum of GroEL-bound cytochrome c was recorded between 280 and 600 nm and compared with the spectra of native and unfolded cytochrome c (Fig. 4). GroEL-bound and native cytochrome c were found to

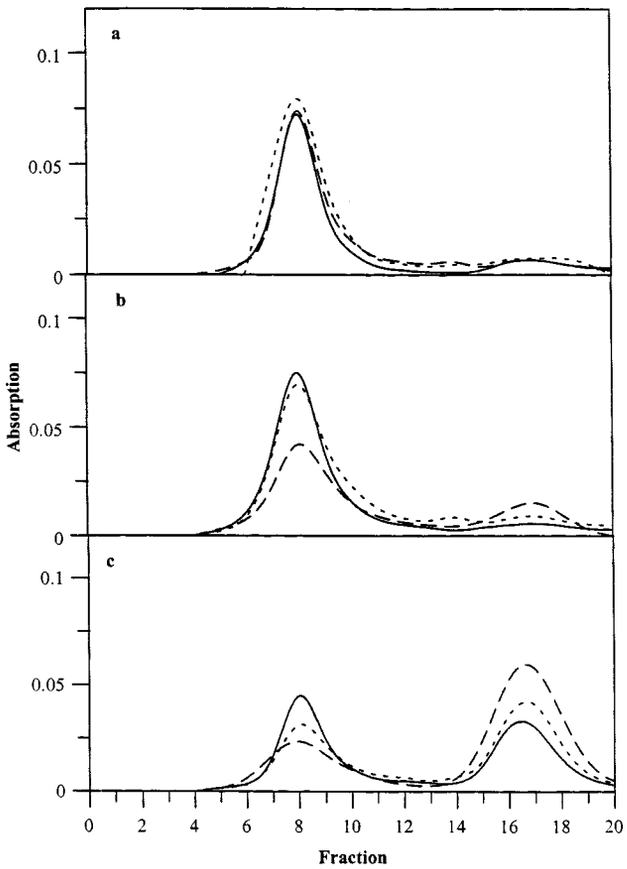


Fig. 3 Thermal stability of GroEL-bound cytochrome c complex. Complex was prepared as in Fig. 2 and then concentrated (20 μM final cytochrome c concentration). Aliquots were incubated at 4 °C (a), 25 °C (b) and 37 °C (c) and then individual samples eluted through a Superose-12 gel filtration column at 1 h (——), 2 h (- - -) and 20 h (– – –). Fractions (1 ml) were analysed for the presence of cytochome c by their absorption at 410 nm. GroEL-bound cytochrome c elutes first (fractions 7–9 approx.); cytochrome c released from binding interaction elutes second (fractions 16–18 approx.).

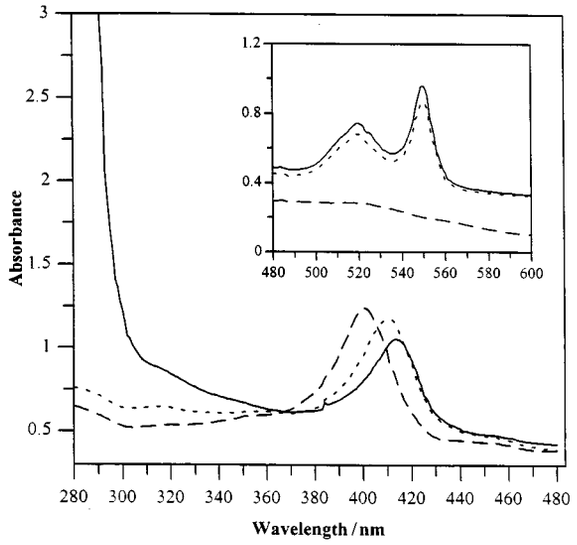


Fig. 4 UV-visible absorption spectra of cytochrome c. Spectra of GroEL-bound cytochrome c (——), native cytochrome c (- - -) and Gu-HCl unfolded cytochrome c (– – –) (all 10 μM cytochrome c concentration) in standard folding buffer A. Inset: detail of α/β bands from spectra recorded at 20 μM cytochrome concentration. All spectra were recorded within a few minutes of equilibration from 4 °C to 25 °C. The spectrum of GroEL-bound cytochrome c was recorded within 1 h after the complex had been prepared and stored at 4 °C.

have some similarities in their UV-visible spectra. Their respective Soret maxima appeared at slightly different wavelengths (416 and 410 nm respectively). By contrast, the α/β bands (π–π* transition) at 520 and 550 nm were essentially identical. Soret maxima characteristically report on changes in the spin state of the heme iron due to changes in the axial ligands.[27] Therefore, the small difference between the observed Soret maxima suggested that there may be differences in the coordination spheres surrounding the heme iron in GroEL-bound and native cytochrome c respectively. This possibility was reinforced when we were unable to detect a weak absorption band at 695 nm in the UV-visible spectrum of the GroEL-bound cytochrome c (results not shown). The 695 nm band is a charge transfer band which is diagnostic for M80 coordination to heme iron,[38] and this band is normally lost when the S–Fe bond between M80 and heme iron is disrupted as is the case in all non-native states of cytochrome c. The spectrum of unfolded cytochrome c (Fig. 4) showed all the expected spectral hall-marks of a disordered structure, namely a displaced Soret maximum (blue-shifted to 403 nm) and an absence of both α/β bands and the charge transfer band. The α/β bands are not normally seen in the UV-visible spectrum of unfolded cytochrome c owing to line-broadening resulting from conformational freedom in the unstructured polypeptide.[32]

The circular dichroism spectrum of GroEL-bound cytochrome c was recorded between 280 and 600 nm and compared directly with the spectra for native and unfolded cytochrome c under identical conditions (Fig. 5). In the case of GroEL-bound cytochrome c, far and near-UV CD proved uninformative owing to the large GroEL absorbtion which overwhelmed any contribution from cytochrome c. However, there was little or no GroEL interference in the Soret region (300–600 nm), therefore visible light CD in the Soret region proved to be much more informative. Soret region CD has been shown in the past to report on the integrity of the heme crevice of cytochrome c.[39] Optical activity results from the coupling of heme π–π* electric dipole transition moments with those of nearby aromatic amino acid residues in the protein.[40] Hence CD spectroscopy involving the heme ligand can be a potent tool to probe the integrity of the native state of cytochrome c. The CD spectra of both GroEL-bound and native cytochrome c both showed strong negative features in the 320–380 nm region, although these features were more strongly negative in the spectrum of the native protein than in that of the GroEL-bound form (Fig. 5). Such negative features derive from the Soret–Cotton effect primarily as a result of heme–polypeptide interactions. The fact that the negative features were more intense in the native spectrum suggested that heme–polypeptide interactions were probably stronger in native than in GroEL-bound cytochrome c, thereby implying that GroEL-bound cytochrome c was less compact than native cytochrome c. In keeping with this analysis, negative features were largely absent from the spectrum of unfolded cytochrome c presumably because of wholesale disruption in the coupling between π–π* transitions of the heme and neighbouring aromatic amino acid residues caused by the loss of protein tertiary structure with unfolding (Fig. 5).

Further information about the nature of GroEL-bound cytochrome c could be deduced by comparing the positive features of the CD spectra of unfolded, GroEL-bound and native cytochrome c in the 380–440 nm Soret region (Fig. 5). The CD spectrum of unfolded cytochrome c showed a single maximum at 400 nm which is the expected spectral hall-mark of cytochrome c unfolded either by chaotropic agents such as Gu-HCl, temperature, extrinsic ligands, pH or carboxymethylation.[41] In comparison, the CD spectra of both GroEL-bound and native cytochrome c showed maxima at 420 nm, the former more intense. Furthermore, the spectrum of GroEL-bound cytochrome c showed an additional maximum at 408 nm. The presence of these two maxima suggested that GroEL-bound cytochrome c may not be stabilised in one but in two different states, one of which was closer to the native state. These could

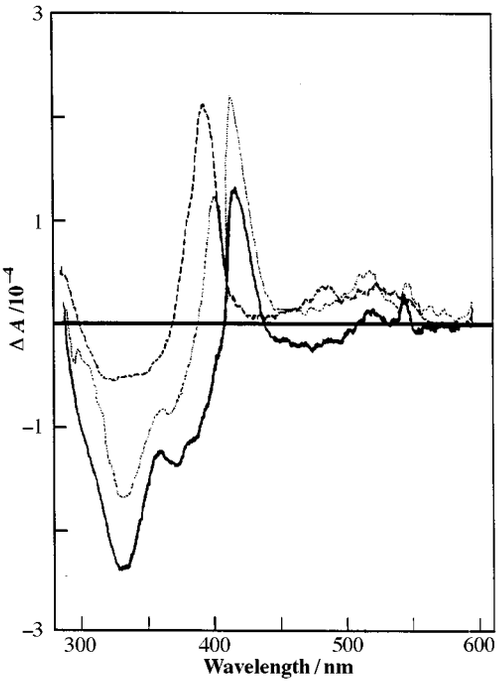


Fig. 5 CD spectra of cytochrome c. Spectra of GroEL-bound cytochrome c (.....) , native cytochrome c (——) and Gu-HCl unfolded cytochrome c (– – –) (all 16 μM cytochrome c concentration) in standard folding buffer A. Spectra were recorded within a few minutes of equilibration from 4 °C to 25 °C, in a 1 cm pathlength cell. The spectrum of GroEL-bound cytochrome c was recorded within 1 h after the complex had been prepared and stored at 4 °C.

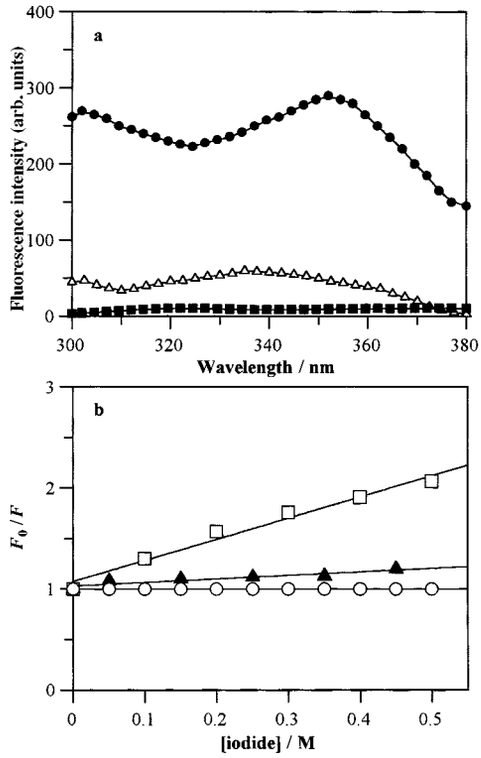


Fig. 6 Aromatic fluorescence spectra of cytochrome c. (a) Aromatic fluorescence spectra of Gu-HCl unfolded cytochrome c (●), GroEL-bound cytochrome c (△) and native cytochrome c (■) (all 1 μM cytochrome c concentration) in standard folding buffer A. Spectra were recorded within a few minutes of equilibration from 4 °C to 25 °C, in a 1 cm pathlength cell with slit widths of 10 nm and an excitation wavelength of 280 nm. The spectrum of GroEL-bound cytochrome c was recorded within 1 h after the complex had been prepared and stored at 4 °C. The spectrum was corrected for GroEL (1 μM homo-oligomer concentration) background fluorescence. (b) Change in the fluorescence intensity (F), relative to the initial intensity (F_0), of the tryptophan (W59) fluorescence maxima of Gu-HCl unfolded cytochrome c (□), GroEL-bound cytochrome c (▲) and native cytochrome c (○) (all 1 μM cytochrome c concentration), as a function of iodide quenching agent concentration. Intensity data was processed with the Stern–Volmer eqn. (2). Quenching constants, K_q, for iodide and acrylamide quenching agents are summarised (Table 1).

conceivably correspond with GroEL-bound forms of folding intermediates I^{H}_{NC} and I* which would certainly accumulate in solution once folding was initiated and persist for long enough (approx. 1 s) to allow for capture and binding to GroEL (Scheme 2). Gervasoni *et al.*[10,42,43] have demonstrated that β-lactamase may bind to GroEL in at least two different intermediate folded states, so our suggestion that at least two different intermediate folded states of a protein may bind to GroEL under manual-mixing conditions and be stabilised simultaneously is not improbable though must be treated with caution in the absence of additional evidence.

The compact nature of GroEL-bound cytochrome c was further established by fluorescence spectroscopy. Using an excitation wavelength of 280 nm which excites both tyrosine and principally tryptophan amino acid residues, a direct comparison was made between the fluorescence spectra of unfolded, GroEL-bound and native cytochrome c in the region 300 to 380 nm (Fig. 6a). In line with previously published data,[32] Gu-HCl unfolded cytochrome c exhibited a strong fluorescence emission spectrum from the aromatic amino acid residues, including a maximum at 353 nm mainly attributable to fluoresence emission from the single tryptophan residue in cytochrome c, tryptophan 59 (W59). By complete contrast, native cytochrome c yielded a broad, weak fluorescence emission spectrum with no clearly discernable maxima in the spectral window observed. The weakness of this emission spectrum is likely due to Förster energy transfer between aromatic residues and the heme group which occurs when both heme group and aromatic residue side-chains are brought into close proximity in the native state.[44] GroEL-bound cytochrome c also yielded only a weak fluorescence emission spectrum, but one with a distinct W59 maximum (336 nm) blue-shifted by 17 nm relative to the observed maximum in unfolded cytochrome c. This blue shift is consistent with the movement of W59 from a polar environment in unfolded cytochrome c, to a buried hydrophobic environment in GroEL-bound cytochrome c, once again confirming that GroEL-bound cytochrome c was in a compact state. However, the very fact that Förster energy transfers were not as efficient in GroEL-bound cytochrome c as in native cytochrome c once again implies that GroEL-bound cytochrome c was in a less compact state than native cytochrome c, in line with the CD data reported above. Steady state fluorescence quenching experiments provided further evidence to confirm the nature of the compact state of GroEL-bound cytochrome c. Quenching experiments were performed using either acrylamide or iodide as quenching agents and the change in fluorescence intensity of the W59 maximum observed as a function of the concentration of each separate quenching agent. Results obtained using iodide are shown (Fig. 6b). Quenching effects were then evaluated using the Stern–Volmer eqn. (2), where F_o and F are fluorescence intensities at the

$$F_o/F = 1 + K_q[Q] \quad (2)$$

tryptophan fluorescence emission maximum in the absence and presence of quenching agent respectively, [Q] is the concentration of quenching agent and K_q is the apparant quenching constant. The complete set of K_q values is given (Table 1). According to these data, native cytochrome c fluorescence was unaffected by the presence of either quenching agent in marked contrast to unfolded cytochrome c where the W59 fluorescence maximum declined linearly with the concentration of either quenching agent. Obviously, W59 in native cytochrome c is buried in the core of the protein and should not be accessible to

Table 1 Fluorescence emission maxima, $\lambda_{max\ em}$, and fluorescence quenching constants, K_q, obtained from fluorescence spectra of Gu-HCl unfolded cytochrome c, GroEL-bound cytochrome c and native cytochrome c (all 1 μM cytochrome c concentration) in standard folding buffer A. Fluorescence quenching constants, K_q, were obtained using the Stern–Volmer eqn. (2). Quencher concentrations were varied from 0–0.6 M. All spectra were recorded within a few minutes after equilibration from 4 °C to 25 °C; spectra of GroEL-bound cytochrome c were all recorded within 1 h after the complex had been prepared and stored at 4 °C.

Cytochrome c	$\lambda_{max\ em}$/nm	K_q(iodide)/M^{-1}	K_q(acrylamide)/M^{-1}
Gu-HCl unfolded	353	2.0	3.8
GroEL-bound	336	0.4	0.5
Native	*a*	0	0

[a] Native cytochrome c produced a broad, flat fluorescence emission spectrum with no clearly discernable maxima.

quenching agents, whilst the opposite is true of W59 in unfolded cytochrome c. The modest effect of quenching agents upon the W59 fluorescence of GroEL-bound cytochrome c, is completely consistent with GroEL-bound cytochrome c being in a compact state close to the native state, but sufficiently flexible for some fluorescence quenching to be observed. Similar fluorescence results have been obtained with GroEL-bound rhodanese, α-glucosidase, and dihydrofolate reductase.[45]

Frequently, when complexes between GroEL and a protein substrate have been studied in the past, specific release of bound protein substrate with the assistance of adenosine 5′-triphosphate (ATP) and the co-chaperone GroES has not been demonstrated. Therefore, the functional relevance of the observations has not been properly established. In the absence of such release data, it is not possible to know whether structural observations of a complex between GroEL and a substrate protein are artifactual or not. Therefore, we devised an ultrafiltration release assay in order to examine the effect of different release conditions on GroEL-bound cytochrome c. In this release assay, GroEL-bound cytochrome c was incubated at 20 °C in standard folding buffer containing either salts ($MgCl_2$ and KCl), ATP and salts, or ATP with GroES and salts. Released cytochrome c was then separated from GroEL-bound cytochrome c by means of 100 kDa centricon concentrators at 4 °C. This concentrator system was chosen so that any remaining GroEL-bound cytochrome c, with a combined molecular weight well in excess of 800 kDa, would be retained in the retentate whilst released cytochrome c (with a molecular weight of approx. 12 kDa) would be separated into the filtrate. Where GroEL and GroES were employed in the presence of ATP and $MgCl_2$, we had observed (unpublished observations) that GroES (approx. 70 kDa) was retained in the retentate, probably by forming a resting state complex **1** with GroEL (see Scheme 1). The percentage recoveries of cytochrome c released from GroEL under the various conditions of incubation was determined showing a clear hierarchy of release conditions with GroES and ATP together being the most efficient agents of release, ATP alone the next best agent and simple incubation the worst (Table 2). In other words, specific release did appear indeed to be taking place. The extent of cytochrome c release under conditions of simple incubation is consistent with both the thermal instability of GroEL-bound cytochrome c analysed previously (Fig. 3), and the fact that the association between GroEL and cytochrome c is governed by significant electrostatic forces which would be weakened by the presence of $MgCl_2$ and KCl in the incubation buffer (Table 2). Cytochrome c released from GroEL with the aid of GroES and ATP was recovered and concentrated (3 kDa centricon concentrators) and then shown to be a functional electron transfer substrate for the enzyme cytochrome c oxidase in a redox-coupled assay (Table 2).[25] The efficiency of electron transfer from cytochrome c to cytochrome c oxidase is well known to depend on the native state conformational integrity of cytochrome c.[46] Therefore, these electron transfer results leave little doubt that released cytochrome c was able to attain a biologically active native state following release from GroEL. Intriguingly, released cytochrome c did show some tendency to aggregate if released from GroEL at concentrations greater than 4 μM, suggesting that cytochrome c was not being initially released from binding interaction with GroEL in the native state but instead in the form of folding intermediates, such as I^H_{NC} or I*, which would then have to complete the folding process to the native state initially in the GroEL-cavity (Scheme 1), and then afterwards in free solution. This observation is perfectly consistent with the results of others, who have demonstrated with a number of other model proteins that GroEL-bound substrate proteins are released from binding interaction with GroEL in non-native states.[18,47]

Table 2 Results of release studies using GroEL-bound cytochrome c together with the corresponding results of redox-coupled assays performed using cytochrome c released from GroEL. GroEL-bound cytochrome c (4 μM cytochrome c concentration) was incubated at 20 °C for 1 h in standard folding buffer A containing 10 mM $MgCl_2$ and 10 mM KCl together with release factors as appropriate. Released cytochrome c was separated from GroEL by means of 100 kDa centricon concentrators at 4 °C and tested for conformational and biological integrity as a substrate for cytochrome c oxidase in a redox coupled assay.

Release factors	Release of cytochrome c (%)	Cytochrome c oxidase activity	
		k_{cat}/s^{-1}	K_m/μM
GroES,[a] ATP[b]	62	4.4	1.4[c]
ATP[b]	25	n/d[d]	
none	6	n/d[d]	

[a] 8 μM homo-oligomer concentration. [b] 2 mM concentration. [c] k_{cat} and K_m values of 3.4 s^{-1} and 3.2 μM, respectively, were found using spontaneously refolded monomeric cytochrome c as a substrate for cytochrome c oxidase. Spontaneously refolded cytochrome c was obtained as described in the legend to Fig. 2. [d] n/d, not determined owing to low concentration of cytochrome c recovered after release.

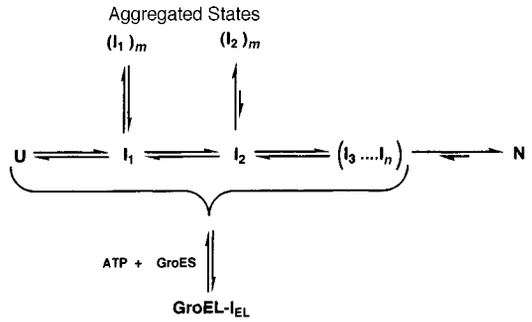
The foregoing data interlock to support the view that GroEL-bound cytochrome c is in a compact state which could correspond to GroEL-bound forms of cytochrome c folding intermediates I^H_{NC} and I* (Scheme 2). Gratifyingly, the GroEL-bound states of some other substrate proteins have also been reported to be compact, non-native states. For instance, the GroEL-bound state of α-lactalbumin has been shown to be either a "molten globule"[48] or a "pre-molten globule" state.[49] Similarly GroEL-bound luciferase was found to be in a molten globule state.[35] However, by contrast Gervasoni *et al.*[10,42,43] have recently demonstrated that GroEL-bound β-lactamase is either truely native-like in state or else disordered and dynamic, depending upon whether heat or Gu-HCl were used to unfold this substrate protein prior to association with GroEL. Furthermore, GroEL-bound dihydrofolate reductase was also shown to possess significant native-like structure.[50] Still other research groups have shown that very small proteins such as cyclophilin or barnase form only transient associations with GroEL and appear to be actively unfolded into disordered, dynamic states by interaction with GroEL.[19] In other words, the degree of structure and conformational mobility of GroEL-bound substrate proteins appears to vary, sometimes substantially, from one model protein to the next. As stated previously, GroEL is known to recognise and interact with a wide range of unfolded states of substrate proteins ranging from "early folding intermediates" and "molten-globules", to "late folding intermediates", not to mention structured but misfolded states.[24,33–35] Therefore, these observed states of GroEL-bound substrate proteins are probably the result of an initial interaction between GroEL and a number of different substrate

protein unfolded states, followed by equilibration. This process of equilibration leads either to a modicum of structural distortion,[10,42,43,49] or alternatively to a great deal of distortion.[19] Given all this variety, we would suggest that the nature of the observed state(s) of a GroEL-bound substrate protein is essentially a function of the requirement to optimise the free energy of association between GroEL and the given substrate protein under the given set of binding conditions. In other words, the observed GroEL-bound state(s) is a minimum energy state(s) appropriate for the substrate protein in question and the conditions of binding. There is unlikely to be any further functional significance than this. Therefore, claims that the general purpose of substrate protein binding to GroEL is either to actively drive the unfolding of misfolded proteins,[13,18–21] or alternatively to actively drive the correct folding of the substrate protein,[21,22] appear to be inappropriate.

Conclusions

Given the above discussion, how might molecular chaperone GroEL and co-chaperone GroES actually assist protein folding/refolding? The theory of protein folding is by no means secure at this stage, therefore it is perhaps a little premature to even discuss this question. Nevertheless, taking evidence from some of the most recent current theoretical and experimental studies, protein folding initially appears to involve a broad continuum of unfolded protein microstates which is reduced by hydrophobic collapse, and other imposed conformational restraints, into a smaller and smaller population of intermediate states which eventually converge in a rate determining manner on the final folded, biologically active native state of the protein.[51] These intermediate states which precede the rate determinining final step(s) of folding frequently appear to be vulnerable to aggregation. Therefore, it is aggregation, along with irreversible misfolding under some conditions, which represent the greatest problems for efficient protein folding.[52,53] Aggregation is a bi-/multimolecular phenomenon whose rate depends upon the *n*th power of protein concentration (where *n* is >2).[52] By contrast, the folding of a single polypeptide is a unimolecular phenomenon (where *n* is 1). Therefore, the rate of aggregation increases with protein concentration whilst the rate of folding remains constant. Hence, if the steady state concentrations of protein folding intermediates could be maintained below a critical threshold for aggregation, and the extrinsic conditions of folding adjusted to minimise or even prevent misfolding, then the yield of correctly folded, biologically active protein would be optimal.

Molecular chaperone GroEL and co-chaperone GroES probably have little control over the extrinsic conditions of protein folding. However, they could control the steady state concentrations of protein folding intermediates simply through the cyclical sequestration and release of substrate protein folding intermediates, which would be possible with the two stroke motor mechanism described previously (Scheme 1). Therefore, in our view, the function of GroEL substrate protein binding interactions of the type we have observed with cytochrome c is simply to sequester and protect a wide range of vulnerable substrate protein folding intermediates so as to prevent them aggregating through interaction of their exposed hydrophobic surfaces. The chaperone system then releases bound substrate protein in a controlled and cyclical manner so as to maintain the steady state concentration of protein folding intermediates below the critical threshold for aggregation. In so doing, substrate protein folding intermediates are encouraged to partition kinetically along routes to correctly folded protein in preference to aggregated states, thereby maximising the yield of correctly folded protein; that is unless the extrinsic folding conditions themselves otherwise prevented this happening by promoting protein misfolding through the formation of incorrect intramolecular interactions (Scheme 3).[24,53] Such misfolded substrate



Scheme 3 Proposed mechanism of GroEL-assisted folding/refolding of proteins. This mechanism assumes that protein folding is initiated at an unfolded state, **U**, which folds through a succession of intermediate states $\mathbf{I_1}$, $\mathbf{I_2}$, $(\mathbf{I_3...I_n})$ before reaching the native state, **N**. States $\mathbf{I_1}$ and $\mathbf{I_2}$ are considered arbitrarily to be unstable to aggregation, forming aggregated states $(\mathbf{I_1})_m$ and $(\mathbf{I_2})_m$ through interaction of their exposed hydrophobic surfaces. GroEL is able to bind to most states of the folding protein, except **N**, forming a GroEL-bound state **GroEL-$\mathbf{I_{EL}}$**. The nature of this state is a function of the requirement to optimise the free energy of association between GroEL and the given unfolded protein state under the given set of binding conditions. Binding interaction with GroEL is reversed in a controlled manner with the assistance of GroES and adenosine 5′-triphosphate (ATP) (see Scheme 1). As a result of this cyclical binding and controlled release into a GroEL cavity and then free solution, steady state concentrations of **U**, $\mathbf{I_1}$, $\mathbf{I_2}$ and $(\mathbf{I_3...I_n})$ are maintained below the critical threshold for aggregation so that these states are free to partition kinetically to **N**; unless the extrinsic folding conditions themselves otherwise prevent this happening by assisting **U**, $\mathbf{I_1}$, $\mathbf{I_2}$ and/or $(\mathbf{I_3...I_n})$ to form incorrect intramolecular interactions which lead to misfolded states unable to form **N**. In principle, these misfolded states could also bind/rebind to GroEL but unless their individual free energies of association exceed the energies of the incorrect interactions, GroEL is unlikely to have the opportunity to rescue these states and enable them to partition kinetically to form further **N**.

proteins could in principle rebind/bind to GroEL but unless the free energy of association were to exceed the combined energy of these incorrect interactions, then the misfolded protein would not necessarily be rescued.[24] This is essentially a passive kinetic partitioning mechanism which would involve no catalysis of productive protein folding pathways or any other significant intervention with such pathways.

There is experimental evidence to support our passive mechanism. For instance, GroEL-bound substrate proteins do frequently appear to be released from GroEL in non-native states, as described above and elsewhere.[18,47] Furthermore, we were able to demonstrate a few years ago that GroEL and GroES do not catalyse the productive protein folding pathway of mitochondrial malate dehydrogenase but instead increase the flux through this pathway by increasing the effective concentration of folding competent intermediates, in other words passive kinetic partitioning.[54] Very recently, Clark and Frieden,[55] have similarly demonstrated an absence of productive folding pathway catalysis in their studies on GroEL-mediated folding of structurally homologous dihydrofolate reductases. In addition, Persson *et al.*[56] have been able to show that the activation energy barrier to the rate determining step of carbonic anhydrase folding is essentially unaltered by the GroEL/GroES machinery. Finally, Walter *et al.*[57] have shown that GroEL does not affect the microscopic rate constant of protein unfolding either. Notably, where GroEL/GroES-mediated catalysis of folding has been described,[58] perceived rate enhancements appear to be very modest and measured in one direction only (back reaction rate enhancements were not verified). There are some reports that GroEL alone will substantially reduce the rate of folding of some proteins.[34] However, this observation has been noted only with small proteins which fold rapidly ($\leqslant$15 s) and associate only transiently with GroEL. Therefore, the general relevance of this observation is a little unclear. One final piece of circumstantial evidence to support the passive nature of the molecular chaperone mechanism

is the very fact that the GroEL/GroES molecular chaperone machinery is normally partnered *in vivo* with other molecular chaperones whose role is apparently to optimise the presentation of unfolded protein substrates to GroEL.[59]

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References

1 (*a*) M. Beissinger and J. Buchner, *Biol. Chem.*, 1998, **379**, 245; (*b*) J. Martin and F.-U. Hartl, *Curr. Opin. Struct. Biol.*, 1997, **7**, 41; (*c*) J. Buchner, *FASEB J.*, 1996, **10**, 10; (*d*) M. J. Gething and J. Sambrook, *Nature*, 1992, **355**, 33.
2 J. Mou, S. Sheng, R. Ho and Z. Shao, *Biophys. J.*, 1996, **71**, 2213.
3 (*a*) N. A. Ranson, H. E. White and H. R. Saibil, *Biochem. J.*, 1998, **333**, 233; (*b*) H. E. White, S. Chen, A. M. Roseman, O. Yifrach, A. Horovitz and H. R. Saibil, *Nat. Struct. Biol.*, 1997, **4**, 690; (*c*) O. Llorca, S. Marco, J. L. Carrascosa and J. M. Valpuesta, *FEBS Lett.*, 1997, **405**, 195; (*d*) A. M. Roseman, S. Chen, H. White, K. Braig and H. R. Saibil, *Cell*, 1996, **87**, 241.
4 (*a*) S. Chen, A. M. Roseman, A. S. Hunter, S. P. Wood, S. G. Burston, N. A. Ranson, A. R. Clarke and H. R. Saibil, *Nature*, 1994, **371**, 261; (*b*) A. Azem, M. Kessel and P. Goloubinoff, *Science*, 1994, **265**, 653; (*c*) M. Schmidt, K. Rutkat, R. Rachel, G. Pfeifer, R. Jaenicke, P. Viitanen, G. Lorimer and J. Buchner, *Science*, 1994, **265**, 656; (*d*) A. Engel, M. K. Hayer-Hartl, K. N. Goldie, G. Pfeifer, R. Hegerl, S. Müller, A. C. R. da Silva, W. Baumeister and F.-U. Hartl, *Science*, 1995, **269**, 832; (*e*) G. N. Chandrasekhar, K. Tilly, C. Woolford, R. Hendrix and C. Georgopoulos, *J. Biol. Chem.*, 1986, **261**, 12414.
5 (*a*) D. C. Boisvert, J. Wang, Z. Otwinowski, A. L. Horwich and P. B. Sigler, *Nat. Struct. Biol.*, 1996, **3**, 170; (*b*) K. Braig, P. D. Adams and A. T. Brünger, *Nat. Struct. Biol.*, 1995, **2**, 1083; (*c*) K. Braig, Z. Otwinowski, R. Hegde, D. C. Boisvert, A. Joachimiak, A. L. Horwich and P. B. Sigler, *Nature*, 1994, **371**, 578.
6 Z. Xu, A. L. Horwich and P. B. Sigler, *Nature*, 1997, **388**, 741.
7 J. F. Hunt, A. J. Weaver, S. J. Landry, L. Gierasch and J. Deisenhofer, *Nature*, 1996, **379**, 37.
8 P. V. Viitanen, A. A. Gatenby and G. H. Lorimer, *Protein Sci.*, 1992, **1**, 363.
9 J. P. Hutchinson, T. C. Oldham, T. S. H. El-Thaher and A. D. Miller, *J. Chem. Soc., Perkin Trans. 2*, 1997, 279.
10 P. Gervasoni, W. Staudenmann, P. James and A. Plückthun, *Biochemistry*, 1998, **37**, 11660.
11 (*a*) S. Perret, R. Zahn, G. Stenberg and A. R. Fersht, *J. Mol. Biol.*, 1997, **269**, 892; (*b*) K. Katsumata, A. Okazaki, G. P. Tsurupa and K. Kuwajima, *J. Mol. Biol.*, 1996, **264**, 643.
12 (*a*) O. Yifrach and A. Horovitz, *J. Mol. Biol.*, 1996, **255**, 356; (*b*) E. Inbar and A. Horovitz, *Biochemistry*, 1997, **36**, 12276.
13 R. A. Staniforth, S. G. Burston, T. Atkinson and A. R. Clarke, *Biochem. J.*, 1994, **300**, 651.
14 H. S. Rye, S. G. Burston, W. A. Fenton, J. M. Beechem, Z. Xu, P. B. Sigler and A. L. Horwich, *Nature*, 1997, **388**, 792.
15 G. Lorimer, *Nature*, 1997, **388**, 720.
16 H. Sparrer and J. Buchner, *J. Biol. Chem.*, 1997, **272**, 14080.
17 H. Sparrer, H. Lilie and J. Buchner, *J. Mol. Biol.*, 1996, **258**, 74.
18 (*a*) H. Taguchi and M. Yoshida, *FEBS Lett.*, 1995, **359**, 195; (*b*) J. S. Weissman, Y. Kashi, W. A. Fenton and A. L. Horwich, *Cell*, 1994, **78**, 693.
19 (*a*) S. E. Nieba-Axmann, M. Ottiger, K. Wüthrich and A. Plückthun, *J. Mol. Biol.*, 1997, **271**, 803; (*b*) R. Zahn, S. Perrett and A. R. Fersht, *J. Mol. Biol.*, 1996, **261**, 43; (*c*) R. Zahn, S. Perrett, G. Stenberg and A. R. Fersht, *Science*, 1996, **271**, 642.
20 (*a*) M. J. Todd, G. H. Lorimer and D. Thirumalai, *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 4030; (*b*) M. J. Todd, P. V. Viitanen and G. H. Lorimer, *Science*, 1994, **265**, 659; (*c*) G. S. Jackson, R. A. Staniforth, D. J. Halsall, T. Atkinson, J. J. Holbrook, A. R. Clarke and S. G. Burston, *Biochemistry*, 1993, **32**, 2554.
21 F. J. Corrales and A. R. Fersht, *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 4509.
22 (*a*) G. Tian, I. E. Vainberg, W. D. Tap, S. A. Lewis and N. J. Cowan, *J. Biol. Chem.*, 1995, **270**, 23910; (*b*) J. Martin, M. Mayhew, T. Langer and F.-U. Hartl, *Nature*, 1993, **366**, 228; (*c*) F. J. Corrales and A. R. Fersht, *Proc. Natl. Acad. Sci., USA*, 1995, **92**, 5326; (*d*) R. Hlodan, P. Tempst and F.-U. Hartl, *Nat. Struct. Biol.*, 1995, **2**, 587; (*e*) M. Mayhew, A. C. R. da Silva, J. Martin, H. Erdjument-Bromage, P. Tempst and F.-U. Hartl, *Nature*, 1996, **379**, 420.
23 E. Margoliash and N. Frohwirt, *Biochem. J.*, 1959, **71**, 570.
24 J. P. Hutchinson, T. S. H. El-Thaher and A. D. Miller, *Biochem. J.*, 1994, **302**, 405.
25 B. A. El-Agez, PhD Thesis, University of Essex, UK, 1992.
26 (*a*) T. J. T. Pinheiro, G. A. Elöve, A. Watts and H. Roder, *Biochemistry*, 1997, **36**, 13122; (*b*) K. M. Pryse, T. G. Bruckman, B. W. Maxfield and E. L. Elson, *Biochemistry*, 1992, **31**, 5127; (*c*) D. T. Haynie and E. Freire, *Proteins*, 1993, **16**, 115; (*d*) G. A. Elöve, A. F. Chaffotte, H. Roder and M. E. Goldberg, *Biochemistry*, 1992, **31**, 6876; (*e*) G. A. Elöve, A. K. Bhuyan and H. Roder, *Biochemistry*, 1994, **33**, 6925; (*f*) K. Kuwajima, H. Yamaya, S. Miwa, S. Sugai and T. Nagamura, *FEBS Lett.*, 1987, **221**, 115.
27 D. N. Brems and E. Stellwagen, *J. Biol. Chem.*, 1983, **258**, 3655.
28 H. Yang and D. L. Smith, *Biochemistry*, 1997, **36**, 14992.
29 W. Colón, G. A. Elöve, L. P. Wakem, F. Sherman and H. Roder, *Biochemistry*, 1996, **35**, 5538.
30 W. Colón, L. P. Wakem, F. Sherman and H. Roder, *Biochemistry*, 1997, **36**, 12535.
31 H. Roder, G. A. Elöve and S. W. Englander, *Nature*, 1988, **335**, 700.
32 G. R. Moore and G. W. Pettigrew, in *Cytochromes c*, Springer-Verlag, Berlin, 1990.
33 (*a*) A. Okazaki, T. Ikura, K. Nikaido and K. Kuwajima, *Nat. Struct. Biol.*, 1994, **1**, 439; (*b*) M. K. Hayer-Hartl, J. J. Ewbank, T. E. Creighton and F.-U. Hartl, *EMBO J.*, 1994, **13**, 3192; (*c*) H. Lilie and J. Buchner, *Proc. Natl. Acad. Sci. USA*, 1995, **92**, 8100; (*d*) K. E. Smith and M. T. Fisher, *J. Biol. Chem.*, 1995, **270**, 21517.
34 T. E. Gray and A. R. Fersht, *J. Mol. Biol.*, 1993, **232**, 1197.
35 G. C. Flynn, C. J. M. Beckers, W. A. Baase and F. W. Dahlquist, *Proc. Natl. Acad. Sci. USA*, 1993, **90**, 10826.
36 M. Hoshino, Y. Kawata and Y. Goto, *J. Mol. Biol.*, 1996, **262**, 575.
37 H. F. Rosenberg, S. J. Ackerman and D. G. Tenen, *J. Biol. Chem.*, 1993, **268**, 4499.
38 Y. Gao, A. D. J. Lee, R. J. P. Williams and G. Williams, *Eur. J. Biochem.*, 1989, **182**, 57.
39 Y. P. Myer, *J. Biol. Chem.*, 1968, **243**, 2115.
40 M. C. Hsu and R. W. Woody, *J. Am. Chem. Soc.*, 1971, **93**, 3515.
41 (*a*) Y. P. Myer, *Biochemistry*, 1968, **7**, 765; (*b*) R. Santucci, A. Giartosio and F. Ascoli, *Biochim. Biophys. Acta*, 1987, **914**, 185; (*c*) R. Santucci, A. Giartosio and F. Ascoli, *Arch. Biochem. Biophys.*, 1989, **275**, 496.
42 P. Gervasoni, W. Staudenmann, P. James, P. Gehrig and A. Plückthun, *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 12189.
43 P. Gervasoni, P. Gehrig and A. Plückthun, *J. Mol. Biol.*, 1998, **275**, 663.
44 J. M. Vanderkooi and M. Erecinska, *Eur. J. Biochem.*, 1975, **60**, 199.
45 (*a*) B. Holl-Neugebauer, R. Rudolph, M. Schmidt. and J. Buchner, *Biochemistry*, 1991, **30**, 11609; (*b*) J. Martin, T. Langer, R. Boteva, A. Schramel, A. L. Horwich and F.-U. Hartl, *Nature*, 1991, **352**, 36; (*c*) J. A. Mendoza, M. C. Butler and P. M. Horowitz, *J. Biol. Chem.*, 1992, **267**, 24648.
46 P. Hildebrandt, F. Vanhecke, G. Buse, T. Soulimane and A. G. Mauk, *Biochemistry*, 1993, **32**, 10912.
47 Z. Lin and E. Eisenstein, *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 1977.
48 C. V. Robinson, M. Groß, S. J. Eyles, J. J. Ewbank, M. Mayhew, F.-U. Hartl, C. M. Dobson and S. E. Radford, *Nature*, 1994, **372**, 646.
49 A. Okazaki, K. Katsumata and K. Kuwajima, *J. Biochem.*, 1997, **121**, 534.
50 (*a*) M. S. Goldberg, J. Zhang, S. Sondek, C. R. Matthews, F. O. Fox and A. L. Horwich, *Proc. Natl. Acad. Sci. USA*, 1997, **94**, 1080; (*b*) M. Groß, C. V. Robinson, M. Mayhew, F.-U. Hartl and S. E. Radford, *Protein Sci.*, 1996, **5**, 2506.
51 (*a*) C. R. Matthews, *Annu. Rev. Biochem.*, 1993, **62**, 653; (*b*) K. A. Dill and H. S. Chan, *Nat. Struct. Biol.*, 1997, **4**, 10; (*c*) R. L. Baldwin, *Nature*, 1994, **369**, 183; (*d*) T. Lazaridis and M. Karplus, *Science*, 1997, **278**, 1928.
52 G. Zettlmeissl, R. Rudolph and R. Jaenicke, *Biochemistry*, 1979, **18**, 5567.
53 (*a*) J. London, C. Skrzynia and M. E. Goldberg, *Eur. J. Biochem.*, 1974, **47**, 409; (*b*) G. Orsini and M. E. Goldberg, *J. Biol. Chem.*, 1978, **253**, 3453; (*c*) T. Kiefhaber, R. Rudolph, H.-H. Kohler and J. Buchner, *Biotechnology*, 1991, **9**, 825.
54 A. D. Miller, K. Maghlaoui, G. Albanese, D. A. Kleinjan and C. Smith, *Biochem. J.*, 1993, **291**, 139.

55 (*a*) A. C. Clark and C. Frieden, *J. Mol. Biol.*, 1997, **268**, 512; (*b*) C. Frieden and A. C. Clark, *Proc. Natl. Acad. Sci. USA*, 1997, **94**, 5535.
56 M. Persson, U. Carlsson and N. Bergenhem, *FEBS Lett.*, 1997, **411**, 43.
57 S. Walter, G. H. Lorimer and F. X. Schmid, *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 9425.
58 (*a*) N. A. Ranson, N. J. Dunster, S. G. Burston and A. R. Clarke, *J. Mol. Biol.*, 1995, **250**, 581; (*b*) R. A. Staniforth, A. Cortes, S. G. Burston, T. Atkinson, J. J. Holbrook and A. R. Clarke, *FEBS Lett.*, 1994, **344**, 129.
59 T. Langer, C. Lu, H. Echols, J. Flanagan, M. K. Hayer and F.-U. Hartl, *Nature*, 1992, **356**, 683.
60 G. W. Bushnell, G. V. Louie and G. D. Brayer, *J. Mol. Biol.*, 1990, **214**, 585.

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