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Semibiological Molecular Machine with an Implemented "AND" Logic Gate for Regulation of Protein Folding

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Abstract: A semibiological molecular machine with an implemented "AND" logic gate was developed, which was capable of controlling the folding process of proteins in response to ATP and light as input stimuli. The molecular design made use of a genetically engineered chaperonin GroEL bearing, at both entrance parts of its cylindrical cavity, cysteine residues, which were functionalized by an azobenzene derivative to construct photoresponsive mechanical gates (azo-GroEL). This engineered chaperonin trapped denatured green fluorescent protein (GFP_{denat}) and prohibited its refolding. However, when hosting azo-GroEL detected ATP (input stimulus 1) and UV light (input stimulus 2) at the same time, it quickly released GFP_{denat} to allow its refolding. In contrast, reception of either input stimulus 1 or 2 resulted in only very slow or no substantial refolding of GFP_{denat}. Implementation of such "AND" logic gate mechanisms in mechanically driven biomolecular systems is an important step toward the design of secured drug delivery systems.

Introduction

Electric devices, designed to operate upon simultaneous reception of multiple input signals, are called "AND" logic gates. Biological systems sometimes utilize molecular logic gate mechanisms for triggering enzymatic functions by two different input signals. For example, in signal transduction systems, the production of c-AMP by type II adenylyl cyclase is triggered by the simultaneous activation of α- and $\beta\gamma$-subunits of stimulatory G-proteins.[1] Although several examples of so-called molecular logic gates have been reported,[2] most of them are designed for chemosensors that emit optical or electrical signals when they detect chemical stimuli. Here, we report a semibiological molecular machine with an implemented "AND" logic gate, which is capable of controlling the folding process of proteins in response to ATP and light as input stimuli. For molecular design, we utilized a genetically engineered chaperonin GroEL bearing, at each entrance part of its cylindrical cavity, cysteine residues, which were functionalized by an azobenzene derivative to construct a photoresponsive mechanical gate (azo-GroEL). GroEL,[3] originating from *Escherichia coli*, is a tubular protein assembly (800 kDa) consisting of 14 identical subunits (57 kDa), where each seven subunits forms a doughnut-like ring, which is stacked to give a double-decker architecture.[4] This cylindrical protein assembly possesses a cavity with a diameter of 4.5 nm, a height of 14.7 nm, and a wall thickness of 4.6 nm. GroEL is known to serve as a molecular chaperone that incorporates denatured proteins and assists their refolding inside the cavity. In the first step, $GroEL_{closed}$ traps denatured proteins at its entrance parts via a hydrophobic interaction, and then GroES, a capping cochaperonin, binds the resulting hybrids to initiate the protein refolding.[5,6] Upon binding with ATP, $GroEL_{closed}$ turns to adopt an open geometry ($GroEL_{open}$),[7] so that refolded proteins are released from the cavity. In the absence of GroES, the trapped proteins are prohibited from refolding and simply released when the $GroEL_{closed}$/protein hybrids bind ATP.

Our genetically and chemically engineered GroEL (azo-GroEL) is programmed to give rise to mechanical motions in response to ATP and light. Namely, ATP triggers an opening motion of the cylindrical protein cavity, while UV and visible lights induce trans-to-cis and cis-to-trans isomerizations of the azobenzene units, respectively (Figure 1a). Consequently, the protein binding sites will change their shape and polarity,[8] thereby changing the binding affinity toward denatured proteins.

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[⊥] N.I. was responsible for TEM microscopy.

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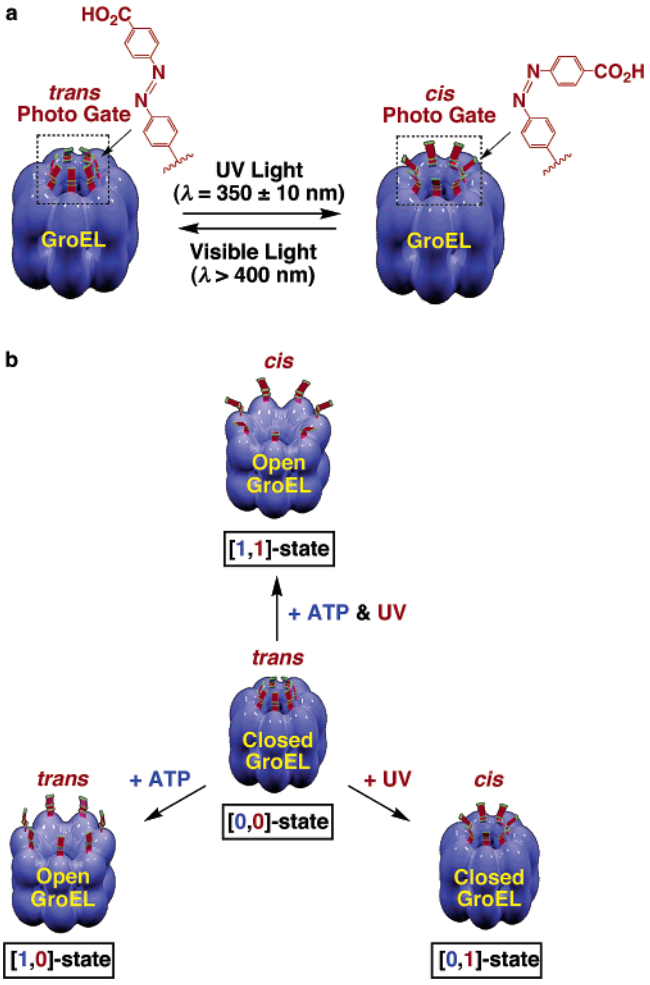

Figure 1. (a) Schematic representations of structural changes of the azobenzene-based photomechanical gates of azo-GroEL upon exposure to UV and visible lights. (b) "AND" logic gate responses of azo-GroEL to ATP combined with UV and visible lights as input stimuli.

As shown in Figure 1b, azo-GroEL can provide four different states by the combination of ATP with UV and visible lights as input stimuli: as-prepared azo-GroEL is defined as the [0,0]-state, where the chaperonin cavity adopts a closed geometry, while the photo gates are composed of *trans*-azobenzene groups (*trans*-azo-GroEL$_{closed}$). Upon trans-to-cis isomerization of the photo gates by UV irradiation, *cis*-azo-GroEL$_{closed}$, defined as the [0,1]-state, is generated, while the addition of ATP to the [0,0]-state results in opening the chaperonin cavity, so that *trans*-azo-GroEL$_{open}$, defined as the [1,0]-state, can be generated. On the other hand, upon irradiation with UV light in the presence of ATP, *cis*-azo-GroEL$_{open}$, defined as the [1,1]-state, is generated. Among these four states, the [1,1]-state is expected to exhibit the lowest affinity toward denatured proteins, considering the higher polarity (lower hydrophobicity) of *cis*-azobenzene than *trans*-azobenzene, along with the open conformation of the chaperone part.

Results and Discussion

Preparation of azo-GroEL. GroEL was genetically mutated in a way that all the cysteine residues (nos. 138, 458, and 519) in each subunit were replaced by alanine, and cysteine was substituted for arginine no. 231 located at the entrance parts (Figure 2a, ^{cys}GroEL). Mutant ^{cys}GroEL (1.55 nmol) thus obtained was functionalized with maleimido-appended *trans*-azobenzene (trans-**1**, Figure 2a) (0.22 μmol, [trans-**1**]/[cysteine] = 10) in a buffer [tris(hydroxymethyl)aminomethane (Tris)/HCl, pH = 7.8, 1 mL],[9] and the reaction mixture was subjected to size-exclusion chromatography (SEC) to allow the isolation of *trans*-azo-GroEL (*trans*-azo-GroEL$_{closed}$). The product displayed an absorption band at λ = 333 nm, characteristic of azobenzene derivatives. Analytical SEC (Tosoh G4000$_{SWXL}$) of GroEL$_{closed}$ carrying trans-**1**, monitored at λ = 280 nm (λ_{max} of GroEL), showed a single peak with an elution volume of 8.9 mL (Figure 2b). This peak was superimposed on an elution peak monitored at λ = 350 nm (λ_{max} of trans-**1**), where the precursor ^{cys}GroEL was totally silent, indicating covalent functionalization of ^{cys}GroEL with the azobenzene groups. As estimated from the absorbance at λ = 333 nm, azo-GroEL bears 11 azobenzene units. By means of multi-angle light scattering (MALS; Figure 3), we successfully evaluated the average molecular weights of ^{cys}GroEL and *trans*-azo-GroEL$_{closed}$ as 820 ± 20 and 830 ± 30 kDa, respectively. Furthermore, circular dichroism spectroscopy (CD; Figure 4) and transmission electron microscopy (TEM) (Figure 5) indicated that *trans*-azo-GroEL$_{closed}$ adopts a cylindrical morphology analogous to ^{cys}GroEL.

Photochemical Responses of azo-GroEL. Upon exposure to UV light (λ = 350 ± 10 nm) at 20 °C in a neutral buffer composed of 3-(*N*-morphorino)propane sulfonic acid (MOPS)/NaOH (50 mM, pH = 7.0), *trans*-azo-GroEL$_{closed}$ showed an increase in intensity of the absorption band at λ = 440 nm at the expense of the absorbance at λ = 330 nm (Figure 6, left), indicating the occurrence of a trans-to-cis isomerization of the azobenzene units (Figure 1a). This absorption spectral change reached a photostationary state within 50 s. On the other hand, exposure of this isomerized azo-GroEL (*cis*-azo-GroEL$_{closed}$) to visible light (λ > 400 nm) resulted in an inverse spectral change until reaching the photostationary state (10 s) (Figure 6, right), as a consequence of the backward (cis-to-trans) isomerization of the azobenzene units (Figure 1a). By means of SEC, MALS, and CD spectroscopy (Figure 4), we confirmed that azo-GroEL preserves its ternary structure even upon repeated isomerization events induced by UV and visible lights.

Azo-GroEL-Mediated Refolding of Green Fluorescent Protein (GFP) in Response to ATP and Light. We chose green fluorescent protein (GFP)[10] as the substrate since the refolding process of GFP can easily be followed by a change in intensity of its characteristic green fluorescence. It is known that native GFP fluoresces at λ_{em} = 511 nm (λ_{ext} = 398 nm) in a neutral buffer, while it turns nonfluorescent upon denaturation under acidic conditions (pH < 4). On the other hand, when acid-denatured GFP (GFP$_{denat}$, pH = 2) is placed under neutral conditions (50 mM MOPS/NaOH containing 100 mM KCl and 1.25 mM Mg(OAc)$_2$, pH 7.0) at 4 °C, it undergoes spontaneous refolding and gradually retrieves the 511 nm green luminescence.[11] To investigate the "AND" logic gate performance of azo-GroEL, we took notice of the fact that GFP$_{denat}$, trapped at the entrance parts of GroEL$_{closed}$, cannot refold even under neutral conditions,[11] while it undergoes spontaneous refolding when detached from the chaperonin by the action of ATP.

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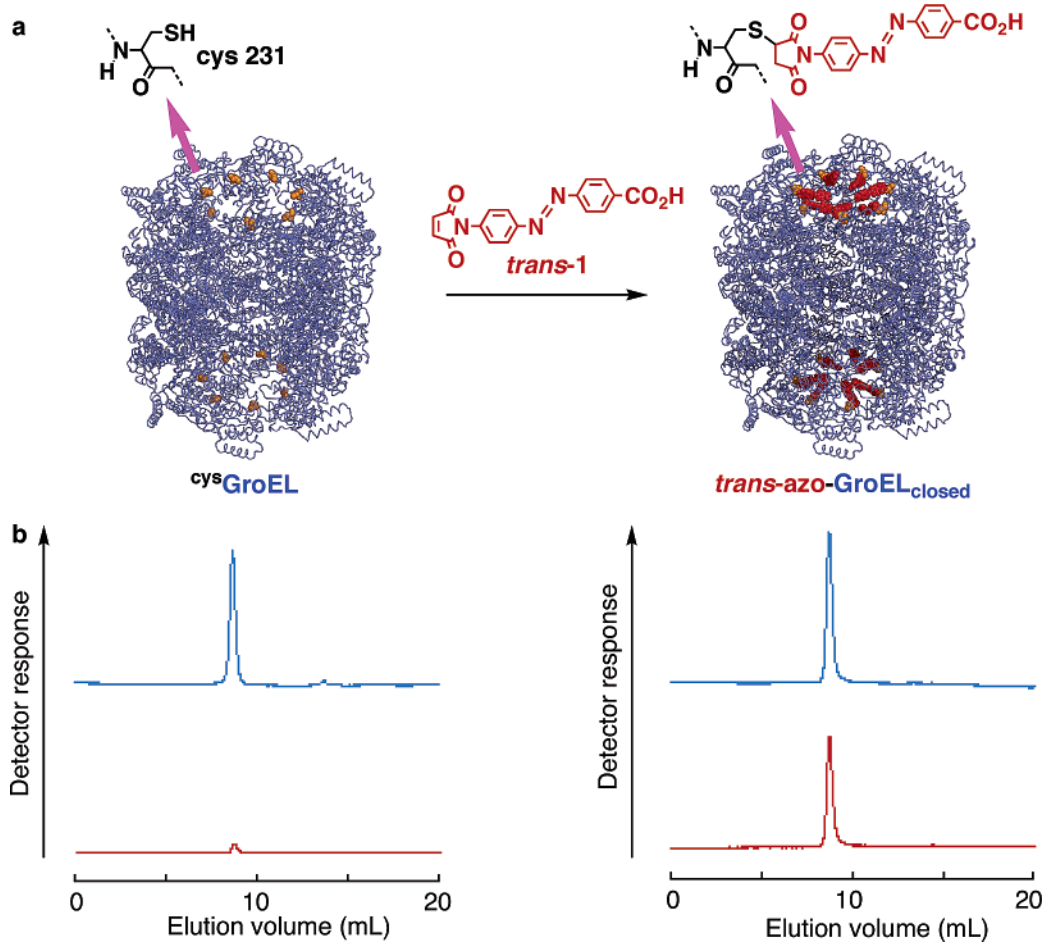

Figure 2. (a) Introduction of an azobenzene-based photomechanical gate onto ^{cys}GroEL at the entrance parts of its cylindrical cavity. ^{cys}GroEL possesses seven cysteine residues (red) at each entrance, which are functionalized with trans-**1**. The three-dimensional structures are based on the crystal structure of GroEL (PDB code: 1DER), with arginine 231 replaced by cysteine. (b) Size-exclusion chromatography (SEC) traces of ^{cys}GroEL (left) and *trans*-azo-GroEL$_{closed}$ (right), monitored at 20 °C by changes in absorbances at $\lambda = 280$ (blue) and 350 nm (red).

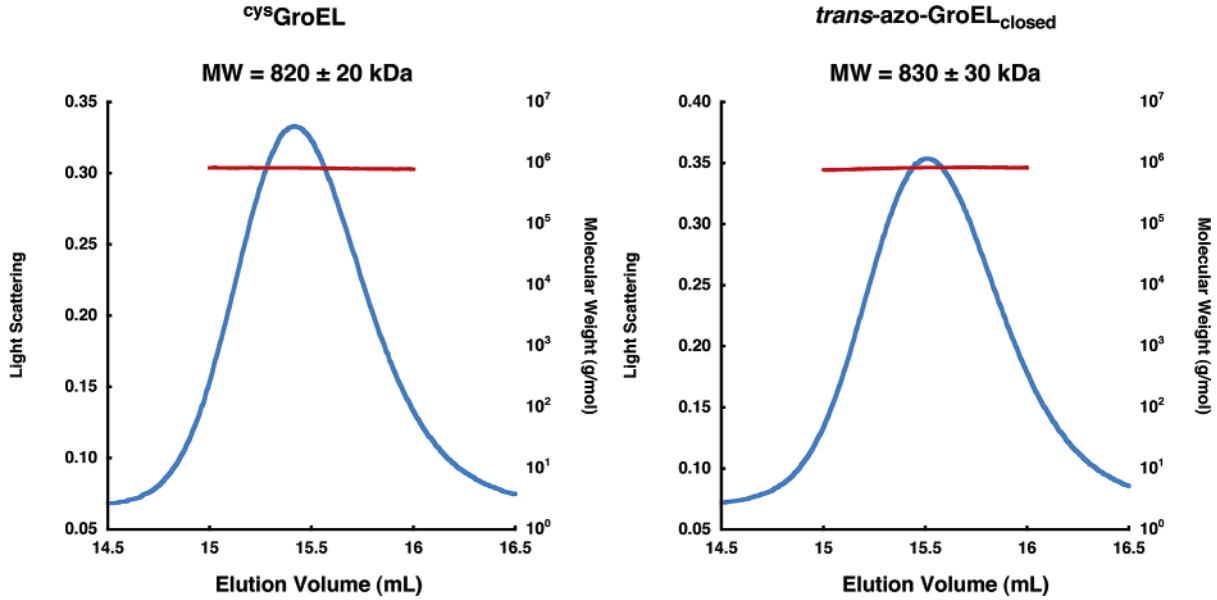

Figure 3. Molecular weight determination of ^{cys}GroEL (left) and *trans*-azo-GroEL$_{closed}$ (right). Analytical SEC traces (Shodex Protein KW-803 and KW-804; eluent: a MOPS/NaOH buffer 50 mM, pH 7.0 containing 100 mM KCl; flow rate: 1.0 mL/min) were recorded on a Shodex model DAWN HPLC system equipped with a multi-angle light scattering (MALS: blue curves)/refractive index (RI) dual detector. The MALS output at each elution volume was converted into molecular weight (red curves) according to eq 1, where c is a protein concentration evaluated from RI intensity, M is a molecular weight, and r is an apparatus-dependent calibration constant, while dc/dn is supposed to be 0.180 on the basis of that of BSA: light scattering intensity $= (\mathrm{d}c/\mathrm{d}n)^2cMr$ ($r = 4.8786 \times 10^{-5}$) (eq 1).

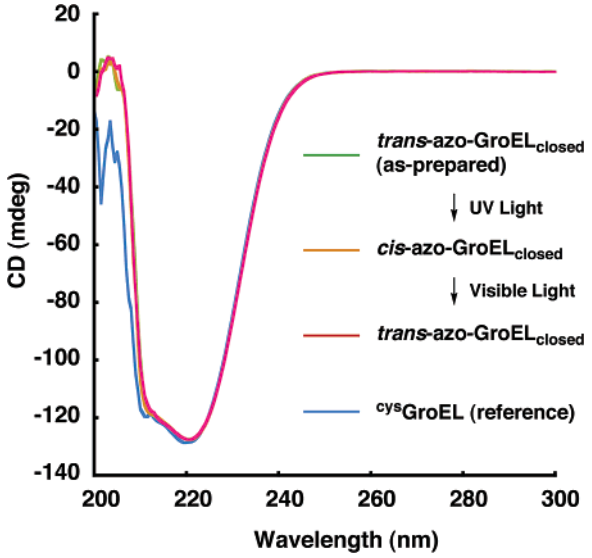

Figure 4. Circular dichroism spectra of *trans*-azo-GroEL$_{closed}$ (as-prepared: green curve), *cis*-azo-GroEL$_{closed}$ (after exposure of *trans*-azo-GroEL$_{closed}$ to UV light (λ = 350 ± 10 nm): orange curve), and *trans*-azo-GroEL$_{closed}$ (after exposure of *cis*-azo-GroEL$_{closed}$ to visible light (λ > 400 nm): red curve), along with that of ^{cys}GroEL (blue curve) as a reference.

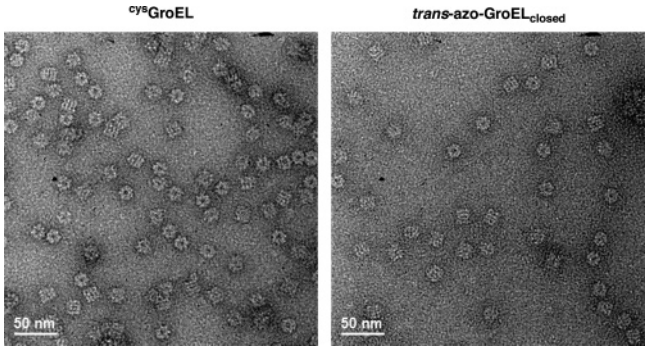

Figure 5. TEM micrographs of ^{cys}GroEL and *trans*-azo-GroEL$_{closed}$. The samples were applied to an electron microscope specimen grid covered with a thin carbon support film that had been hydrophilized by ion bombardment. After being dried with pre-water-soaked filter paper, the grid was negatively stained with 1% uranyl acetate. TEM micrographs were then recorded on a PHILIPS Tecnai 20F transmission electron microscopy operating at an anode voltage of 120 kV.

We found that the trans and cis isomers of azo-GroEL$_{closed}$ both inhibit refolding of GFP$_{denat}$ in a neutral buffer (125 nM in MOPS/NaOH, pH 7.0). For example, when *trans*-azo-GroEL$_{closed}$ ([0,0-state], Figure 1b) or *cis*-azo-GroEL$_{closed}$ ([0,1]-state) was present, GFP$_{denat}$ hardly retrieved the green fluorescence activity (Figure 7a, red diamonds and orange triangles, respectively). On the other hand, upon the addition of ATP (25 μM) to GFP$_{denat}$–bound *cis*-azo-GroEL$_{closed}$ ([0,1]-state), the resultant buffer solution containing [1,1]-state of azo-GroEL rapidly retrieved the 511 nm green fluorescence due to the refolding of GFP (Figure 7a, blue squares). Of interest, when *trans*-azo-GroEL$_{open}$ ([1,0]-state) was used in place of the cis isomer ([1,1]-state), the rate of this fluorescence recovery was notably slower by a factor of 3 (Figure 7a, green circles). Since the refolding of GFP$_{denat}$ is considered to start when GFP$_{denat}$ is detached from the chaperone part, the observed difference in the rates of fluorescence recovery indicates that the affinities of *trans*- and *cis*-azo-GroEL$_{open}$ toward GFP$_{denat}$ are different from one another.

Having these observations in mind, we conducted sequential irradiation of a mixture of GFP$_{denat}$ and azo-GroEL with UV and visible lights in the absence and presence of ATP. Thus, *cis*-azo-GroEL$_{closed}$ ([0,1]-state in Figure 1b) (125 nM), initially generated, was mixed with GFP$_{denat}$, and after a 10 min incubation (Figure 7b, A), the mixture was exposed to visible light (λ > 400 nm) for 1 min at 4 °C to allow photoisomerization of the azobenzene groups. However, the fluorescence at λ_{em} = 511 nm was not enhanced, indicating that GFP$_{denat}$ remains hybridized with resultant *trans*-azo-GroEL$_{closed}$ ([0,0]-state) and is prohibited from refolding (Figure 7b, B). No release of GFP$_{denat}$ again took place when resulting *trans*-azo-GroEL$_{closed}$ was allowed to isomerize backward to the cis form by UV irradiation (λ = 350 ± 10 nm) for 2 min (Figure 7b, C). Therefore, in the absence of ATP, where the chaperone part is conformationally closed, the release of GFP$_{denat}$ from azo-GroEL$_{closed}$ hardly occurs, irrespective of whether the azobenzene groups adopt the trans ([0,0]-state) or cis ([0,1]-state) configuration. In sharp contrast, when ATP (250 μM, [ATP]/[azo-GroEL] = 2000) was added to GFP$_{denat}$-bound *cis*-azo-GroEL$_{closed}$ ([0,1]-state in Figure 1b; 125 nM in MOPS/NaOH, pH 7.0) to generate the [1,1]-state, GFP$_{denat}$ was immediately detached from the chaperonin (Figure 7b, D). On the other hand, when the resulting buffer solution was exposed for 1 min to visible light (λ > 400 nm) to induce the cis-to-trans isomerization of the azobenzene groups ([1,0]-state), a notable retardation of the release of GFP$_{denat}$ resulted (Figure 7b, E). Here, the apparent rate dropped to one-third as much as that before irradiation with visible light. Furthermore, switching of the [1,0]-state back to the [1,1]-state by exposure to UV light (λ = 350

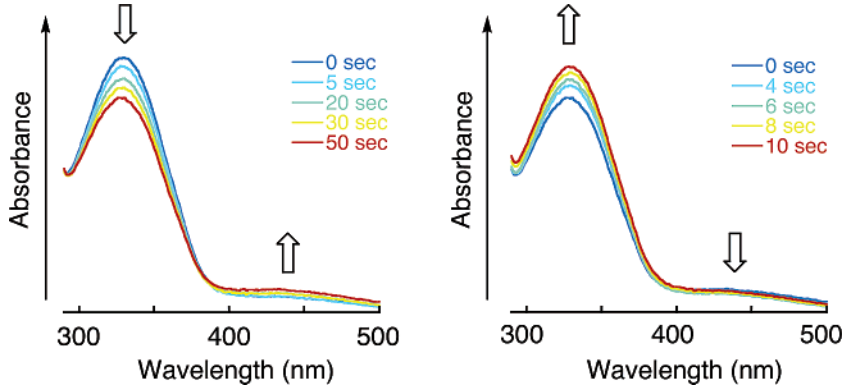

Figure 6. Photoinduced structural changes of the azobenzene-based photomechanical gates of azo-GroEL$_{closed}$. Electronic absorption spectral changes at 20 °C upon exposure of azo-GroEL$_{closed}$ to UV (λ = 350 ± 10 nm; left) and visible light (λ > 400 nm; right).

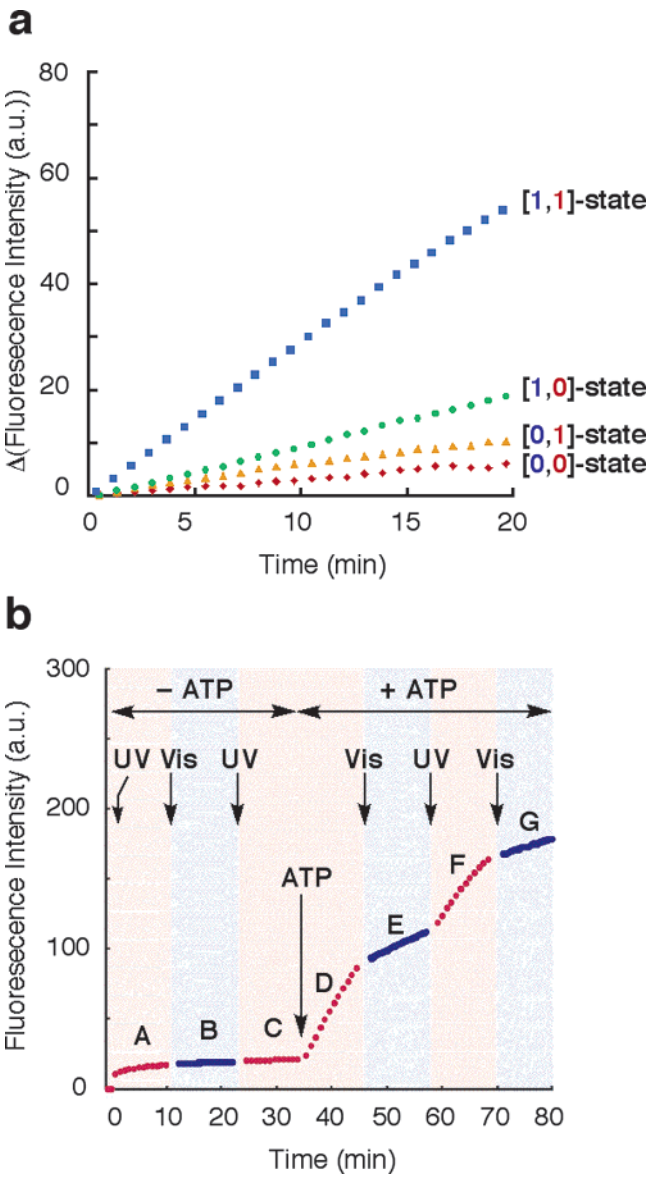

Figure 7. "AND" logic gate performance of engineered chaperonin azo-GroEL in response to ATP combined with UV and visible lights, as investigated by refolding of acid-denatured green fluorescent protein (GFP_{denat}). (a) Changes in fluorescence intensity at $\lambda_{em} = 511$ nm ($\lambda_{ext} = 398$ nm) in the presence of azo-GroEL at the [0,0]-state (*trans*-azo-$GroEL_{closed}$; without ATP in the dark; red diamonds), [0,1]-state (*cis*-azo-$GroEL_{closed}$; without ATP under irradiation with UV light ($\lambda = 350 \pm 10$ nm) for 60 s; orange triangles), [1,0]-state (*trans*-azo-$GroEL_{open}$; with ATP in the dark; light green circles), and [1,1]-state (*cis*-azo-$GroEL_{open}$; with ATP under irradiation with UV light for 60 s; blue squares). (b) Changes in fluorescence intensity at $\lambda_{em} = 511$ nm ($\lambda_{ext} = 398$ nm) upon sequential irradiation with UV ($\lambda = 350 \pm 10$ nm, initial 120 s in red areas) and visible lights ($\lambda > 400$ nm, initial 60 s in blue areas), before and after the addition of ATP. A long arrow indicates the addition of ATP, while short arrows denote the irradiation with UV and visible lights.

$\pm$ 10 nm) for 2 min resulted in an accelerated release of GFP_{denat} (Figure 7b, F). Such photoresponsive retardation and acceleration could be repeated until ATP in the system was completely consumed into ADP. These observations indicate that the release of GFP_{denat} can be controlled by light, but only when the chaperone part is conformationally opened by the action of ATP.

ATPase Activities of azo-GroEL. For the photoresponsive release of GFP_{denat} from azo-$GroEL_{open}$ (Figure 7), one may consider if the binding of *cis*-azo-$GroEL_{closed}$ with ATP, leading to the conformational change of the chaperone part, might be faster than that of *trans*-azo-$GroEL_{closed}$. However, the hydrolysis rate of ATP (ATPase activity) with *cis*-azo-$GroEL_{closed}$ (7.9 min^{-1}) at 4 °C was comparable to that with *trans*-azo-$GroEL_{closed}$ (8.3 min^{-1}). Therefore, the photoresponsive protein refolding with azo-GroEL in the presence of ATP indeed originates from the difference between the release rates of GFP_{denat} from *trans*- and *cis*-azo-$GroEL_{open}$. As described already, $GroEL_{closed}$ utilizes a hydrophobic interaction to trap denatured proteins. Therefore, the faster release of GFP_{denat} from *cis*-azo-$GroEL_{open}$ (Figure 7) suggests that the binding site of the [1,1]-state in Figure 1b is geometrically more relaxed and also possesses a higher polarity than *trans*-azo-$GroEL_{open}$ ([1,0]-state in Figure 1b). Consequently, the hydrophobic interaction with GFP_{denat} is depressed upon photo switching of the [1,0]-state to the [1,1]-state, leading to the photoresponsive release and subsequent refolding of GFP_{denat} (Figure 7).

Conclusions

We have demonstrated that genetically and chemically engineered chaperonin azo-GroEL, bearing photoresponsive mechanical gates at the entrance parts of its cylindrical cavity, serves as a semibiological molecular machine with an implemented "AND" logic gate, which is capable of controlling the folding process of proteins in response to ATP and light as input stimuli. This engineered chaperonin traps denatured green fluorescent protein (GFP_{denat}) and prohibits its refolding. However, when hosting azo-GroEL simultaneously detects ATP and UV light, it quickly releases GFP_{denat} to allow its refolding. In contrast, reception of either of the previous two input stimuli results in only very slow or no substantial refolding of GFP_{denat}. We believe that such logic gate conceptions based on nanobiotechnologies[12,13] are not only important for the advancement of molecular machinery but can also be integrated into the design of secured drug release systems.

Experimental Procedures

General. ^{cys}GroEL was expressed and isolated as described in a previous paper.[14] Electronic absorption, circular dichroism (CD), and infrared (IR) spectra were recorded on a JASCO type V-560 spectrometer, a JASCO type J-720 spectropolarimeter, and a JASCO type FT/IR-610 spectrometer, respectively. ^{1}H NMR spectra were recorded on JEOL type GSX-270 and GSX-500 spectrometers. Matrix-assisted laser desorption/ionization time-of-flight mass (MALDI-TOF-MS) spectrometry was performed with dithranol as a matrix on an Applied Biosystems BioSpectrometry Workstation model Voyager-DE STR spectrometer. Analytical SEC was performed at 20 °C using a 7.8 mm-ϕ $\times$ 300 mm long silica gel column (TOSOH TSKgel BioAssist $G4SW_{XL}$ or $G4000SW_{XL}$) on a JASCO type PU-980 HPLC pump, equipped with a JASCO type UV-2077 variable-wavelength UV−vis detector.

4-((*E*)-(4-((*Z*)-3-Carboxyacrylamido)phenyl)diazenyl)benzoic Acid (2). Maleic anhydride (49 mg, 500 μmol) was added to a mixture of 4-[(4-aminophenyl)azo]benzoic acid[15] (108 mg, 448 μmol) and AcOH (40 mL). After being stirred at 25 °C for 24 h, the reaction mixture

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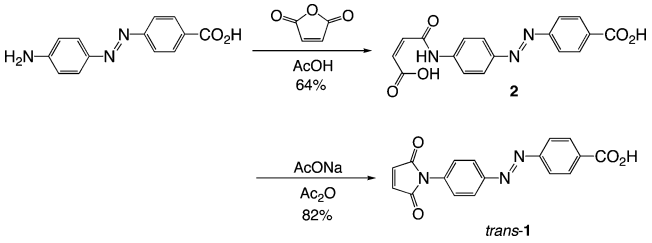
Scheme 1. Synthesis of **trans-1**



was filtered to allow isolation of **2** as a brownish red powder in 64% yield (98 mg, 289 μmol). ^{1}H NMR (270.05 MHz; DMSO-d_6, 20 °C; ppm) d 12.69 (s, 1H, CO_2H), 10.70 (s, 1H, N*H*), 8.12, 7.86 (dd, 4H, Ar-*H*), 7.95, 7.92 (dd, 4H, Ar-*H*), 6.41 (d, 2H, HC═C*H*—CONH), 6.34 (d, 2H, HO_2C—C*H*═CH). MALDI-TOF-MS (dithranol): *m*/*z* 340 ([M + H^+] calcd: 340).

***trans*-4-[(4-Maleimidephenyl)azo]benzoic Acid (1).** AcONa (115 mg, 1.40 mmol) was added to a mixture of **2** (935 mg, 2.76 mmol) and Ac_2O (10 mL) at 25 °C under N_2. After being stirred at 100 °C for 20 min, the reaction mixture was poured into iced water (15 g) and extracted with $CHCl_3$ (60 mL × 5). The combined organic extract was dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure to leave a red powdery substance, which was recrystallized from EtOH to afford trans-**1**, contaminated with 10% cis-**1**, as a reddish brown powder in 82% yield (726 mg, 2.26 mmol). ^{1}H NMR (270.05 MHz; DMSO-d_6, 20 °C; ppm) d 13.03 (s, 1H, CO_2H), 8.15, 7.98 (dd, 4H, Ar-*H*), 8.04, 7.62 (dd, 4H, Ar-*H*), 7.24 (s, 2H, *H*C═C*H*).

Preparation of *trans*-azo-GroEL$_{closed}$. To a Tris/HCl buffer (50 mM, pH 7.8) solution (1 mL) of ^{cys}GroEL (1.55 μM) was added a dimethyl sulfoxide solution (4.4 μL) of trans-**1** (50 mM), and the mixture was incubated at 4 °C.[9] After 1 h, 2-mercaptoethanol (29.6 mmol) was added to the resulting solution, and the reaction mixture was immediately subjected to size-exclusion chromatography (SEC) using a MOPS/NaOH buffer (50 mM, pH 7.0 containing 100 mM KCl) as an eluent on a PD-10 desalting column (Amersham Bioscience), affording a buffer solution (2 mL) of *trans*-azo-GroEL$_{closed}$ (0.5 μM).

Complexation of GFP$_{denat}$ with *trans*-azo-GroEL$_{closed}$. A Tris/HCl buffer (25 mM, pH 7.5) solution (3 μL) of native GFP (52 μM) was added to a mixture of a Tris/HCl buffer (50 mM, pH 7.5, 4 μL), hydrochloric acid (0.1 M, 3.1 μL), dithiothreitol (10 mM, 2.5 μL), EDTA (3 mM, 2.5 μL), and water (9.9 μL) at 4 °C. The mixture was incubated at 20 °C for 5 min, to afford an acidic buffer solution (25 μL) of denatured GFP (GFP$_{denat}$; 6.25 μM, pH 2).[11] The solution of GFP$_{denat}$ (20 μL), thus prepared, was added at 4 °C to a MOPS/NaOH buffer solution (50 mM containing 100 mM KCl, 1.25 mM $Mg(OAc)_2$, 1.25 mM DTT, and 0.125 mg/mL BSA, pH 7.0)[11] (2 mL) of *trans*-azo-GroEL$_{closed}$ or *cis*-azo-GroEL$_{closed}$ (0.125 μM) in a 10 mm thick quartz cell, and the mixture was subjected to fluorescence spectroscopy on a JASCO model FP-777W spectrophotometer.

Photoisomerization. Photoisomerization of the azobenzene groups of azo-GroEL, in the presence or absence of GFP$_{denat}$, was carried out in a 10 mm thick quartz cell at 4 or 20 °C upon exposure of a MOPS/NaOH buffer solution (50 mM, pH 7.0 containing 100 mM KCl) of azo-GroEL$_{closed}$ or azo-GroEL$_{open}$ (0.125 or 0.24 μM) to a 150-W xenon arc lamp through a band-pass filter (Kenko; $\lambda = 350 \pm 10$ nm) for UV irradiation and a cut filter (Kenko; $\lambda > 400$ nm) for visible light irradiation.

ATPase Activity Assay.[16] An aqueous solution (20 μL) of $MgCl_2$ (50 mM) was added to a MOPS/NaOH buffer (50 mM, pH 7.0 containing 100 mM KCl) solution (138 μL) of *trans*-azo-GroEL$_{closed}$ or *cis*-azo-GroEL$_{closed}$ (0.1 μM). To the resulting solution, after being incubated at 4 °C for 10 min, was added an aqueous solution (40 μL) of ATP (100 μM), and the mixture was incubated at 4 °C. After 10 min, perchloric acid (70%, 9 μL) was added to the reaction mixture, and the resulting solution was incubated at 4 °C for 10 min and then centrifuged for 4 min at 15 000 rpm. To the supernatant solution (80 μL), thus obtained, was added an aqueous solution (20 μL) of a color reagent[17] (a mixture of malachite green (1 mM), hexaammonium heptamolybdate tetrahydrate (3%), Tween #20 (0.17%), and sulfuric acid (13%)), and the mixture was allowed to stand at 25 °C for 10 min. The phosphate concentration of the mixture was evaluated on the basis of the absorbance at $\lambda = 630$ nm.

Molecular Weight Determination of ^{cys}GroEL and *trans*-Azo-GroEL$_{closed}$. Analytical size-exclusion chromatography (SEC; Shodex Protein KW-803 and KW-804) traces of ^{cys}GroEL and *trans*-azo-GroEL$_{closed}$ were obtained using a refractive index (RI)/multi-angle light scattering (MALS) dual detector.[18,19]

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