Conformational Changes at the Nucleotide Binding of GroEL Induced by Binding of Protein Substrates

LUMINESCENCE STUDIES*

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2'-Deoxy-3'-anthranilyl adenosine-5'-triphosphate (ANT-dATP) coordinated to Tb$^{3+}$ was used as an environmentally sensitive probe of the nucleotide-binding site of GroEL. Tb$^{3+}$-ANT-dATP recognizes the nucleotide-binding site of GroEL and inhibits ATPase activity. Sensitized luminescence, arising from resonance energy transfer from the anthranilyl moiety to Tb$^{3+}$, is substantially enhanced in the presence of GroEL. Binding of denatured mitochondrial malate dehydrogenase to the apical domain of GroEL causes a red shift in the fluorescence emitted by anthranilyl and further enhancement in the phosphorescence emitted by Tb$^{3+}$ upon excitation at 320 nm.

It is suggested that binding of the protein substrate initiates domain movement, which is extended to the nucleotide-binding site. The luminescence results are discussed in reference to the structure of GroEL derived from x-ray crystallographic studies.

Electron microscopic studies of two-dimensional crystals have shown that Escherichia coli chaperonin GroEL is a cylindrical homo-oligomer composed of two rings, each containing seven 57-kDa subunits (1).

More recently the three-dimensional structure of GroEL (2), solved to 2.8 Å resolution, has given a more detailed insight into the domain structure of the GroEL subunit; the apical domain forming the opening of the central cavity contains the substrate-binding site, whereas the largest equatorial domain provides a nucleotide-binding site.

GroEL is known to bind at least half of the E. coli proteins in their denatured states (3). It is thus likely that flexibility is required to bind a large variety of proteins differing in primary, secondary and tertiary structure.

Several lines of experimental evidence suggest that binding of nucleotides to GroEL induces conformational changes in the apical domain, which influences the affinity of peptides and unfolded proteins (4-6). ATP-induced conformational changes have been detected with fluorometric techniques in a chemically modified form of GroEL (7) or in GroEL mutants containing tryptophan residues in the apical domain (8). Although it has been shown that binding of protein substrates stimulate ATPase activity(4), it is not known whether the same binding process is responsible for conformational changes at the level of the nucleotide-binding site in the equatorial domain.

Does the binding of the protein substrate alter the conformation of the nucleotide-binding site? In an attempt to detect coupling between the apical and equatorial domains of GroEL, the luminescence properties of a probe coordinated to the nucleotide-binding site were investigated in detail. The ATP analog 2-deoxy-3-anthranilyladenosine-5'-triphosphate (ANT-dATP)1 coordinated to the ion Tb$^{3+}$ was used for these experiments, because it forms a tightly bound complex with GroEL, which does not undergo a process of hydrolysis, resulting in the formation of ADP and phosphate. Moreover, the donor-acceptor pair, anthranilyl-Tb$^{3+}$ is ideally suited for energy transfer experiments, because the absorption band of the acceptor overlaps the emission band of the donor to yield a critical distance of transfer of 5 Å (9). The fluorescence band of anthranilyl, maximum at around 410 nm, is easily distinguished from the four phosphorescence bands of Tb$^{3+}$, which are centered at 490, 545, 580, and 620 nm (10). The results obtained with two different spectroscopic methods, i.e. fluorescence and phosphorescence, covering the time range 2 ns to 1ms, lend support to the contention that the interaction of unfolded proteins with GroEL perturbs the microenvironment of the nucleotide site.

EXPERIMENTAL PROCEDURES

Purification of Proteins—The E. coli GroEL gene (11) was expressed in E. coli MC1061 cells from the pND5 plasmid (12). The protein was purified by a modification of the procedure (13) originally published in Ref. 14. Protein concentration was determined by the Lowry method (24). Truncated GroEL was prepared by thermolysin digestion of E. coli GroEL as described by Makino et al. (15). The fragment of 35 kDa was isolated by gel filtration chromatography as described in Ref. 15.

Mitochondrial malate dehydrogenase (mMDH) purchased from Boehringer Mannheim was purified by DEAE-cellulose chromatography using a linear gradient from 20 to 100 mM of Tris-HCl, pH 7.5. The specific activity of the enzyme was 1400 units/mg of protein. The assay buffer for mMDH was composed of 100 mM Tris-HCl, pH 7.5, 0.5 mM oxaloacetate, and 0.2 mM NADH. The initial rates for conversion of NADH to NAD were determined by changes in absorbance at 340 nm for 1 min at 25°C.

The concentration on mMDH was calculated using $A_{280} = 2.5$ for a 1% solution with a subunit molecular mass of 35 kDa (16).

Unfolding Experiments—mMDH at a concentration of 1 mg/ml was treated with 3 mM guanidinium chloride in 100 mM Tris-HCl buffer, pH 7.0, for 1 h at 37°C. For refolding experiments, the denatured enzyme was diluted to a final concentration of 0.02 mg/ml, or it was filtered through a small Sephadex G-25 column to remove the denaturing agent. Partially folded mMDH does not recover its catalytic activity in the absence of GroEL, Mg-ATP and GroES.

Synthesis of ANT-dATP—It was prepared essentially as described by Hiratsuka (17) for the ribonucleotides. The product was purified by two chromatography steps using a Sephadex LH-20 column (2 × 56 cm) equilibrated with water and a DEAE-cellulose column (2 × 30 cm) equilibrated with 0.2 M triethy lammonium bicarbonate, pH 7.5, and eluted with a gradient of 0.2–0.5 M triethy lammonium bicarbonate, pH 7.5. The purified compound has an absorption coefficient of 4700 M$^{-1}$ cm$^{-1}$ at 330 nm.

ATPase Assays—ATPase assays for purified GroEL were performed exactly as described by Bais (18) using 0.5 mM ATP containing 1 μCi of [γ-32P]ATP in the standard assay buffer (20 mM Tris-HCl, pH 7, 2 mM

1 The abbreviations used are: ANT-dATP, 2-deoxy-3-anthranilyladenosine-5'-triphosphate; mMDH, mitochondrial malate dehydrogenase; ANS, 1-anilinonaphthalene-8-sulfonate.

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magnesium acetate, 25 mM KCl, 1 mM dithiothreitol).

The turnover rate of MgATP was 0.030 mol/subunit/s. Tb³⁺, at a concentration of 5 μM, inhibits completely the hydrolysis of MgATP (Table II).

Luminescence Spectroscopy—A Perkin-Elmer model 50 S spectrofluorometer was used in the luminescence measurements. For fluorescence measurements of the anthraniloyl chromophore, the excitation wavelength was 320 nm. The excitation and emission slits were set at 2.5 nm. For the study of Tb³⁺-sensitized luminescence, the sample was excited with a pulsed xenon flash lamp with pulse width at half-maximum height of 10 μs. The decay time was 1 ms, and the gate time was 10 ms. The excitation and emission slits for excitation at 320 nm were at 5 nm. Phosphorescence decay times were measured with a precision of 5%, and the decay curves were fitted to single or double exponential functions using statgraphics (Statistical Graphic System STSC Inc.).

Energy Transfer Measurements—They were performed on Tb³⁺-ANT-dATP coordinated to GroEL at pH 7 in 20 mM Tris-HCl buffer, pH 7, containing 50 mM KCl.

The Efficiency of Sensitized Luminescence Was Calculated with the Aid of Equation 1,

\[ E = \frac{L_{Tb}^{3+} \cdot Q_{max}}{L_{ANT-dATP} \cdot Q_{Tb}^{3+}} \]  

(Eq. 1)

where \( L_{Tb}^{3+} \) and \( L_{ANT-dATP} \) are the integrated areas for Tb³⁺-phosphorescence and ANT-dATP fluorescence, respectively, corrected for instrumental response. \( Q_{max} = 0.12 \) and \( Q_{Tb}^{3+} = 0.28 \) are the quantum yields determined as described in Ref. 9.

Due to the value of \( L_{Tb}^{3+}/L_{ANT-dATP} = 0.013 \) for Tb ANT-dATP, the efficiency of energy transfer is low.

The critical distance of transfer \( R_{0} = 5 \) Å for the pair anthraniloyl-Tb³⁺ was determined with the aid of Förster’s equation as described in Ref. 9.

Fitting of Experimental Data—Experimental data were fitted using the software Kaleidograph (Albeck Software) and Statgraphics (Statistical Graphic System STSC, Inc.).

RESULTS AND DISCUSSION

Binding of ANT-dATP to GroEL—The affinity of Tb³⁺ for ANT-dATP varies between 0.2 and 0.02 μM, depending upon the pH of the medium. At pH 7, a dissociation constant of 0.05 μM has been determined for the coordinated complex (9).

The anthraniloyl moiety of Tb³⁺-ANT-dATP exhibits a fluorescence band at around 410 nm and phosphorescence bands at 490, 545, 580, and 620 nm due to sensitized luminescence of Tb³⁺ upon excitation at 320 nm (9).

Upon binding to GroEL, the fluorescence intensity remains practically unchanged (Fig. 1), although the emission anisotropy of the derivatized nucleotide in the presence of protein undergoes a significant change from \( A_{0} = 0.03 \) for free ligand to \( A_{m} = 0.15 \) for bound ligand (Table II).

When the interaction of the ATP analog with the chaperone was investigated by sensitized luminescence spectroscopy, it was found that the characteristic emission band of Tb³⁺ at 490, 545, 580, and 620 nm was at least 2-fold enhanced with respect to free Tb³⁺-ANT-dATP excited at the same wavelength of 320 nm (Fig. 2).

Under this set of experimental conditions, i.e. excitation at 320 nm and micromolar concentrations of Tb³⁺, the luminescence of free Tb³⁺ ions could not be detected.

The enhancement of sensitized luminescence could be attributed to a change in the relative position or orientation of the donor-acceptor pair induced by changes in the microenvironment where ATP binds. This change in efficiency of sensitized luminescence was exploited to determine the stoichiometry of binding. As can be seen from the results included in Fig. 3, the sensitized luminescence at 550 nm increases as a function of Tb³⁺-ANT-dATP concentration until a maximum value of the intensity is attained at a mixing molar ratio of approximately 1 mol of subunit/mol of Tb³⁺-ANT-dATP.

A truncated subunit obtained by limited thermolysin proteolysis of GroEL failed to bind Tb³⁺-ANT-dATP, suggesting that the nucleotide binding structure of the chaperone has been disrupted by proteolysis (Fig. 2). This is consistent with the observation that the truncated protein of 35 kDa stimulates rhodanese activity without the participation of MgATP (15).

In an effort to measure the dissociation constant of Tb³⁺-ANT-dATP bound to GroEL, sensitized luminescence measurements were performed at GroEL concentrations ranging from 0.1 to 1 μM. At the lowest concentration of protein tested, i.e. 0.1 μM, the addition of 1.4 μM Tb³⁺-ANT-dATP yields a 2-fold increase in sensitized luminescence when compared with free Tb³⁺-ANT-dATP. Due to weak signals of the sensitized luminescence at protein concentrations below 0.1 μM, it was difficult to determine the dissociation constant of Tb³⁺-ANT-dATP coordinated to the protein. However, it is inferred that Tb³⁺-ANT-dATP binds more tightly than MgATP, whose dissociation constant is above 1 μM(4).

Interaction of Denatured Mitochondrial Malate Dehydrogenase with GroEL—To ascertain whether binding of a denatured

### Table I

| Sample            | \( k_{cat} \) |
|-------------------|---------------|
| GroEL + MgATP     | 0.03 s⁻¹      |
| GroEL + MgATP + mMDH | 0.3 s⁻¹      |
| GroEL + MgATP + TbCl₃ (5 μM) | 0.0 s⁻¹ |

### Table II

| Sample                  | Anisotropy | Energy transfer (efficiency) | Phosphorescence decay (ms) |
|-------------------------|------------|-----------------------------|---------------------------|
| Tb³⁺ - ANT-dATP         | 0.03       | 0.0065                      | 1.0                       |
| Tb³⁺ - ANT-dATP + GroEL | 0.15       | 0.011                       | 1.10                      |
| Tb³⁺ - ANT-dATP + GroEL + mMDH | 0.16       | 0.0182                      | 1.15                      |
protein to GroEL influences the spectroscopic properties of a probe positioned at the nucleotide-binding site, the fluorescence and luminescence properties of Tb$^{3+}$-ANT-dATP coordinated to GroEL were measured in the absence and presence of denatured mMDH, respectively.

Several reasons led us to choose denatured mMDH for the luminescence experiments. Denaturation of mMDH by guanidinium chloride, followed by dilution with 20 mM Tris-HCl buffer, pH 7, does not restore the physical and biological properties of the undenatured enzyme (19, 20).

Indeed, the formation of partially folded species are easily detected by circular dichroism, fluorescence spectroscopy, and high performance liquid chromatography (20). Spontaneous recovery of catalytic activity occurs in the presence of GroEL, ATP, and GroES. GroEL alone inhibits the spontaneous refolding of mMDH (21).

When partially folded mMDH was added to a solution of GroEL containing an equimolar amount of Tb$^{3+}$-ANT-dATP, it was found that the emission band of the anthraniloyl chromophore was enhanced with a concomitant red shift in the band position (Fig. 1). A control containing partially folded mMDH and Tb$^{3+}$-ANT-dATP gave an emission band identical to that of free Tb$^{3+}$-ANT-dATP, indicating that the fluorescence shift is not due to binding of the ATP analog to the protein substrate. Similar enhancement of anthraniloyl fluorescence was observed when samples of partially folded mMDH, passed through a Sephadex G-25 column to remove guanidinium chloride, were allowed to interact with Tb$^{3+}$-ANT-dATP-GroEL as shown by the results included in Fig. 4. Hence, changes in anthraniloyl fluorescence induced by addition of partially folded mMDH could not be attributed to perturbation of the structure of GroEL by the denaturing agent.

The increase in fluorescence intensity at 410 nm was used to determine the stoichiometry of binding. As can be seen from the results included in Fig. 4, the fluorescence emitted by the anthraniloyl chromophore increases as a function of denatured mMDH concentration until it reaches a maximum at a molar mixing ration of GroEL to partially folded mMDH of approximately 1:1.

The binding of partially folded mMDH to GroEL not only influences the fluorescence emitted by the anthraniloyl chromophore, but also the sensitized luminescence of Tb$^{3+}$ as shown by the results included in Fig. 2.

It might be argued that the increase in fluorescence and sensitized luminescence of Tb$^{3+}$-ANT-dATP is due to the formation of a complex in which the denatured protein interacts directly with the ATP-binding sites of GroEL. Although denatured mMDH does not bind ANT-dATP, the possibility exists that upon binding to GroEL additional hydrophobic binding sites on the denatured protein are exposed to interactions with anthraniloyl chromophores. To test this hypothesis, the exposure of hydrophobic groups in partially folded mMDH was followed by measuring changes in the fluorescence emission of

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**FIG. 2. Sensitized luminescence of Tb$^{3+}$-ANT-dATP.** Phosphorescence spectra of solutions containing Tb$^{3+}$-ANT-dATP (14 µM) in the absence (—) and presence of GroEL (1 µM) (———) at pH 7 in 20 mM Tris-HCl (50 mM KCl). Phosphorescence spectra recorded after addition of denatured mMDH (0.5 µM) to a solution containing Tb$^{3+}$-ANT-dATP (14 µM) and GroEL (1 µM) (---). The phosphorescence spectrum of Tb$^{3+}$-ANT-dATP (14 µM) in the presence of truncated GroEL (20 µM) is included in the figure (×××) (bottom). A pulsating light source was used for excitation at 320 nm.

**FIG. 3. Changes in the sensitized luminescence as a function of Tb$^{3+}$-ANT-dATP concentration.** Results obtained when a solution of GroEL (1 µM) in 20 mM Tris-HCl buffer, pH 7, was mixed with increasing concentrations of Tb$^{3+}$-ANT-dATP and the luminescence read at 550 nm upon excitation at 320 nm. $L - L_{o}$ is the difference of luminescence in the presence and absence of GroEL, respectively.

**FIG. 4. Changes in the fluorescence intensity of bound Tb$^{3+}$-ANT-dATP as a function of denatured mMDH concentration.** mMDH (1 mg/ml) was denatured in the presence of 3 M guanidinium chloride at pH 7 for 1 h at 37°C. It was divided into two fractions; one of the fractions was passed through Sephadex G-25 to eliminate traces of the denaturing agent. A solution of GroEL (1 µM) containing Tb$^{3+}$-ANT-dATP (14 µM) in 20 mM Tris-HCl, 50 mM KCl, pH 7, was mixed with increasing concentrations of denatured mMDH and the fluorescence measured at 410 nm upon excitation at 320 nm. Results were obtained with samples contaminated with guanidinium chloride (○) and with partially folded mMDH passed through a gel filtration column prior to the titrations (●).
the extrinsic probe, 1-anilinonaphthalene-8-sulfonate (ANS), which is known to recognize hydrophobic clusters in proteins. Fig. 5 shows the results obtained when the denatured protein, in the absence and presence of GroEL, was allowed to interact with 40-fold molar excess of ANS. A pronounced increase in the fluorescence yield of the extrinsic probe is due to the presence of denatured mMDH. Further increase is observed in the presence of GroEL, but this enhancement of fluorescence is due to binding of ANS to the chaperonin as shown by the results included in Fig. 5. Thus, it appears that binding of partially folded mMDH to GroEL does not result in the exposure of additional hydrophobic sites. Moreover, it should be noted that the binding of partially folded mMDH to Tb$^{3+}$-ANT-dATP-GroEL results in 20% increase in anthraniloyl fluorescence, even when only 6 mol of Tb$^{3+}$-ANT-dATP are bound to 1 mol of GroEL (results not shown). According to x-ray crystallographic studies (2), the diameter of the central cavity of GroEL is about 47 Å and the length 146 Å. If the volume occupied by an unfolded protein is about 30% greater than its native counterpart, then the central cavity would accept a protein of 70 kDa, which is twice the size of denatured mMDH.

If, on the other hand, binding of partially folded mMDH occurs exclusively at the outer GroEL surface, as has been proposed for other protein substrates (22, 23), then it is impossible to explain direct interaction of the unfolded protein with the ATP-binding sites.

The most likely explanation for the change in the luminescence properties of bound Tb$^{3+}$-ANT-dATP, elicited by binding of unfolded forms of the protein substrate, must be sought in conformational changes of the equatorial domains of GroEL. The functional role played by conformational changes of GroEL in the rearrangement of the protein substrate can be discussed in reference to the structure provided by x-ray crystallography (2).

According to these studies, the equatorial domain is highly α-helical and well ordered. It provides most of the side-to-side contacts between subunits in the ring and most of the contacts between the rings across the equatorial plane. The ATP-binding site resides in the equatorial domain of each subunit of GroEL. Hence, subtle changes in interdomain contacts might alter the flexibility of the chaperonin, which is required to facilitate the folding of the protein substrate.

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