Protein substrate binding induces conformational changes in the chaperonin GroEL. A suggested mechanism for unfoldase activity.

Per Hammarström¹, Malin Persson¹, Rikard Owenius², Mikael Lindgren² and Uno Carlsson¹

¹IFM-Department of Chemistry and ²Chemical Physics, Linköping University, S-581 83 Linköping, Sweden

Correspondence should be addressed to U.C., E-mail: ucn@ifm.liu.se

Abbreviations: AEDANS-GroEL, GroEL labeled with 5-(((2-iodoacytlyl)amino)-ethyl)amino)naphtalene-1-sulfonic acid; EPR, electron paramagnetic resonance; GuHCl, guanidine hydrochloride; HCA IIₚᵢₜ, pseudo-wild type human carbonic anhydrase II with a C206S mutation; IAF-GroEL, GroEL labeled with 6-iodoacetamidofluorescein; IPSL-GroEL, GroEL labeled with N-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide.

Running title: Conformational changes induced in GroEL by protein binding
ABSTRACT

Chaperonins are molecules that assist proteins during folding and protect them from irreversible aggregation. We studied the chaperonin GroEL and its interaction with the enzyme human carbonic anhydrase II (HCA II), which induces unfolding of the enzyme. We focused on conformational changes that occur in GroEL during formation of the GroEL-HCA II complex. We measured the rate of GroEL cysteine reactivity towards iodo[2-14C]acetic acid and found that the cysteines become more accessible during binding of a cysteine free mutant of HCA II. Spin labeling of GroEL with N-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide (IPSL) revealed that this additional binding occurred because buried cysteine residues become accessible during HCA II binding. Also, a GroEL variant labeled with 6-iodoacetamidofluorescein (IAF) exhibited decreased fluorescence anisotropy upon HCA II binding, which resembles the effect of GroES/ATP binding. Furthermore, by producing cysteine-modified GroEL with the spin label IPSL and the fluorescent label 5-(((2-iodoacetyl)amino)-ethyl)amino)naphthalene-1-sulfonic acid (IAEDANS), we detected increases in spin-label mobility and fluorescence intensity in GroEL upon HCA II binding. Together, these results show that conformational changes occur in the chaperonin as a consequence of protein substrate binding. Together with previous results on the unfoldase activity of GroEL we suggest that the chaperonin opens up as the substrate protein binds. This opening mechanism may induce stretching of the protein, which would account for reported unfoldase activity of GroEL and might explain how GroEL can actively chaperone proteins larger than HCA II.
INTRODUCTION

Proteins can refold spontaneously if the unfolded state is exposed to conditions that favor the folded conformation. However, it has been found that under many circumstances productive protein folding is not achieved as easily as previously postulated (1, 2). In living cells, molecular chaperones facilitate protein folding by preventing misfolding and aggregation. We have previously shown that renaturing of fully GuHCl-unfolded human carbonic anhydrase II (HCA II), yields approximately 70% native protein under optimal refolding conditions. However, the E. coli chaperonin GroEL can assist HCA II during refolding, increasing the yield to 100%, and it can also protect thermally denatured HCA II from irreversible aggregation. This process does not require ATP or GroES binding (3). Previous investigations have shown that GroEL possesses unfoldase activity (4-8). It has also been reported that GroEL can in a passive mass action manner bind to partially unfolded proteins as they are populated (9). During the GroEL-HCA II interaction, GroEL does not only passively bind to a population of partially unfolded proteins but causes further unfolding of the substrate upon binding (8). This action is achieved by GroEL alone and does not require the complete GroEL/ES/ATP system, as, for example, is seen for RuBisCO (10, 11). Recently, a chaperone-percolator model has been suggested, in which the chaperones do not generally unfold their targets, but by a multidirectional expansion preferentially loosen the core structure and during expansion water molecules enter the hydrophobic core of the protein substrate (12).

It is possible that the energy of binding of the chaperone to the substrate protein is used to unfold the substrate and thereby give it a new chance to refold correctly (5), however the detailed mechanism is not known. Furthermore, this unfoldase activity could require a substantial conformational change in GroEL enabling unfolding of the protein substrate. It was recently shown that binding of peptides to GroEL induces conformational changes in the
substrate binding region in the apical domain. This structural plasticity was suggested to account for the promiscuous recognition of protein substrates (13).

The present study focuses on how GroEL-assisted refolding of HCA II is achieved. In previous folding studies of HCA II (14-16), we used carboxymethylation of cysteine residues to probe side chain accessibility/compactness of surrounding structure. Here, a similar approach is used to probe accessibility of cysteines in GroEL as a means of monitoring conformational changes. Each subunit of GroEL contains three cysteine residues, Cys 138, Cys 458, and Cys 519, which are all situated in the equatorial and intermediate domains (Fig.1). We found an increased rate of carboxymethylation of cysteine residues in the intermediate/equatorial domain of GroEL, indicating conformational changes in the chaperonin upon binding of partially unfolded HCA II. According to spin-labeling experiments this increase in cysteine accessibility could be attributed to buried cysteine residues. We also analyzed fluorescence anisotropy and fluorescence resonance energy homotransfer from fluorescein-labeled GroEL, as well as the spectroscopic characteristics of IAEDANS- and IPSL-labeled GroEL, to investigate the conformational changes in GroEL upon binding of HCA II. The results of these measurements taken together with the reactivity data show that the accessibility of GroEL cysteines and the dynamics at these sites increase when the protein substrate is bound. By these and previous results (8) we suggest that the mechanism of the GroEL machinery is as follows: the chaperonin expands upon binding to a substrate protein, which further unfolds the substrate protein in a process similar to the stretching of an elastic sticker attached to a balloon during inflation; this action smoothes out the surface of the folding energy landscape and thereby allows the protein to fold correctly.
EXPERIMENTAL PROCEDURES

Materials—Iodo[2-14C]acetic acid (54mCi/mmol) was obtained from Amersham. Aqua safe 300 plus scintillation cocktail was purchased from Zinsser Analytic. Reagent grade GuHCl was obtained from Pierce. 5-((((2-iodoacetyl)amino)-ethyl)amino)naphthalene-1-sulfonic acid, 1,5-IAEDANS and 6-iodacetamido-fluorescein, 6-IAF, were purchased from Molecular Probes and N-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide, IPSL, was obtained from Sigma. All other chemicals were of the highest available grade.

Protein production and purification—GroEL was purified as previously described (17). HCA II<sub>pwt</sub>, a C206S mutant of cloned HCA II, was produced as previously described (15). The GroEL concentration was determined by the Biorad Bradford BSA assay. HCA II<sub>pwt</sub> concentration was determined by absorbance measurements using ε<sub>280nm</sub> = 54800 M<sup>-1</sup> cm<sup>-1</sup> (18).

Incubation buffer—In all measurements concerning the interaction between HCA II<sub>pwt</sub> and GroEL, an incubation buffer was used containing 200 mM GuHCl and 100 mM Tris H<sub>2</sub>SO<sub>4</sub>, pH 7.5.

14C-carboxymethylation of GroEL—0.1 nmol of GroEL was incubated in a final volume of 100 µl of incubation buffer at 20 °C and 50 °C in the presence and absence of HCA II<sub>pwt</sub> for 1 h with 41 molar excess of iodo[2-14C]acetic acid over GroEL tetradecamer. The reaction was quenched by addition of 2-mercaptoethanol to a final concentration of 1.3 M and the proteins were precipitated with 100 µl of 50 % w:w trichloroacetic acid, 0.2 % w:w deoxycholate and 300 µl of ice cold distilled water and was left on ice for 30 minutes followed by
centrifugation. The precipitate was washed 4 times with 200 µl of ice cold distilled water and
was thereafter dissolved in 200 µl of 5.9 M GuHCl. The protein solution was incubated in 15
ml of scintillation cocktail (Aqua safe 300 plus) and the cpm value from each sample was
measured in a Beckman LS-6500 scintillator. This procedure was repeated for 3 different
samples both in the presence and absence of equimolar concentrations of HCA II<sub>pwt</sub>. 
Incorporation of iodo[2-<sup>14</sup>C]acetic acid was also measured on HCA II<sub>pwt</sub> (3 samples) at 50 °C
as a control. This value was then subtracted from that obtained from GroEL-HCAII<sub>pwt</sub>
labeling. An additional control experiment was made on extensively unfolded HCA II<sub>pwt</sub>
to probe for non specific modification by iodo[2-<sup>14</sup>C]acetic acid, by unfolding HCA II<sub>pwt</sub> in 5.0
M GuHCl at 50 °C. This cpm h<sup>-1</sup> value (104 cpm h<sup>-1</sup>) was lower than that obtained in 0.2 M
GuHCl (molten globule state of HCA II<sub>pwt</sub>, which was 337 cpm h<sup>-1</sup>).

6-IAF labeling and fluorescence measurements of IAF-GroEL— Labeling with 6-
iodoacetamidofluorescein, 6-IAF, was done by adding 0.75 µM GroEL to a 1400 µl solution
of 90 mM Tris H<sub>2</sub>SO<sub>4</sub>, pH 7.5, containing a 400-fold molar excess of label. The reaction was
quenched by addition of a 2-fold molar excess of 2-mercaptoethanol over reagent and IAF-
GroEL was purified by gel filtration on a PD-10 column equilibrated with 10 mM Tris H<sub>2</sub>SO<sub>4</sub>,
pH 7.5. The degree of labeling was obtained by absorbance measurements at 495 nm for 6-
IAF using ε<sub>495</sub> = 81000 M<sup>-1</sup>cm<sup>-1</sup> (19). Fluorescence spectra were recorded on a Hitachi F-
4500 spectrofluorimeter equipped with a thermostatted sample cell connected to a circulating
water bath. Fluorescence anisotropy measurements were conducted with excitation in the
range 460-505 nm and recording of the emission spectra in the range 510-600 nm using 5 nm
and 2.5 nm slits for excitation and emission, respectively. Fluorescence polarization spectra
were recorded by the use of sheet polarizers and the spectra were corrected for unequal
transmission efficiencies of vertically and horizontally polarized light. Measurements were conducted on samples containing 18 nM of tetradecameric IAF-GroEL. To some of the samples a 1:1 molar ratio of HCA II or a 2:1 molar ratio of GroES and 1 mM Mg-ATP were added to the IAF-GroEL solution. Spectra were recorded after incubation at the temperature of interest for 1 h. IAF-GroEL and unlabeled GroEL was subjected to gel filtration on a Sephacryl S-100 HR gel filtration column (of length 28 cm and diameter 2 cm).

*Fluorescein-fluorescein fluorescence resonance energy homotransfer (homo-FRET)* — Fluorophores, such as fluorescein, with small Stokes shifts can undergo fluorescence self-transfer, also known as fluorescence resonance energy homotransfer, homo-FRET, which is detected by depolarization of the emission light (20). FRET is sensitive to the distance between donor and acceptor sites and therefore homo-FRET can be used to estimate the distance between fluorescein molecules. A thorough description of the background of fluorescein-fluorescein homo-FRET, was reported by Hamman et al. (21) and we briefly summarize some of the useful equations below.

A steady state expression for the efficiency of energy transfer (E) as a function of fluorescence anisotropy is described by:

\[ E = \frac{2(r_0 - <r>)}{r_0} \]  
(1)

where \(<r>\) is the observed anisotropy and \(r_0\) is the anisotropy in the absence of energy transfer. \(E\) is dependent on the distance, \(R\), between the fluorophores as described by:

\[ E = \frac{R^6}{R_0^6 + R^6} \]  
(2)
where $R_0$ is the Förster radius, i.e. the distance between the probes for 50% energy transfer. $R_0$ was determined by Hamman et al. (21) to be 40 Å for the fluorescein-fluorescein pair.

1,5-IAEDANS labeling and fluorescence measurements of AEDANS-GroEL—1 μM of GroEL was added to 500 μl of 100 mM Tris-H$_2$SO$_4$, pH 7.5 containing a 420-fold molar excess of 5-(((2-iodoacetyl)amino)-ethyl)amino)naphthalene-1-sulfonic acid, 1,5-IAEDANS. The reaction was allowed to proceed over night in the dark and excess reagent was removed by 5 rounds of dialysis versus 10 mM Tris-H$_2$SO$_4$, pH 7.5 for 3 days. The degree of labeling was obtained by absorbance measurements at 337 nm for AEDANS using $\varepsilon_{337} = 6100$ M$^{-1}$cm$^{-1}$.

Samples containing 80 nM tetradecameric AEDANS-GroEL in incubation buffer was measured under various conditions. Fluorescence spectra were recorded between 380-600 nm after 1 h of incubation at the temperature of interest by excitation at 350 nm, using 5 nm slits for both excitation and emission light.

Spin labeling and EPR measurements of IPSL-GroEL—Cysteine modification experiments: 1.2 μM of GroEL was mixed in a total volume of 500 μl of 100 mM Tris-H$_2$SO$_4$, pH 7.5 containing 200 mM GuHCl. In one experiment a 1:1 molar ratio of HCA II$_{pwt}$ was added. The samples were incubated for 1 h at 50 °C in the presence of a 400-fold molar excess of N-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide, IPSL. The reaction was then quenched by addition of a 2-fold molar excess of 2-mercaptoethanol over IPSL and the samples were then dialyzed for 3 days with 4 changes versus 300 ml of 10 mM Tris-H$_2$SO$_4$, pH 7.5. Before the EPR measurements were conducted, a 1:1 molar ratio of HCA II$_{pwt}$ was added to GroEL labeled in the absence of HCA II$_{pwt}$ in order have identical protein mixtures. The EPR measurements were conducted at 20 °C.
In another experiment 3.5 µM of GroEL was mixed in 500 µl of 50 mM Tris-H$_2$SO$_4$, pH 7.5 containing a 400-fold molar excess of N-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide, IPSL. The reaction was allowed to proceed over night at 20 °C in the dark and excess reagent was removed by 5 rounds of dialysis versus 10 mM Tris-H$_2$SO$_4$, pH 7.5 for 3 days. Samples for EPR measurements were prepared with 0.66 µM IPSL-GroEL in incubation buffer and the degree of labeling was estimated from the EPR signal. Spectra were recorded after 1 h of incubation at the temperature of interest. The EPR spectrometer, measurements and temperature control were set up as previously described (8).

*Chaperone assay*—We also evaluated the chaperone activity of IAF-GroEL, AEDANS-GroEL and IPSL-GroEL on HCA II$_{pwt}$ under the equivalent conditions as the corresponding fluorescence and EPR measurements. The enzyme was heat denatured at 50 °C in incubation buffer for 1 h in the absence and presence of equimolar concentrations of GroEL variants and was subsequently cooled down to 20 °C to refold. This procedure has previously been described by Persson et al. (17), however a 2-fold molar excess of GroEL was used in those experiments.
RESULTS

By heating it was impossible to induce the molten-globule conformation of HCA II at a temperature that GroEL will withstand without being heat denatured, because a temperature of 60-65°C was required to complete the transition to the molten globule from the native state of HCA II (8). Therefore, all experiments on GroEL in this study were performed in buffers containing 0.2 M GuHCl as in the cited study. By this GuHCl inclusion the molten-globule state was reached at 50°C, and at this temperature HCA II was chaperoned by GroEL, whereas at 60°C no chaperone activity was detected (8).

Another reason for performing the experiments in low concentrations of GuHCl is that previous cited GroEL-mediated refolding studies on HCA II have been performed in this medium (3, 17, 22), permitting us to make comparisons under similar conditions. It has previously been demonstrated that the function of GroEL can be influenced by GuHCl (23). In that study it was shown that low concentrations of GuHCl decreased the ATPase activity of GroEL and destabilized the GroEL/ES complex, thus relieving the inhibition of GroEL ATPase by GroES. However, the refolding of HCA II can be efficiently assisted by GroEL without GroES and ATP (3). Since GroEL alone is used in this study the known effects by GuHCl on the chaperonin should be off-set. In a control experiment both the spontaneous and the GroEL-mediated refolding behavior of HCA II was also shown to be very similar in urea (0.6 M) and GuHCl (0.2 M) (data not shown). In our GroES binding studies (Figure 3A) we do not include GuHCl, because of the reported effect by GuHCl on the GroEL/ES complex (23).

\[^{14}\text{C-carboxymethylation of GroEL cysteines.}\] Figure 2 summarizes the results of iodo[^{14}\text{C}]acetic acid labeling experiments. At 20 °C, only minor modification of GroEL was achieved and there was no significant difference between results obtained with GroEL alone
or in the presence of HCA II\textsubscript{pwt}. However, at 50 °C in the presence of HCA II\textsubscript{pwt}, as compared to GroEL alone, there was a 45% increase in the rate of incorporation of radioactivity, which indicates increased cysteine modification in GroEL in the presence of protein substrate. This can be attributed only to GroEL, since the small rate of nonspecific incorporation of iodo[2-\textsuperscript{14}C]acetic acid to the cysteine-free (C206S) HCA II\textsubscript{pwt} was subtracted from that of the GroEL-HCA II\textsubscript{pwt} complex.

*Degree of fluorescence and spin labeling of GroEL*— GroEL incorporated 5.5 6-IAF labels per GroEL tetradecamer. Furthermore, on average, GroEL was modified with 14 IPSL spin labels. Using 1,5-IAEDANS, the degree of labeling was 12 AEDANS labels per GroEL tetradecamer. From the stoichiometry of the labeling one single cysteine per subunit of GroEL might have been modified, however, a distribution of labeled sites cannot be excluded. These findings are very similar to the results previously presented by Hansen and Gafni (24).

*Chaperone activity of AEDANS-, IAF-, and IPSL-GroEL*—Both AEDANS-GroEL and IPSL-GroEL actively protect HCA II\textsubscript{pwt} from thermal aggregation, as shown in Table 1, which agrees with earlier results obtained with unlabeled GroEL (17). IAF-GroEL had the same effect as unlabeled GroEL on binding and inhibition of thermally unfolded HCA II\textsubscript{pwt}. However, the release mechanism seemed to be impaired, because only a minute amount of HCA II\textsubscript{pwt} was recovered (Table 1). Furthermore, the yield of active HCA II was lower in the presence of IAF-GroEL than in the spontaneous reaction, which has also been reported for fluorescein-maleimide-labeled GroEL (25). Addition of GroES and ATP did not induce further release of HCA II\textsubscript{pwt} (data not shown).
**Gel filtration experiments**—In light of the impaired chaperone activity of IAF-GroEL, we subjected the chaperonin to gel filtration to determine whether its oligomeric structure had undergone dissociation as a result of the fluorescein labeling, as was recently reported for GroEL with extensively labeled cysteines (25, 26). The oligomeric structure was found to be intact, because no monomeric GroEL could be detected (data not shown). The same results have been reported for GroEL labeled with 7 fluorescein-maleimide labels per tetradecamer (25).

**IAF-Fluorescence anisotropy measurements**—It was possible to use fluorescence anisotropy of fluorescein-labeled GroEL to detect conformational changes in GroEL due to the short lifetime of the fluorophore (approximately 4 ns; (27)). In the binding studies of HCA IIₚᵦ, we added 200 mM GuHCl to the samples (incubation buffer; see above). As can be seen in Figure 3 (compare the solid-line curve with filled circles in A with the upper solid-line curve with the same symbols in B), the anisotropy decreased. Thus, that amount of denaturant apparently increased the mobility of the fluorescein labels attached to GroEL. However, most importantly, at 50 °C the anisotropy was smaller in the presence (Fig. 3 B; lower curve: open circles, dotted line) than in the absence (Fig. 3B; lower curve: filled circles, solid line) of HCA IIₚᵦ, indicating that a conformational change occurs in GroEL upon binding of the protein substrate.

**Fluorescein-fluorescein distances in IAF-GroEL as determined by fluorescence resonance energy homotransfer (homo-FRET)**—Fluorescein has a very small Stokes shift, thus its absorption and emission spectra overlap, and it can therefore display fluorescence resonance energy homotransfer if two fluorophores are within 20–70 Å of each other (21). Fluorescence anisotropy of IAF-GroEL plotted versus the excitation wavelength is shown in Figure 3 A
The red-edge excitation anisotropy dependence of IAF-GroEL indicates homo-FRET in the protein. This is evident, because the anisotropy increases at longer wavelengths (at the red-edge of the excitation spectra), where homo-FRET fails at low energy excitation (20, 28). The Förster radius is 40 Å for the fluorescein-fluorescein pair (21). The dependence on excitation wavelength was not detected for unfolded GroEL in 6 M GuHCl (Fig. 3A; filled triangles, solid line), which demonstrates that the homo-FRET requires intact GroEL structure. The fluorescence anisotropy in the absence of energy transfer, r01, must be known in order to determine the interprobe distances in IAF-GroEL (equation 1 in Experimental Procedures). The red-edge excitation increase in anisotropy will end at this value (20), hence we used fluorescence anisotropy in the interval 495–505 nm for linear curve fitting and, after extrapolation, to estimate the r01 value in the interval 522–528 nm (21). Thereafter, the r01 estimated values could be used together with the measured fluorescence anisotropy in the interval 460–480 nm to estimate the interprobe distances within the IAF-GroEL protein. This gave values of about 37 Å, which shows that IAF-GroEL is oligomeric, otherwise homo-FRET would not be possible. Figure 3A also illustrates the detected fluorescence anisotropy of IAF-GroEL alone at 23 °C (filled circles, solid line) and IAF-GroEL in complex with GroES and ATP (open circles, dotted line). The fluorescence anisotropy decreased uniformly over the entire excitation region upon binding of GroES at 23 °C (compare the upper two curves in Fig. 3A), thus no major changes in interprobe distance were expected.

Almost identical curves were noted for the fluorescence anisotropy of IAF-GroEL, with or without the presence of HCA IIpwt at 20 °C (Fig 3B, upper curves). Notably the fluorescence anisotropy here becomes different, upon an increase in temperature; lower for the sample containing HCA IIpwt. Thermally unfolded HCA II is known to interact more strongly with
GroEL than the conformation it takes at 20 °C, and it can be concluded that the change in fluorescence anisotropy is a result of the GroEL-HCA II interaction. However, no definitive conclusions can be drawn regarding the effects of such binding on the magnitude of the change in distance between the labeled sites in GroEL. The sensitivity of these measurements allows assessment of only relatively large changes in distance (5–10 Å), that either separates or bring the sites together. Therefore, the complexity of the system, comprising 5.5 labels per tetradecamer (attached to 42 possible cysteines), makes it difficult to determine how the inter-probe distances in IAF-GroEL is affected by a conformational change. However, it is safe to ascertain that the average mobility of the fluorescent labels is larger, both when contacted to GroES/ATP (23 °C) and HCA II_pwt (50 °C), as judged from the decrease in anisotropy.

_AEDANS fluorescence_— Fluorescence spectra of AEDANS-GroEL are presented in Fig. 4, and the fluorescence data are summarized in Table 2. The spectra recorded at 20 °C (Fig. 4, upper spectra) are centered around 484–485 nm and reflect a somewhat hydrophobic environment (equivalent to 40% v/v ethanol/water; (29)). Interestingly, the fluorescence quantum yield of AEDANS-GroEL was 0.51 times the quantum yield of AEDANS-mercaptoethanol, implying that the AEDANS labels are quenched when bound to GroEL. The fluorescence anisotropy measurements presented in Table 2 show that the fluorophores are very mobile in the chaperone, which indicates that local motion is the only contributing component in these spectra, since GroEL is a very large protein (800 kDa). We found no differences in anisotropy between GroEL alone and in complex with HCA II_pwt, hence conclusions cannot be drawn about local mobility changes, due to the long lifetime of the excited state of AEDANS (15 ns). Therefore local mobility was instead monitored by fluorescein anisotropy, spin labeling, and EPR measurements (see below). The fluorescence
intensity is presented in Table 2 as a comparison of the fluorescence of AEDANS-GroEL alone, at the different temperatures, and to that of AEDANS-GroEL in the presence of HCA II<sub>pwt</sub>. The fluorescence intensities were almost identical for AEDANS-GroEL at 20 °C with and without protein substrate (Fig. 4; upper spectra). When the AEDANS fluorescence spectra were recorded at 50 °C, a peak was detected around 487 nm (Fig. 4; lower spectra). No significant differences in peak positions were found in the presence or absence of HCA II<sub>pwt</sub>. We observed a small increase in intensity (3.4%) for AEDANS-GroEL at 50 °C in the presence of HCA II<sub>pwt</sub> (Fig. 4; lower spectra, dotted curve).

**EPR spectra from IPSL-GroEL**—Compared to <sup>14</sup>C-carboxymethylation of the cysteines in GroEL, for which an increased rate of modification was detected in the presence of HCA II<sub>pwt</sub>, we noted that binding of HCA II<sub>pwt</sub> led to only a slight increase in the amount of incorporated IPSL (6%), as determined from the integrated signal (Fig. 5). However, there was a significant difference in EPR lineshape (see Fig. 5), revealing that buried cysteine residues become accessible for modification when GroEL binds HCA II.

EPR spectra were also recorded for IPSL-GroEL in the presence and absence of HCA II<sub>pwt</sub> at 20 and 50 °C, respectively (Fig. 6). At 20 °C, spin labels in IPSL-GroEL showed greater mobility when HCA II<sub>pwt</sub> was present (Fig. 6A). At 50 °C, the spin labels were more mobile due to increased thermal motion (Fig. 6B), although the effect in the presence of HCA II<sub>pwt</sub> was not as pronounced at this temperature as at 20 °C. The increased dynamics at 50 °C decreased the sensitivity of the EPR spectrum; despite that, it is still obvious that the spin labels in IPSL-GroEL were more mobile in the presence than in the absence of HCA II<sub>pwt</sub>. 

DISCUSSION

Using EPR (8) and fluorescence resonance energy transfer (30, 31), we have shown that HCA II must exhibit molten globule-like structure to be able to bind to GroEL, and binding results in loosening up the hydrophobic core and further unfolding of the protein substrate. It is possible that a large, enforced unfolding of the substrate protein requires a considerable conformational change in the chaperone. Falke et al., (32) have obtained electron microscopy images that indicate that the GroEL structure opens upon binding of a large protein substrate, glutamine synthetase. Also, in retrospect, examining cryo-electron-microscopy images of GroEL and GroEL in complex with malate dehydrogenase (MDH), published by Chen et al. (33), a slightly expanded structure of the chaperonin can be detected in the MDH-GroEL complex (we compared Fig. 2 B and Fig. 2 E in the cited article by Chen et al. (33) and estimated that the diameter of GroEL increased by 5% upon binding of MDH). Binding of different substrate proteins were also shown to induce various GroEL conformations, indicated by the fragmentation pattern and rate of trypsin induced proteolysis (34). In addition, it was recently shown that there is a significant level of allosteric interaction between the GroEL/ES system and its protein substrate (35).

We employed chemical labeling with iodo[2-^{14}C]acetic acid to monitor the degree of accessibility of cysteine residues in GroEL. At 20 °C, a very low rate of cysteine modification was detected, which shows that these residues are buried in GroEL. However, the rate of the modification of the cysteines was increased at 50 °C, possibly due to a higher reaction rate and increased dynamics of the protein molecule at this elevated temperature. Binding of HCA II_{pwt} led to a 45% increase in the rate of carboxymethylation of the cysteine residues, indicating that GroEL had opened up (Fig. 2). Presumably, binding of the protein substrate to the apical domain would impose a steric hindrance that would prevent the reagent from gaining access to the interior of GroEL, therefore the observed increase in the rate of
modification of the cysteines is somewhat surprising. The three cysteine residues in each subunit, Cys 138, Cys 458, and Cys 519, are all embedded in the intermediate and equatorial domains of GroEL (Fig. 1). To determine whether different cysteines in GroEL are labeled in the presence and the absence of HCA II<sub>pwt</sub>, we used the sensitivity of the EPR spectrum to the structural environment of the spin-labeled sites. Indeed, we detected a significant difference in the EPR lineshape when GroEL was spin labeled at 50 °C with and without HCA II<sub>pwt</sub> (Fig. 5). A broad component that is indicative of buried spin labels was found for IPSL-GroEL labeled in the presence, but not in the absence, of HCA II<sub>pwt</sub>. This demonstrates that not only does protein substrate binding increase the rate of modification (revealed by the rate of carboxymethylation), but it even renders buried cysteines accessible for modification. We also prepared a fluorescein-labeled variant of GroEL, and found that it did not assist in refolding of HCA II<sub>pwt</sub>, but it did bind HCA II<sub>pwt</sub> at 50 °C, because it decreased the yield of refolding (Table 1). Moreover, the gel filtration experiments showed that the oligomeric structure of this GroEL variant was intact. Thus, this variant should represent a good model for the characterization of the GroEL-HCA II<sub>pwt</sub> complex. We assume that the reagents we used can label any of the three cysteines in the intermediate and equatorial domains, although it is unlikely that Cys 458 is labeled by IPSL and 1,5-IAEDANS, as discussed below. Under mildly denaturing conditions that separated GroEL into monomers, Horowitz et al. (36) reported that Cys 519 in the equatorial domain was specifically labeled by 6-iodoacetamidofluorescein. We were able to incorporate 5.5 labels of 6-IAF per GroEL tetradecamer by incubating the protein with the reagent for 60 h. In a recent investigation by Jai and Horowitz (25), it was shown that Cys 138 and Cys 458, but not Cys 519, could be modified with fluorescein-maleimide (under conditions similar to those used in our study). Consequently, all three cysteines can to some extent be accessible for modification.
The fluorescein measurements yielded a large amount of data that are relevant for structural interpretation. The Förster radius of the fluorescein-fluorescein pair was 40 Å, and we detected fluorescence resonance energy homotransfer in IAF-GroEL (Fig. 3, A and B). We estimated the average distance between the fluorescein labels to 37 Å, which is well within the size of the GroEL protein. Considering unfolded IAF-GroEL in 6 M GuHCl, the fluorescence anisotropy did not seem to depend on excitation wavelength (Fig. 3 A), which demonstrates that the homo-FRET required intact IAF-GroEL structure. Interestingly, the anisotropy decreased substantially in the IAF-GroEL/GroES/ATP complex. However, the decreased anisotropy occurred in parallel over the entire excitation interval, indicating that the change in anisotropy in IAF-GroEL alone and in the IAF-GroEL/GroES/ATP complex was due to increased mobility of the labels around the attachment sites and not to large changes in distances between labeled sites in the GroEL molecule. These results demonstrate that fluorescein anisotropy is a sensitive parameter for detection of conformational changes in the intermediate/equatorial domain of GroEL.

Studying IAF-GroEL in the presence and absence of HCA II_pwt, we found almost no difference in fluorescence anisotropy at 20 °C, whereas at 50 °C with added HCA II_pwt, complex formation with the protein substrate decreased the anisotropy of the bound fluorescein, indicating that the intermediate/equatorial part of GroEL is more flexible when the protein substrate is bound. Binding of GroES has previously been shown to induce expansion of the GroEL cavity (37). Because the change in fluorescence anisotropy is qualitatively similar to that seen for the IAF-GroEL/GroES/ATP complex, we suggest that GroEL expands and opens up upon binding of HCA II_pwt.

In a fluorescence anisotropy study performed by Gorovits and Horowitz (38), more rigid fluorophores were found in the GroEL-rhodanese complex than in the chaperone alone. This
was noted for pyrene labels attached to lysine residues in GroEL. An inflation of the chaperone or a direct contact with the protein substrate could explain these results.

We also prepared fully active fluorescent- and spin-labeled variants of GroEL. The labeled variants were fully capable of protecting HCA II\textsubscript{pwt} from thermal aggregation and, evidently, were therefore able to bind HCA II\textsubscript{pwt} at 50 °C. This implies that the oligomeric structure of GroEL is intact in these derivatives, and not dissociated, as previously reported for GroEL extensively modified at Cys 458 with 4,4’-dithiopyridine (26) and fluorescein-maleimide (25). Accordingly, it is unlikely that IPSL and 1,5-IAEDANS are attached to Cys 458.

Spectroscopic characterization of AEDANS-GroEL has been performed by Hansen and Gafni (24) and the results of that study were very similar to ours. Most important in our study is that we compared the structure of GroEL in the presence and the absence of HCA II\textsubscript{pwt}. No differences between the fluorescence spectra were found under these conditions at 20 °C. Moreover, despite evident HCA II\textsubscript{pwt} binding at 50 °C, no changes in Stokes shift were detected for the AEDANS-GroEL-HCA II\textsubscript{pwt} complex, as compared to AEDANS-GroEL alone. However, a small increase (3.4%) in AEDANS fluorescence intensity was observed at 50 °C during formation of the GroEL-HCA II\textsubscript{pwt} complex, which might indicate a conformational change in the chaperone in the vicinity of the label. Notably, the fluorescence of AEDANS-labeled GroEL was found to be strongly quenched, and protein substrate binding apparently decreased this quenching effect.

EPR spectra were recorded for IPSL-GroEL to detect possible differences in mobility in the equatorial/intermediate domain in the presence or absence of protein substrate. At 20 °C, more mobile spin labels were found in the presence of HCA II\textsubscript{pwt} than for IPSL-GroEL alone (Fig. 6A). At 50 °C, the mobility of the spin labels increased (Fig. 6B), which was expected due to increased thermal motion at this higher temperature. The EPR spectra of IPSL-GroEL at 50 °C with or without HCA II\textsubscript{pwt} are very similar, whereas the spin labels were slightly
more mobile in the presence than in the absence of HCA II_pwt (Fig. 6B). This indicates that the GroEL structure is very dynamic, which is important for the allostERIC interactions between the subunits. These results are consistent with previous findings of conformational changes in the equatorial domain upon protein substrate binding (39).
CONCLUDING REMARKS

We employed five different methods to monitor conformational changes occurring around cysteine residues in GroEL upon binding of HCA II_{pwt}. Together, our findings indicate that the chaperonin opens up as a result of binding of the protein substrate.

The interaction between HCA II and GroEL causes further unfolding of HCA II, and this is probably achieved by opening of the chaperonin, which thereby stretches the protein substrate and pulls it apart. The unfoldase activity of GroEL is required to give a misfolded protein a new chance to fold productively. The opening mechanism of GroEL as it binds to protein substrates may also be a way for the chaperone to accommodate large substrates. The molecular weight of HCA II is only 30 kDa, and it might be that more substantial opening effects occur in GroEL upon binding of larger protein substrates.

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REFERENCES


Figure Legends

Figure 1. The structure of GroEL showing the cysteine residues. (A) Close-up of a GroEL subunit, with arrows indicating Cys 138, Cys 458, and Cys 519; coordinates from Braig et al. (40), with the pdb code 1GRL. (B) One ring of GroEL showing one cysteine in each subunit; coordinates from Braig et al. (41), with the pdb code 1OEL.

Figure 2. Rate of incorporation of iodo[2-14C]acetic acid into GroEL. The experiments were performed for one hour at 20 °C and 50 °C in the absence or the presence of HCA IIprot. The error bars are standard deviations from three different experiments.

Figure 3. Excitation anisotropy spectra of IAF-GroEL and fluorescein. (A) IAF-GroEL alone (filled circles, solid line) and in the presence of 2:1 GroES and 1 mM Mg-ATP (open circles, dotted line). Both samples dissolved in 0.1 M Tris H2SO4 (pH 7.5) at 23 °C. Also shown is the anisotropy of IAF-GroEL in 6 M GuHCl containing 0.1 M Tris H2SO4 (pH 7.5) at 23 °C (filled triangles, solid line) and IAF-mercaptoethanol (open triangles, dashed line). (B). IAF-GroEL with (open circles, dashed line) and without (filled circles, solid lines) HCA IIprot in the incubation buffer. The upper and lower curves were recorded at 20 and 50 °C, respectively.

Figure 4. Fluorescence spectra of AEDANS-GroEL. Recorded at 20 °C (upper spectra) and 50 °C (lower spectra) for GroEL alone (solid curves) and in the presence of HCA IIprot (dashed curves). Experimental details are described in the Experimental procedures section.
Figure 5. Spin-label incorporation of GroEL. EPR absorption spectra of GroEL labeled with IPSL at 50 °C in the presence (solid line) or absence (dotted line) of HCA II<sub>pwt</sub>. For both samples, the spectra of the purified modified GroEL were recorded at 20 °C in the presence of HCA II<sub>pwt</sub> (1:1). The arrows indicate the broad component.

Figure 6. EPR spectra of IPSL-GroEL. Recorded at 20 °C (A) and 50 °C (B), alone (solid lines) and in the presence of HCA II<sub>pwt</sub> (dashed lines). Experimental details are described in the Experimental procedures section.
TABLE 1. Reactivation of heat denatured HCA II\textsubscript{pwt} in presence and absence of equimolar amounts of AEDANS-GroEL, IAF-GroEL and IPSL-GroEL.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>denaturation/reactivation\textsuperscript{a) }</th>
<th>yield after 3h of reactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCA II\textsubscript{pwt}</td>
<td>d, 50 °C for 1h/ r, 20 °C</td>
<td>30 %</td>
</tr>
<tr>
<td>HCA II\textsubscript{pwt}, AEDANS-GroEL</td>
<td>d, 50 °C for 1h/ r, 20 °C</td>
<td>77 %</td>
</tr>
<tr>
<td>HCA II\textsubscript{pwt}, IAF-GroEL</td>
<td>d, 50 °C for 1h/ r, 20 °C</td>
<td>16 %</td>
</tr>
<tr>
<td>HCA II\textsubscript{pwt}, IPSL-GroEL</td>
<td>d, 50 °C for 1h/ r, 20 °C</td>
<td>77 %</td>
</tr>
</tbody>
</table>

\textsuperscript{a) }Denaturation, d, for 1 h in 50 °C followed by refolding, r, by lowering the temperature to 20 °C.

TABLE 2. Fluorescence data from AEDANS-GroEL in absence and presence of equimolar amounts of HCA II substrate, for details, see the Experimental Procedures section.

<table>
<thead>
<tr>
<th>Sample</th>
<th>peak (nm)</th>
<th>Intensity (%)\textsuperscript{a) }</th>
<th>Anisotropy</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 °C AEDANS-GroEL</td>
<td>485.2</td>
<td>100</td>
<td>0.05</td>
</tr>
<tr>
<td>20 °C AEDANS-GroEL+ HCA II\textsubscript{pwt}</td>
<td>484.4</td>
<td>99.8</td>
<td>0.05</td>
</tr>
<tr>
<td>50 °C AEDANS-GroEL</td>
<td>486.8</td>
<td>100</td>
<td>0.02</td>
</tr>
<tr>
<td>50 °C AEDANS-GroEL+ HCA II\textsubscript{pwt}</td>
<td>487.0</td>
<td>103.4</td>
<td>0.02</td>
</tr>
</tbody>
</table>

\textsuperscript{a) }Normalized to AEDANS-GroEL alone at the given temperature.
Protein substrate binding induces conformational changes in the chaperonin GroEL. A suggested mechanism for unfoldase activity
Per Hammarström, Malin Persson, Rikard Owenius, Mikael Lindgren and Uno Carlsson
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