Conformational Changes Generated in GroEL during ATP Hydrolysis as Seen by Time-resolved Infrared Spectroscopy*

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Changes in the vibrational spectrum of the chaperonin GroEL in the presence of ADP and ATP have been followed as a function of time using rapid scan Fourier transform infrared spectroscopy. The interaction of nucleotides with GroEL was triggered by the photochemical release of the ligands from their corresponding biologically inactive precursors (caged nucleotides; P-nitrophenyl nucleotide). Binding of either ADP or ATP induced the appearance of small differential signals in the amide I band of the protein, sensitive to protein secondary structure, suggesting a subtle and localized change in protein conformation. Moreover, conformational changes associated with ATP hydrolysis were detected that differed markedly from those observed upon nucleotide binding. Both, high-amplitude absorbance changes and difference bands attributable to modifications in the interaction between oppositely charged residues were observed during ATP hydrolysis. Once this process had occurred, the protein relaxed to an ADP-like conformation. Our results suggest that the secondary structure as well as salt bridges of GroEL are modified during ATP hydrolysis, as compared with the ATP and ADP bound protein states.

Chaperonin GroEL from Escherichia coli facilitates protein folding in vivo and in vitro in an ATP-regulated manner (1, 2). GroEL consists of two heptameric rings composed of identical subunits that form a double toroid structure. Each subunit is organized into two major domains, the apical domain being involved in GroES and substrate binding, and the equatorial one holding the nucleotide-binding site and most of the intra- and inter-ring contacts, both linked by a small and flexible intermediate domain (3).

Numerous ligands have been shown to modulate the conformation of GroEL (4, 5). Among them, nucleotides can modify the chemical properties, structure and substrate-binding affinity of the central cavity of the protein that binds the substrate (1, 6). These modifications depend on the nature of the bound nucleotide. The cooperative binding of ATP switches GroEL from a high affinity state for non-folded proteins (T) to a protein release state (R) which has low affinity for substrate proteins (7). ATP hydrolysis drives the oligomer cavity through alternating states with different affinities for unfolded protein, and it has been recently demonstrated that nucleotide hydrolysis is required to release GroES, once ATP binds to the adjacent protein ring (8). Electron microscopy (EM) studies have shown that ATP induces large conformational changes in GroEL (5, 9). Supporting these findings, proteolysis studies have revealed a conformational change in the apical GroEL domain in response to nucleotide binding (4, 10). Moreover, ATP hydrolysis has also been correlated with an increased exposure of hydrophobic patches at the chaperonin surface (11).

Although the importance of the molecular events associated to the nucleotide-induced allosteric transition of GroEL has been widely recognized, they remain as yet mostly unknown (12). In an effort to obtain such information, we have applied time-resolved infrared difference spectroscopy combined with the use of caged nucleotides. This approach allows to monitor subtle changes in the vibrational spectrum between two protein states whose interconversion can be triggered in the infrared cuvette. Moreover, time-resolved IR spectroscopy makes it possible to follow in real-time the formation of intermediates in the ATPase reaction cycle of GroEL, with the following advantages. (i) It is a non-invasive technique, i.e. there is no need to label the protein, and therefore the possible alteration of the protein structure by the label is avoided. (ii) It allows to follow, at the same time, the kinetic parameters of overall protein conformational changes, and modifications of specific protein groups as a consequence of nucleotide binding and hydrolysis (13, 14). Our results demonstrate that GroEL undergoes a transient conformational transition during ATP hydrolysis, that differs from the one observed upon ATP or ADP binding.

MATERIALS AND METHODS

Sample Preparation—GroEL was overexpressed from E. coli and purified as described previously (15). The protein was concentrated with microconcentration filters (Centricon-50 (Amicon)), and the buffer was exchanged by repeated concentration and dilution steps with buffer 100 mM imidazole, 100 mM NaCl, 30 mM MgCl2 prepared in H2O or D2O, pH or pd 7.0. Infrared samples were prepared by drying 1 μl of caged nucleotide (20 mM) and 1 μl of 40 mM KCl onto a CaF2 window with a trough of 8-μm depth and 8-mm diameter. For the samples containing DTT, an additional 1 μl of 20 mM DTT was dripped onto the window. They

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1 The abbreviations used are: EM, electron microscopy; DTT, dithiothreitol; IR, infrared.
were rehydrated with the same volume of GroEL dissolved in the above buffer and the samples were sealed with a second flat window and thermostated at 25 °C during the experiment. Protein subunit concentration was 0.8 mM, as determined by the bicinchoninic acid assay (Sigma). Control experiments were measured on identical samples, prepared without GroEL.

Infrared Spectroscopy—Infrared measurements were carried out on a modified Bruker IFS 66 spectrophotometer equipped with a HgCdTe detector. Data were acquired with double sided interferograms in a forward-backward mode at a spectral resolution of 4 cm⁻¹. One interferometer cycle corresponding to two spectra needed 65 ms for completion. After recording a blank difference spectrum to control for the signal-to-noise ratio and base-line stability, the experiment started by measuring a reference spectrum coadded from 300 scans which characterized the unperturbed sample. Photolysis of caged nucleotides was triggered with a Xenon flash tube with high UV output. The voltage of the flash power supply was adjusted to release approximately 1.5 mM nucleotide/flash. After the flash, 20 spectra at 2 scans each, 20 spectra at 4 scans each, 10 spectra at 10 scans each, 20 spectra at 20 scans each, and 20 spectra at 100 scans each were recorded. To additionally improve the signal-to-noise ratio, signals obtained from different samples were averaged after normalizing the solvent-subtracted reference spectra of the protein to the same absorbance value in the amide I (0.4 absorbance unit in D₂O) or amide II (0.06 absorbance unit in H₂O) band. The difference spectra were not subjected to smoothing or other resolution enhancement procedures, such as deconvolution or derivation. The noise level in these spectra, as confirmed from control experiments, was estimated to be around 5 × 10⁻⁵ absorbance unit at frequencies above 1750 cm⁻¹, where no signals appear, and approximately 10⁻⁴ absorbance unit at around 1650 in the H₂O samples due to the strong absorbance from H-O-H bending modes. These noise levels confirmed the reliability of the experimentally observed weak signals.

Kinetic Analysis—To fit the IR absorbance changes to a kinetic model, we used integrated band intensities. Integration was performed with respect to a baseline that was drawn between two limits at each side of the band. To avoid the overlapping effect expected in a complex difference profile, in some cases the integration boundaries were chosen so that the resulting area was characteristic of a single band. To determine the rate constants, several bands with high signal-to-noise ratio were selected. For the kinetic analysis the time slots of spectra recording were represented by their average times.

ATPase Hydrolysis—The ATPase activity of GroEL was assayed at 25 °C, using malachite green to measure the amount of inorganic phosphate released upon ATP hydrolysis as previously reported (16). The reaction was started by adding GroEL final oligomer concentration 0.49 μM to the assay solution containing 50 mM imidazole, 40 mM KCl, 30 mM MgCl₂, 100 mM NaCl, pH 7.0, and 1.5 mM free or caged ATP. Aliquots of the reaction mixture were diluted with 3 volumes of 1 M imidazole, was estimated to be around 5

ATPase activity measurements clearly indicate that caged ATP is not modified by GroEL, and that hydrolysis only occurs after ATP release from the cage (Fig. 1). Furthermore, they also demonstrate that the presence of the free cage does not significantly affect the ATPase activity of the protein. However, they do not rule out the possible interaction between the caged ligands and the protein. In order to test whether binding of caged ADP and ATP to GroEL was taking place before nucleotide release from the cage, two types of experiments were performed. First, filtration experiments pointed out that, unlike free nucleotides which can bind tightly to half of the 14 available sites in GroEL (Kd < 15 μM; 17), none of the caged nucleotides were able to bind to the protein, since all the initially added caged ligands appeared in the filtrate (data not shown). Second, electron microscopy clearly showed that neither caged ATP nor caged ADP were able to induce the formation of GroES-GroEL asymmetric complexes, in contrast to the free nucleotides (5) (data not shown). Therefore, we conclude that caged nucleotides do not bind to GroEL.

ATP-induced Conformational Changes—The interaction between ATP and GroEL is accomplished by triggering the release of the ligand from an inactive (caged) photolabile analog with a UV flash, which does not disturb the sample (18). The spectrum recorded before illuminating the sample is the reference spectrum recorded 3.6 s after release of ATP-induced Conformational Changes—

Binding and Hydrolysis of Caged Nucleotides by GroEL—Prior to the spectroscopic characterization of the interaction of nucleotides with GroEL, it is necessary to ensure that their caged analogs are not bound or hydrolyzed by the protein.
Nucleotide-induced Conformational Changes in GroEL

Thus leaving the 1800–1300 cm\(^{-1}\) band (mainly due to amide-backbone NH groups) shifts from polypeptide backbone from bands due to amino acid side chains. The amide I modes (1700–1610 cm\(^{-1}\)) helps to distinguish bands caused by amide modes of the protein backbone from bands due to amino acid side chains absorbing in this spectral region, i.e. those from Asn, Gln, Lys, Arg, show larger shifts (23, 24). Deuteration of the sample induces the following noticeable changes in the difference spectrum of the protein. 1) The absorbance changes at 1692, 1654, and 1629 cm\(^{-1}\) are downshifted to 1686, 1648, and 1607 cm\(^{-1}\), respectively. 2) The intensity of the differential signal observed in H\(_2\)O at 1556(+)/1546(–) is strongly reduced. 3) The signal at 1511(+)/1508(–) cm\(^{-1}\) almost completely disappears. The small shift observed for the 1692 and 1654 cm\(^{-1}\) bands suggests that they are due to changes in the amide I absorbance of the polypeptide backbone. The existence of an ATP-induced conformational change is also indicated by the 1556(+)/1546(–) differential feature, which can be assigned to the amide I mode of the protein, because it is virtually not detected upon deuteration. Instead, a band is observed at 1469 cm\(^{-1}\), which may represent the amide II’ mode. A possible interpretation of the 1568 cm\(^{-1}\) band is the appearance of carboxylate group(s) upon ATP binding and/or hydrolysis. Indeed, a band at this position has been assigned to -COO\(^-\) groups of Glu or Asp residues and it has been shown to be influenced very little, as compared with other side chain vibrations, by deuteration (23, 24). This could be achieved either by a change in the ionization state of a Glu or Asp residue(s), or a modification of the interaction between oppositely charged residues, i.e. salt bridges. If the former hypothesis would hold, the appearance of this positive band should be accompanied by a negative counterpart at around 1700–1730 cm\(^{-1}\), that would indicate the disappearance of a -COOH group(s). Although a signal at this position is not observed in the difference IR spectra, we have carried out the same experiments in the presence of DTT to eliminate the photolysis signal that appears at 1692 cm\(^{-1}\) (H\(_2\)O) and 1686 cm\(^{-1}\) (D\(_2\)O), that may partially overlap with a COOH vibration. The results (Fig. 2, insets) demonstrate the presence of a weak negative signal at 1700 cm\(^{-1}\) in H\(_2\)O that is shifted upon deuteration to 1695 cm\(^{-1}\). The position of this differential feature, downshifted from the characteristic frequency of the -COOH groups, makes its assignment to a carboxylic group unlikely. It is important to note that the positions and amplitudes of all the differential signals mentioned above are maintained, indicating that the presence of DTT does not modify the structural transition brought about by the interaction of ATP with GroEL.

Alternatively, if a modification of the interaction between oppositely charged residues were responsible for the 1568 cm\(^{-1}\) positive signal, “marker” differential bands for Arg and/or Lys residues should also be observed in the infrared difference spectra. These bands appear in the 1700–1500 cm\(^{-1}\) spectral region, where they can overlap with vibrations from the amide I and amide II modes of the protein. Based on studies of model compounds (23, 24), the protonated side chain of arginine residues gives rise to two signals at around 1673 and 1633 cm\(^{-1}\) in H\(_2\)O that shift to 1607 and 1586 cm\(^{-1}\) in D\(_2\)O, while the NH\(_3\)\(^+\) modes of Lys at around 1513 cm\(^{-1}\) disappear upon deuteration. Due to the possible overlap with protein backbone modes, the assignment of the differential features at around 1673 cm\(^{-1}\) to Arg residues is not straightforward. However, the putative assignment of the 1629 cm\(^{-1}\) signal to these residues is supported by the fact that it is virtually abolished upon deuteration. Furthermore, the absorbance change at 1607 cm\(^{-1}\) in D\(_2\)O may be attributed to Arg side chains, and possible contributions from protein backbone signals are not likely. These results, together with the disappearance of the 1511(+)/1508(–) cm\(^{-1}\) feature upon deuteration, suggest that both Arg and Lys residues could experience a change in their chemical environments during the interaction of ATP with GroEL. The alternative assignment of the 1511(+)/1508(–) cm\(^{-1}\) spectral feature,
observed only in H\textsubscript{2}O, to Tyr residues can be reasonably discarded because if this were the case neither the position nor the intensity of these vibrations should be significantly affected by deuteration (23, 24).

To distinguish the possible different conformational states generated as a consequence of the interaction of ATP with GroEL, we have analyzed the time dependence of the absorbance changes in the infrared difference spectrum of the protein (Fig. 3). Release of ATP from the cage occurs within the first 33 ms after the flash, as judged by the time course of the intensity of the photolysis bands (see the negative band at 1526 cm\textsuperscript{-1}). The corresponding infrared difference spectrum shows a positive differential signal at 1648 cm\textsuperscript{-1}, attributable to the protein, which is maintained in the spectra recorded during the first 0.23 s after the flash. At longer time intervals, up to 7 s, the intensity of this and other positive (1607 and 1568 cm\textsuperscript{-1}) and negative (1673 and around 1620 cm\textsuperscript{-1}) differential features increases. The amplitude of these absorbance changes is maintained during approximately 60 s, a time interval that most likely corresponds to a multiple turnover reaction where the enzyme is in the steady-state (note that the [ATP]/[GroEL subunit] is approximately 2). This interpretation would be in accordance with kinetic studies using pyrene-labeled GroEL (17). Afterward, a slow structural change drives GroEL into a conformation analogous to that exhibited in the presence of ADP (see below). As compared with the IR difference spectra obtained within the 10–60-s interval, the spectrum of this conformation lacks the 1568 cm\textsuperscript{-1} signal and shows a positive signal at 1627 cm\textsuperscript{-1}, both characteristic of the ADP-bound state (see Fig. 3, top trace, for a comparison). The analysis of the time course of the absorbance changes after ATP release is shown in Fig. 4. The selected signals are tentatively assigned to alterations of the amide I mode of the polypeptide backbone (1648 cm\textsuperscript{-1}), of acidic amino acid side chains (1568 cm\textsuperscript{-1}), and of phosphate vibrations (1273 cm\textsuperscript{-1}). To properly analyze the time course of these signals, two time constants were required. Their values for the fast phase are 0.4, 0.55, and 0.8 s for the 1273, 1648, and 1568 cm\textsuperscript{-1} bands, respectively, while those of the slow phase are 3.3 ± 0.3 s for all bands.

ADP-induced Conformational Changes—A comparison of the difference spectra recorded 3.6 s after the photolysis flash, in the presence (solid lines) and absence (dashed lines) of GroEL, reveals that ADP binding to the protein induces the appearance of absorbance changes in the 1700–1610 cm\textsuperscript{-1} spectral region (amide I band) that cannot be attributed to the photolysis reaction (Fig. 5). These absorbance changes are located at 1647 and 1628 cm\textsuperscript{-1} in both H\textsubscript{2}O and D\textsubscript{2}O. The possible contribution of amino acid side chains to these differential signals can be better considered by analyzing the effect of deuteration on the difference spectrum. As stated before, the absence of significant shifts in band position upon deuteration allows assignment of the observed major absorbance changes to peptide C=O groups. It is important to note that none of these bands shows the kinetic behavior observed in the ATP-containing samples (data not shown), and that they reach their maximum amplitude 0.42 s after nucleotide release to the medium. More significantly, the ADP-induced difference spectra do not display absorbance changes attributable to basic and acidic amino acid side chains, as observed in the presence of ATP.

**DISCUSSION**

There are a number of experimental evidences indicating that the conformational rearrangements induced in GroEL by the physiologically relevant nucleotides might have functional implications. EM studies have shown that the apical domains of GroEL move differently in the presence of ATP or ADP (5, 9). Moreover, only in the presence of ATP and during its hydrolysis does GroEL seem to transiently expose additional hydrophobic residues as judged from bis-1-anilino-8-naphthalenesulfonate binding experiments (11). Functional studies with a disulfide GroEL mutant have also shown that nucleotide hydrolysis is coupled to a spatial rearrangement of the protein particle which, in turn, promotes release of the substrate (25). So far, these experimental evidences have been analyzed in terms of changes in the tertiary and/or quaternary structures of the protein, paying little attention to its secondary structure. This is mainly due to limitations of the spectroscopic techniques to monitor local rather than overall conformational changes (26).

In this context, “reaction-induced infrared difference spectroscopy” provides an appropriate tool to analyze the small and localized structural changes associated with binding to and/or hydrolysis of different nucleotides by GroEL.

Before discussing the differences between the GroEL conformational states generated in the presence of ATP or ADP, we shall analyze the time dependence of the ATP-induced absorbance changes. This might help to distinguish the consequences of nucleotide binding from its subsequent hydrolysis on GroEL conformation. As shown by the intensity of the photolysis bands, nucleotide release to the medium occurs within the first 33 ms after the flash. Under the experimental conditions used in this work (high protein and nucleotide concentration, 25 °C) ATP binding to GroEL is strongly favored. This is supported by

![Fig. 3. Time dependence of the IR difference spectrum of GroEL in the presence of ATP. Measurements were performed after release of 1.5 mM ATP in a sample containing 0.8 mM GroEL subunit, at 25 °C in D\textsubscript{2}O buffer. Spectra were recorded at the times indicated below each trace after the photolysis flash. Average of 11 different experiments. The spectrum labeled ADP (top, thick trace) was recorded 15 s after ADP release in the presence of GroEL.](image-url)
the fact that the ADP-induced absorbance changes reach their maximum amplitude 0.42 s after nucleotide release from the cage. Considering that the affinity of GroEL for both nucleotides is similar, it is reasonable to assume that the spectra recorded in the presence of ATP before this time would correspond to the ATP-bound GroEL state, without contributions from ATP hydrolysis. The corresponding difference spectra indicate that ATP binding to GroEL induces a small rearrangement of its secondary structure elements which, in contrast to what is found during nucleotide hydrolysis, does not affect basic and acidic amino acid side chains. Although the rate constant of the initial event compares well with the one described for ATP binding to pyrene-labeled GroEL (17, 27), it should be mentioned that under our experimental conditions overlapping of the binding and hydrolysis steps might obscure the kinetic analysis of the binding process.

During the subsequent ATP hydrolysis step, the protein experiences a more pronounced conformational change, as evidenced by a 3-fold increase in the intensity of differential bands attributable to the polypeptide backbone. Moreover, our results also show the appearance of specific signals tentatively assigned to acidic and basic residues changing their chemical environment, indicating that ATP hydrolysis is probably coupled to modifications of salt bridges of the protein. The transient character of this conformational state is demonstrated by the recovery of the absorbance changes characteristic of the ADP-bound state after nucleotide hydrolysis. There are several salt bridges involved in intra- and intersubunits contacts within the same protein ring, as well as in inter-ring interactions. It has been shown that the intrasubunit salt bridge between Glu-409 and Arg-501, at the hinge between the equatorial and the apical domain and close to the ATP-binding site, becomes significantly weaker during the ATP-induced allosteric transitions of GroEL (28). The ion pair formed by Glu-434 and Lys-105, involved in intra-ring communication, is also believed to change in the presence of ATP, since the nucleotide greatly reduces the electron density at this contact site (9). Salt bridges also provide numerous contact sites between neighboring subunits. Based on EM and modeling studies, it has been proposed that during the ATP-induced transition from the T to the R state, several salt bridges between subunit apical domains are modified (29). A conclusive assignment of the experimentally observed difference signals to specific amino acids will require the characterization of selected mutants. The time constant of the slow process is twice lower than the one estimated from ATPase activity and fluorescence measurements (17, 30). This discrepancy might well be caused by the higher protein and ATP concentrations used in this work, as it has been shown for the Ca$^{2+}$-ATPase from sarcoplasmic reticulum (14).

The difference between the ADP- and ATP-bound states of GroEL is difficult to ascertain, due to the small amplitude of the absorbance changes (~1% of the amide I band intensity). This, nevertheless, suggests that the conformational change induced in GroEL upon nucleotide binding is localized, and it might reflect modifications within existing secondary structure elements rather than a net change of secondary structure (14). However, the conformation of the active, ATP-hydrolyzing state of GroEL is clearly distinct from those of the nucleotide-bound states. The amplitude of the major absorbance changes within the amide I increases 3 and 4 times as compared with those observed upon ATP and ADP binding, respectively. The position of the strongest absorbance change in H$_2$O (1654 cm$^{-1}$) and D$_2$O (1648 cm$^{-1}$) has been described for solvent-exposed α-helical structures (31, 32). Less intense differential signals are characteristic of turns (1686, 1673 cm$^{-1}$ in D$_2$O) and β-structures (negative band, at approximately 1626 cm$^{-1}$).
in D$_2$O). The fact that a differential signal attributable to the amide II mode of the protein also appears during ATP hydrolysis supports the existence of this transient GroEL conformation.

The role of ATP hydrolysis in GroEL function still remains unclear. On one hand, kinetic and structural data indicate that ATP binding is responsible for the conformational changes in GroEL that release the bound substrate, while nucleotide hydrolysis simply resets the system. On the other hand, EM (5, 9) and biochemical data (11) indicate that the ADP-bound state is structurally different from the unliganded and ATP-hydrolyzing state. Recent experiments have shown that ATP hydrolysis in the cis ring weakens the binding of GroEL and primes it for release, after ATP binding to the trans ring (8). In this context, our results suggest that during ATP hydrolysis, GroEL undergoes a unique conformational transition, different from those caused by ATP or ADP-binding, which involves changes in solvent-exposed secondary structure elements and modifications of salt bridges of the protein.

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