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J. Am. Chem. Soc., **2008**, 130 (14), 4580-4581 • DOI: 10.1021/ja078020p

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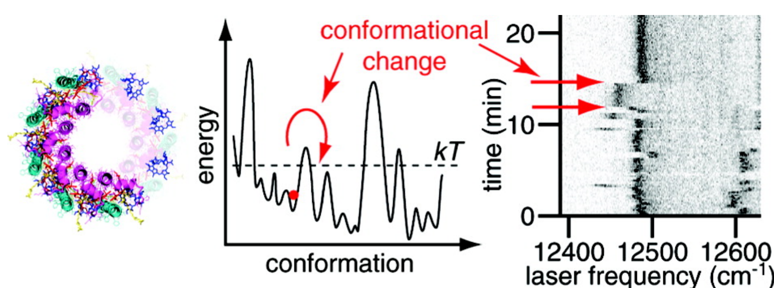

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Published on Web 03/18/2008

How Deep Is the Potential Well Confining a Protein in a Specific Conformation? A Single-Molecule Study on Temperature Dependence of Conformational Change between 5 and 18 K

Hiroyuki Oikawa,[†] Satoru Fujiyoshi,[†,‡] Takehisa Dewa,[§] Mamoru Nango,[§] and Michio Matsushita*[,†,‡]

Department of Physics, Tokyo Institute of Technology, Meguro 152-8551, Japan, CREST, JST, Tokyo, Japan, and Department of Life and Materials Engineering, Nagoya Institute of Technology, Showa-ku, Nagoya 466-8555, Japan

Received October 19, 2007; E-mail: matsushita@phys.titech.ac.jp

Under physiological conditions, a protein is continuously changing its conformation. The conformation at a given time corresponds to one of the local minima on the potential energy surface of the protein, and they are separated by barriers lower than the thermal energy at room temperature. Since the property of a protein depends on the instantaneous conformations and the conformational dynamics, the knowledge about the individual minima and barriers is important to understand the function of a protein. An experimental method to study the minima and barriers is to observe individual proteins at low temperatures where the conformational change is slow enough to be followed. The depth of the potential well will be known from the temperature dependence of the conformational dynamics of a single protein. Fluorescence excitation spectroscopy of single molecules has been successfully applied to photosynthetic pigment−protein complexes to elucidate the structure of the system.[1] A conformational change of the complex was also observed at liquid helium temperature as discrete jumps of absorption wavelength of the single chromophores of the complex.[2] In this report we present the temperature dependence of the conformational change of single proteins for the first time between 5 and 18 K. From the fluorescence excitation spectrum of single chromophores the conformational change is found to be either a thermally activated motion over a potential barrier of around 100 J/mol or a tunneling of a proton through a thermally inaccessible high barrier.

As a pigment−protein complex for a temperature-dependence study we took a light-harvesting 2 (LH2) complex of *Rhodopseudomonas* (*Rps.*) *acidophila.* The complex has nine equivalent binding sites of bacteriochlorophyll *a* (BChl *a*) molecules that give rise to the absorption band around 800 nm which is called B800 band (Figure 1). We focus on these B800 chromophores as a local probe of the conformation of the protein since the excitation of the B800 band is basically localized on the individual chromophores.[3]

For the optical measurements of single LH2 complexes, detergent micelles of LH2 were spatially isolated in a thin polymer film. The film was prepared on a fused quartz substrate by spin-coating a 0.1 nM solution of LH2 containing 1% w/w polyvinyl alcohol, 0.8% *n*-octyl-β-D-glucopyranoside, and Tris-HCl buffer (pH = 8). The fluorescence excitation spectrum of the single complexes was measured with a home-built laser-scanning confocal microscope. The wavelength of a continuous-wave laser with the line width of ca. 1 cm^{-1} was scanned at a rate of 60 cm^{-1}/s. The incident laser power on the sample was ca. 10 W/cm^2. The direction of the polarization of the laser was kept constant throughout the measurements. The temperature of the sample was controlled by a helium gas-flow cryostat (Oxford Inst. Optistat SXM). Against temperature change the sample holder was made stable enough for the same complex to be followed in the confocal image.

[†] Tokyo Institute of Technology.
[‡] CREST.
[§] Nagoya Institute of Technology.

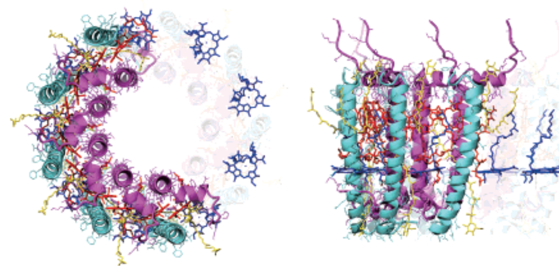

Figure 1. X-ray crystal structure of LH2 complex from *Rps. acidophila*. The complex consists of circularly arranged nine identical subunits. For four subunits on the right-hand side, only the B800 chromophores are highlighted for the sake of clarity. Data from the Protein Data Bank, identification code: 1nkz.

Figure 2 shows a typical temporal behavior of the B800 band in the fluorescence excitation spectrum of a single LH2 complex at four different temperatures. The two-dimensional plots in the figure represent fluorescence intensity as a function of laser frequency and time. Since the sample was excited by linearly polarized light, about half of the nine B800 chromophores were observed. In the 2D plot at 4.7 K four to five spectral peaks can be followed vertically along time. Each spectral peak represents the absorption spectrum of an individual B800 chromophore. The absorption frequency of some chromophores makes a discrete jump of tens of wavenumbers at an interval of a couple of minutes. A simultaneous jump of more than two chromophores has not been observed. Similar changes of the absorption frequency were reported at 1.4 K for the LH2 complex from *Rhodospirillum molischianum*.[4]

At 4.7 K the majority of the spectral jumps were found to be thermally induced and a light-induced effect was not dominant, since the rate of the spectral jump did not change when the laser power was reduced from ca. 10 to ca. 3 W/cm^2.

To quantify the spectral behavior, the absorption frequency of each chromophore was determined by fitting every single scan to a Lorentzian profile. The trajectories of the absorption frequency of the four chromophores are shown in Figure 2 by colored lines. The red line is not present at 18 K since the signal is too weak to be fitted.

The rate of the spectral change of a chromophore is determined by counting the number of changes in a unit time. In the trajectories of the absorption frequency the fitting error causes artificial frequency changes. In order to exclude the artificial changes, we count as a spectral jump only the changes of the absorption frequency that are more than 5 cm^{-1} in two consecutive scans (details of the analysis are given in the Suporting Information). In

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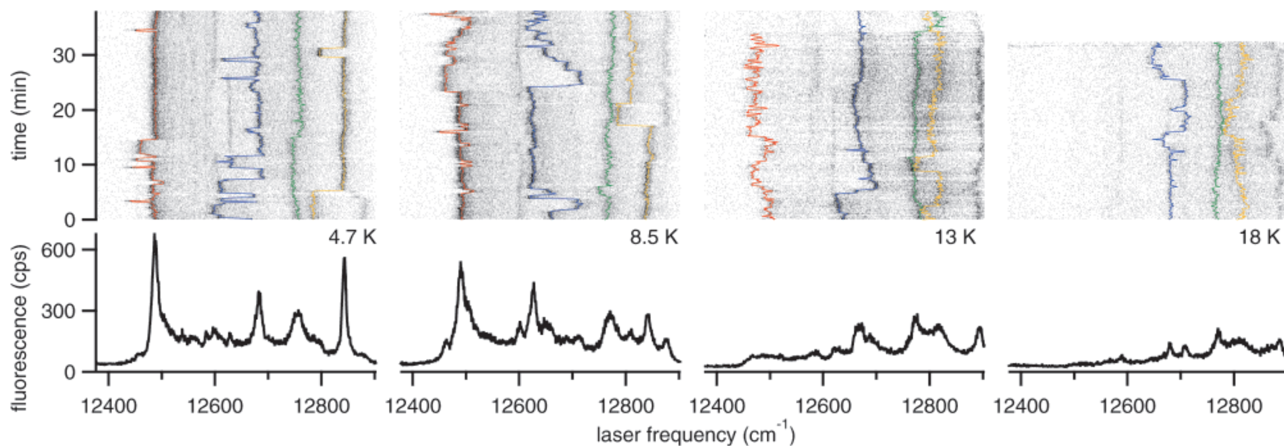

Figure 2. Temperature dependence of the B800 band in the fluorescence excitation spectrum of a single LH2 complex. The 2D plots represent fluorescence intensity in grayscale along laser frequency in the horizontal axis and time in the vertical. The time-averaged spectrum is shown below the 2D plot. Four colored lines represent the trajectory of the absorption frequency determined by fitting every single scan to a Lorentzian profile.

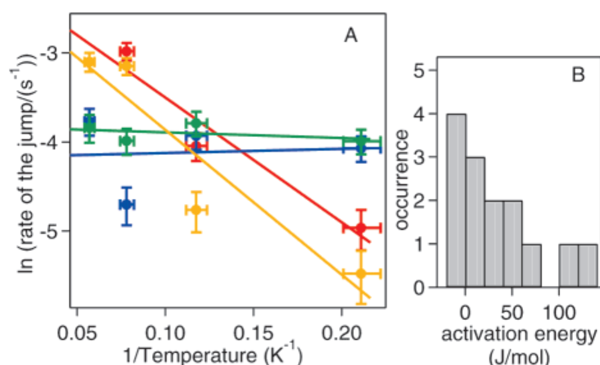

Figure 3. (A) Arrhenius plot of the rate of the spectral jumps of the B800 chromophores. The four colors correspond to the colors in Figure 2. (B) Distribution of the activation energy that are derived from the slope of an Arrhenius plot of the rate of the spectral jump of 14 chromophores.

an Arrhenius plot of Figure 3A the rate of the spectral jumps are plotted in logarithmic scale against the reciprocal of temperature.

As for the temperature dependence of the spectral behavior, the four colored trajectories are classified into two groups. The red and yellow trajectries represent one group in which the rate of the spectral change increases with temperature. They have a negative slope in the Arrhenius plot, giving rise to an activation energy of 1×10^2 J/mol. The blue and green trajectories represent the other group in which the rate is independent of temperature. For the blue trajectory the number of the large spectral jumps decreases with temperature, but the total number of the jumps stays constant. For the green there is no noticiable difference in spectral behavior between different temperatures. They lie flat in the Arrhenius plot with an activation energy of about zero.

In addition to the four chromophores shown in Figures 2 and 3A the same analysis was applied to 10 choromophores out of 2 LH2 complexes (see Supporting Information for the experimental data that are not shown here). The activation energy of the total 14 chromophores determined from the slope in an Arrhenius plot is presented in Figure 3B as a histogram. The distribution consists of the two groups. The first group having an activation energy of ca. 1×10^2 J/mol probes thermally activated conformational changes over a potential barrier. In the second group having approximately zero activation energy, since the rate was independent of temperature below 18 K, the barrier must be much higher than the thermal energy corresponding to 18 K. A candidate of such a temperature-independent motion in the protein environment is tunneling of a proton of a hydrogen bond around the chromophore. There is a hydrogen bond between the B800 BChl *a* molecule and Arg20 residue of a β-apoprotein.[5] There are also hydrogen bonds between the ligand of central Mg^{2+} in BChl *a* and amino acid residues of the apoproteins.[5]

To conclude, for a conformational change of protein which influences the absorption wavelength of chromophores, the temperature dependence of the spectral change of a single chromophore makes a distinction between a temperature-dependent motion of thermal activation over a potential barrier and a temperature-independent motion of tunneling through a barrier. The method would become a powerful tool to unravel functioning proteins.

Acknowledgment. This work was supported by Grants-in-Aid for Scientific Research, (B) from JSPS (No. 16340121) and on Priority Areas from MEXT (19056011), Yamada Science Foundation, The Mitsubishi Foundation, and 21st Century COE program on "Nanometer-Scale Quantum Physics". M.N. thanks Prof. Richard J. Cogdell for the kind gift of photosynthetic bacteria, *Rps. acidophila.*

Supporting Information Available: Determination of the rate of change of the absorption frequency of a single chromophore and the experimental data of the complexes that are not shown but included in the histogram of Figure 3B. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA078020P