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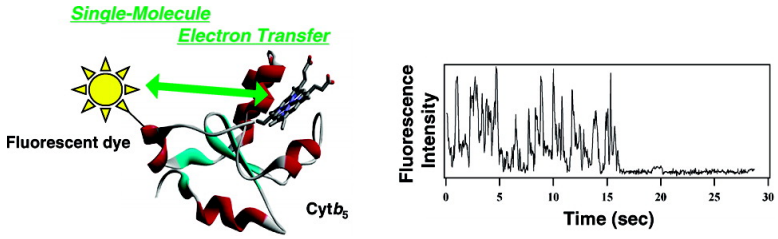

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Electron Transfer Reaction in a Single Protein Molecule Observed by Total Internal Reflection Fluorescence Microscopy

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Abstract: To observe an electron transfer (ET) process in a single protein molecule, we constructed a model system, Alexa-HCytb_5, in which cytochrome b_5 (Cytb_5) is modified with a fluorescent probe, Alexa Fluor 647 dye. In this model system, intramolecular transfer of an electron from the Alexa dye to heme in Cytb_5 is supposed to oxidize the probe and quench its fluorescence, and the ET reaction at the single-molecule level can be monitored as the intermittent change in the fluorescence intensity. Alexa-HCytb_5 was fixed on the glass surface, and illumination of laser light by the total internal reflection resulted in blinking of the fluorescence from the single Alexa-HCytb_5 molecule in the time scale of several hundred milliseconds. Each Alexa-HCytb_5 molecule is characterized by its own rate constant of the blinking, corresponding to the ET rate constant at the single-molecule level, and its variation ranges between 1 and 10 s^{-1}. The current system thus enables us to visualize the ET reaction in the single protein molecule, and the protein ET reaction was found to be explained by the distribution of the rate constants. On the basis of the Marcus theory, we suggest that the origin of this rate distribution is the distance change associated with the structural fluctuation in the protein molecule.

Introduction

It has been increasingly recognized that structural fluctuation in proteins at physiological temperature plays essential roles in regulating various types of enzymatic reactions.[1] Among these, electron transfer (ET) reaction is supposed to be one of the most sensitive processes to fluctuations of the protein structure because the rate constants of ET reactions in proteins are exponentially dependent on the distance between the electron donor and acceptor (D−A distance). In fact, in the nanosecond time scale, molecular dynamics (MD) simulations on ET proteins have proposed that less than 1 Å fluctuation of the ET pathway(s) can affect the reaction rate constant through the modulation of the interference among the multiple ET pathways.[2] Because amplitude of the structural fluctuation (i.e., the distance change) becomes larger in the second or longer time scale,[3] it is quite interesting to examine effects of the slow structural fluctuation on the ET reactions by the MD methods; however, the computer simulation has been difficult in the long time scale over microseconds. Despite this, the fact that the D−A distance change sometimes reaches several angstroms by the structural fluctuation[4] prompts us to examine whether the ET reaction rate would be also changing by the fluctuation. In other words, the structural fluctuation can be regarded as the thermal transition among the conformational substates, and the transitions among the conformational substates can occur on a second time scale or longer. We thus expect that ET rate constants will be varied as a protein molecule temporally and slowly (∼seconds) changes its conformation.

Possible effects of structural fluctuations on ET reactions have been reported by changing some physical parameters, such as pressure[5,6] and viscosity,[7] both of which can perturb the rates of the conformational transition; however, not only the structural fluctuation but also other static properties, such as the protein stability, can be affected. Besides, the structural fluctuations are not necessarily synchronized over all protein molecules in solution, and its effects on the observed kinetics will be ensemble-averaged. Alternatively, effects of structural fluctuation on protein folding kinetics,[8] enzymatic reactions,[9] protein−

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DNA interaction,[10] and conformational changes of DNA[11] have been increasingly reported by using spectroscopic techniques at the single-molecule level. Single-molecule experiments can circumvent the necessity of synchronization of many molecules, which facilitates the characterization of the structural fluctuation in the reactions. We thus utilize the single-molecule fluorescence technique to examine effects of the structural fluctuation on the ET reaction.

It is also interesting to note that the cellular concentration of a typical ET protein is not as condensed as that used for the ensemble-averaging experiments (~millimolar) and has been reported as 0.02−0.05 nmol/mg of total cellular proteins.[12] Given that a cell, in general, contains 1−10 mg of protein total/mL,[13] the cellular concentration of the ET protein would be 0.02−0.5 pM. The subpicomolar range of the protein concentration corresponds to the experimental conditions at the single-molecule level,[14] which might also illuminate in vivo mechanisms of the protein ET reactions.

To observe the single-molecule ET reaction, we constructed a model system, in which a fluorescent probe, Alexa 647, is attached to one of the well-characterized hemoproteins, cytochrome b_5 (Cytb_5). The dynamical behavior of Cytb_5 has been extensively studied by using NMR and MD simulations.[15−17] In this model system, transfer of an electron from the fluorescent probe to heme is considered to result in oxidation of the probe and quenching of its fluorescence. Changes in the fluorescence intensity can thus serve as a beacon for the single-molecule ET reaction. From analysis of the ET reaction at the single protein molecule, we can conclude that the structural fluctuation affects the ET rate constant mainly through fluctuation of the D−A distance.

Experimental Procedures

Protein Preparation and Purification. All of the mutations were performed by the QuikChange Site-Directed Mutagenesis Kit (Stratagene). DNA sequencing was conducted at each step of gene manipulations by using an ABI 3100 Genetic Analyzer. The gene of rat hepatic cytochrome b_5 (Cytb_5), originally included in pUC13 plasmid,[18] was re-cloned into pET15b to make an expression gene for 6 × histidine-tagged cytochrome b_5 (HCytb_5).

HCytb_5 was expressed in an *Escherichia coli* strain, BL21. After harvested, cells were lysed in 50 mM Tris−HCl, 1 mM EDTA, pH 8.0, with lysozyme, DNase, and RNase.[18] Crude HCytb_5 was purified by a Ni−NTA agarose column (Qiagen), and finally, purified HCytb_5 gave an A_{413}/A_{280} ratio of 5.8, which was stored at −80 °C.

Modification with Fluorescent Probe. The fluorescent probe, Alexa Fluor 647 carboxylic acid succinimidyl ester, was purchased from Molecular Probes.[19] Using the Alexa Fluor 647 Protein Labeling Kit, the modification reaction started with 100 μM HCytb_5 in 500 μL of 10 mM Na−Pi, pH 8.0. After incubation for 1 h at room temperature, the reaction mixture was quenched by exchanging the buffer into 10 mM Na−Pi, pH 6.0, with Centrifugal Filter Device (Millipore), and the unbound dye was further removed by the gel filtration column, PD-10 (Amersham Pharmacia), equilibrated with 10 mM Na−Pi, pH 6.0. To obtain the 1:1 modified proteins with the fluorescent probe, the crude reaction mixture was loaded onto a HiTrapQ anion-exchange column (Amersham Pharmacia), equilibrated with 10 mM Na−Pi, pH 8.0. The sample was eluted with the increasing proportion of the elution buffer, 10 mM Na−Pi, 1 M NaCl, pH 8.0. The degree of the labeling was determined by using the electronic absorption spectrum: $\epsilon_{412} = 117$ mM^{-1} cm^{-1} in HCytb_5, and $\epsilon_{650} = 239$ mM^{-1} cm^{-1} in the Alexa Fluor 647 dye. Electronic absorption and fluorescence spectra were obtained by using a Perkin-Elmer Lambda 18 and Perkin-Elmer LS-55, respectively.

Single-Molecule Detection. Glass coverslips (26 × 56 × 1 mm) were purchased from Hikari-Kobo Co. Ltd. and cleaned by sonication in 5% nonfluorescent detergent (Fujiwara Factory Co. Ltd.). Coverslips were further sonicated in acetone and distilled water. Before measurements, MiliQ water was flushed on the surface of the coverslips, which was allowed for air-drying.

The small chamber was made on cleaned coverslips using 25-μm-thick polyester film and coverglass.[20] Fifteen microliters of streptavidin (1 mg/mL, Nacalai tesque) in the phosphate buffer was flowed into this chamber, which was incubated for 5 min at room temperature. After washing the chamber with the buffer solution, 1 mg/mL biotin-X-NTA (Molecular Probes Co. Ltd.) with 100 mM $NiCl_2$ was introduced into the chamber and left for 5 min. Excess biotin-X-NTA and $NiCl_2$ were removed by the buffer solution, and the 100 pM protein solution was subsequently introduced into the chamber. After a 5 min incubation at room temperature, excess protein was also washed out by the buffer solution. The polyester films used for making the chamber were carefully removed, and the edge of the coverglass was sealed by the transparent nail polish. These sample preparations were done in the clean-chamber.[20]

Total internal reflection fluorescence microscopy (TIRFM) was applied to the examination of the single-molecule experiments in this study.[21] The linearly polarized 632 nm line output of a He−Cd laser (Model 05-LHP-928; Melles Griot, Carlsbad, CA) was modulated to circularly polarized light by a quarter-wave plate and attenuated by a neutral density filter. After passing through a focusing lens and a cubic prism, the laser beam was totally reflected at the incident angle of 69° to the norm at the coverslip−sample solution interface. An evanescent field was produced with a penetration depth of 130−160 nm. The fluorescence emission from the specimen was collected with an oil-immersion microscope objective (1.40 NA, 100×, PlanApo; OLYMPUS, Tokyo, Japan), filtered by a barrier filter (570DF30; Omega Optical, Brattleboro, VT), and then focused by a relay lens onto the faceplate of an image intensifier (Model VS4-1845; Video Scope International, Sterling, VA) coupled to an SIT camera (C2400−08; Hamamatsu Photonics, Shizuoka, Japan). The images were recorded on a video cassette recorder with the video frame rate (33 ms) for the further analysis.[20]

The pictures recorded on the videotape were converted into the electronic movie file by using the MTV1000 video capture board (Canopus Co. Ltd.). Changes in the fluorescence intensity of the spots were analyzed as the mean gray scale of the region of interest, which was performed by ImageJ software (http://rsb.info.nih.gov/ij/).

Results and Discussion

Characterization of the Alexa-Modified HCytb_5. The Alexa Fluor 647 dye contains an amine-reactive succinimidyl ester group and is able to specifically modify the amino groups in

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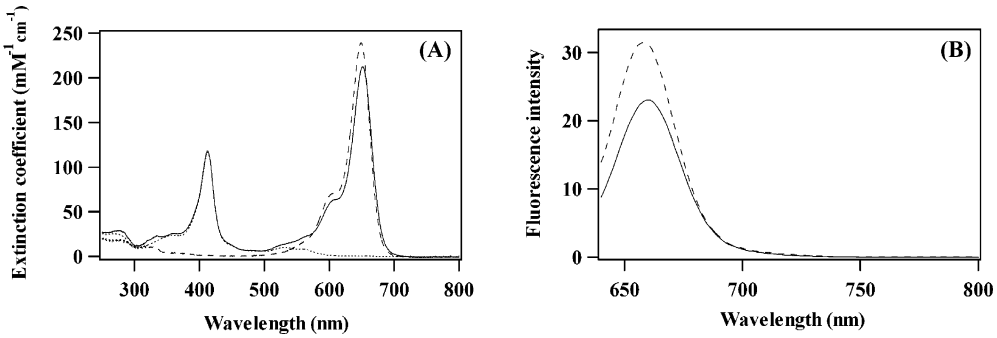


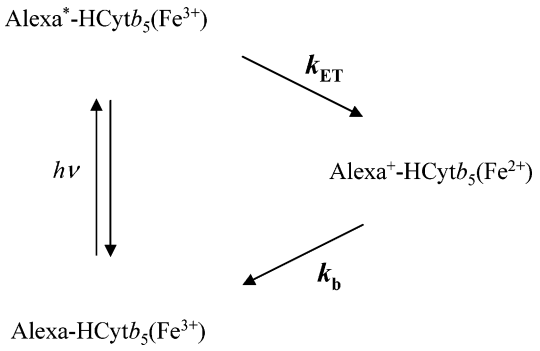
Figure 1. (A) Electronic absorption spectra of HCytb_5 (dotted curve), Alexa 647 dye (broken curve), and Alexa-HCytb_5 (solid curve) in 10 mM Na−Pi, pH 8.0 at room temperature. (B) Fluorescence spectra of Alexa 647 dye (broken curve) and Alexa-HCytb_5 (solid curve). Excitation wavelength was 632 nm. Buffer condition is the same as that in the electronic absorption spectra.

proteins.[19] The 1:1 modification of HCytb_5 with the Alexa dye was confirmed by electronic absorption spectroscopy. Figure 1A compares the absorption spectra before and after the modification of HCytb_5 with the dye. Cytb_5 has an intense absorption peak at 414 nm, while the Alexa dye absorbs the light at 649 nm. The spectrum of modified HCytb_5 (Alexa-HCytb_5) can be well reproduced by the simple addition of the spectra for HCytb_5 and the Alexa dye, indicating that the labeling ratio of the dye to HCytb_5 is equal to unity. Although 11 amino groups on Cytb_5, including the ϵ-amino group in Lys residues and the α-amino group at the N-terminus, could be modified by the Alexa succinimidyl ester, it has been reported that three Lys residues are reactive toward chemical modifications.[22] Our preliminary tryptic peptide mapping using MALDI-TOF mass spectrometry has indicated that Lys5, Lys34, Lys72, and Lys86 are candidates for the modification site for the dye (data not shown). While the unambiguous identification of the modification site needs further detailed studies, we have confirmed that the 1:1 complex between the dye and HCytb_5 is preferably formed after incubation for an hour (Figure 1A). This indicates the relatively homogeneous modification; that is, one of the Lys residues is dominantly modified with the dye. According to the calculation of the solvent accessibility of Lys residues in Cytb_5,[23] Lys34 shows the highest accessibility and can be considered as the most possible site for the modification.

By attaching the Alexa dye to HCytb_5, we can introduce fluorescence into the nonfluorescent hemoprotein, HCytb_5. Alexa-HCytb_5 exhibits a similar fluorescence spectrum with the dye alone except for its reduced intensity (Figure 1B); both of the Alexa dye and Alexa-HCytb_5 show an intense peak centered at 660 nm. While the reduced intensity in Alexa-HCytb_5 could be due to the fluorescence quenching through the energy transfer from the excited dye to heme in HCytb_5,[24] the Förster radius, R_0, at which energy transfer is 50% efficient, is relatively short and calculated as 14 Å in Alexa-HCytb_5, suggesting inefficient energy transfer.[25] Given that the distance, R, between heme and the modified Lys residue(s) is about 20 Å (22, 20, 17, and 31 Å for Lys5, Lys34, Lys72, Lys86, respectively), efficiency of energy transfer, E, is 0.1 by using the following equation:[24]

$$E = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6} \qquad (1)$$

Scheme 1



By comparison, the donor−acceptor pair often used for the energy transfer experiments, such as the dyes (Cy3 and Cy5), has more than 0.9 of the energy transfer efficiency.[24] It is thus unlikely that the reduction of the fluorescence intensity observed here is mainly due to the energy transfer process. Alternatively, the electron transfer from the dye to heme can quench the fluorescence, due to the lower redox potential in the excited state of the fluorescent dye molecule than that in the heme of Cytb_5 (∼5 mV).[26,27] Namely, as shown in Scheme 1, the Alexa dye on the protein surface is at first excited by the laser light to form Alexa*, which gives the intense fluorescent signal. At the next step, fluorescence is quenched through the intramolecular ET reaction from Alexa* to heme (Fe^{3+}), which results in the nonfluorescent and oxidized state, Alexa$^+$, and the reduced state of the heme iron, Cytb_5(Fe^{2+}). Then, an electron returns to Alexa$^+$ from Cytb_5(Fe^{2+}) due to the high redox potential of the

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(25) The Förster distance, R_0, can be calculated from the extinction coefficient, $\epsilon_A(\lambda)$, of acceptor (Cytb_5) and fluorescence emission intensity, $f_D(\lambda)$, of donor (Alexa) by using the following equations (ref 24):

$$R_0(\text{Å}) = \{8.8 \times 10^{23} \cdot \kappa^2 \cdot n^4 \cdot QY_D \cdot J(\lambda)\}^{1/6}$$

$$J(\text{M}^{-1}\,\text{cm}^3) = \frac{\int \epsilon_A(\lambda) \cdot f_D(\lambda) \cdot \lambda^4 d\lambda}{\int f_D(\lambda) d\lambda}$$

where κ^2, n, and QY_D are the dipole orientational factor, refractive index, and fluorescence quantum yield, respectively. κ^2 is set to $^2/_3$, which has been often used as an approximation and is the value for randomly oriented donors and acceptors. QY_D has not been determined for the Alexa dye used here and is assumed to be no more than 0.5 on the basis of the QY_D values of the general fluorescent probes. J value was calculated from the Cytb_5 absorption and the Alexa fluorescence spectrum (Figure 1).
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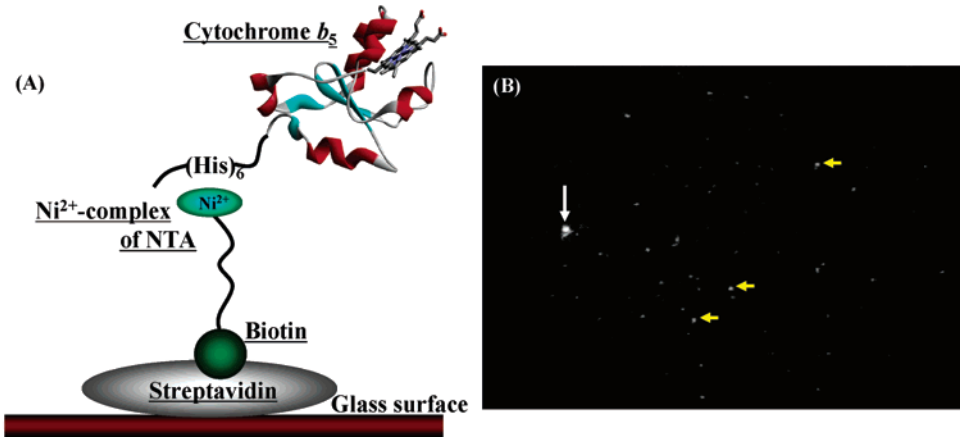

Figure 2. (A) Schematic representation of fixation of Alexa-HCytb_5 on glass surface. (B) Typical frame image of Alexa-HCytb_5 taken at the video rate by the total internal reflection fluorescence microscopy. Alexa-HCytb_5 (100 pM) was introduced in the chamber on the glass coverslip, and the unbound protein molecules were washed out with 10 mM Na−Pi, pH 8.0. Laser light at 632 nm was illuminated and totally reflected at the coverslip−sample solution interface (see Experimental Procedures). The fluorescence spot from an Alexa-HCytb_5 molecule is indicated as a yellow arrow, and three examples are shown here. These fluorescence spots continuously change their intensity and blink. Also, the fluorescence spot from dusts can be sometimes observed and is indicated by a white arrow.

fluorescent dye molecule,[28,29] after which the laser irradiation on the sample can restore the system to the fluorescent state again. On the basis of this scheme, we expect that the ET reaction in the single protein molecule can be observed as the interchange between the fluorescent and nonfluorescent states.

Single-Molecule Fluorescence from Alexa-HCytb_5. In the single-molecule fluorescence microscopy, fixation of protein molecules on the glass surface is required.[21] For this purpose, the histidine tag in HCytb_5 is bound to the Ni complex of biotin-X-NTA, and the biotin group can specifically interact with streptavidin. Streptavidin is strongly adsorbed on the glass surface, and thereby Alexa-HCytb_5 can be attached there (Figure 2A). At the glass surface, illumination of the laser light by the total internal reflection produces an evanescent field with the 1/*e* penetration depth of about 150 nm, which has a significant advantage of imaging with a high ratio of the fluorescence to the background. Figure 2B shows a typical image of the fluorescent spots from the Alexa-HCytb_5 molecules under the continuous excitation with the 632 nm laser light. The observed spots do not change their positions during the measurements, and less numbers of the fluorescent spots were seen when the sample concentration was decreased from 100 to 10 pM (data not shown). These results suggest the successful fixation of Alexa-HCytb_5 on the glass surface (Figure 2A), and the evanescent field can excite the Alexa dye in the Alexa-HCytb_5 molecule. Figure 2B represents three typical fluorescent spots (indicated by yellow arrows) from the respective Alexa-HCytb_5 molecules. While fluorescence (or scattered light) due to dusts can also be observed (white arrow in Figure 2B) and last more than 10 min, the fluorescent spots from Alexa-HCytb_5 molecules change their intensity and blink with the passing of time up to 1 min.

We also confirmed that the observed fluorescence is emitted from a single Alexa-HCytb_5 molecule. When the intensity change of the fluorescent spot, $I(t)$, is plotted against time, t, a typical trace shows drastic and jagged changes in its intensity between the fluorescent and nonfluorescent states, after which the fluorescent spots are abruptly bleached. Three representative traces of the fluorescent intensity changes in the spots are shown in Figure 3A. The bleaching is largely due to the photodecomposition of the dye molecule by the laser light, and the time until the fluorescence bleaching is consistent with the previous studies (10−60 s).[11,21,30] While in the ensemble-averaging experiments, the fluorescence bleaching results in an exponential decay of its intensity, the fluorescence from a single molecule is supposed to be abruptly bleached at the single step. As shown by the arrows in Figure 3A, photobleaching abruptly occurs at a single step, which strongly suggests that the fluorescence seen as a spot is a single-molecule event.

Until fluorescence from a spot is abruptly bleached, its fluorescence intensity seems to be "blinking" in a time scale of hundreds millisecond (Figure 3A). In this study, the fluorescent blinking is the temporary disappearance and return of fluorescence before permanent photobleaching. There appears to be several different fluorescent states, which would be partly due to the limitation of the time resolution (33 ms) in our experimental setup. When the lifetime of the excited state is shorter than the video frame rate, reduced intensity of the fluorescence would be recorded (vide infra).[31] The erratic variations in the fluorescence intensity among the fluorescent state may also result from molecular dynamics that can change the absorption coefficient by changing the orientation of the dye relative to the excitation light[32] because the Alexa-HCytb_5 molecule can pivot on the biotin-X-NTA axis (Figure 2A). Although we still investigate the exact reason that several apparent fluorescent states exist, the fluorescent blinking shown in Figure 3A is expected to be due to the ET reaction between the Alexa and heme (Scheme 1). Blinking of the fluorescence at a single-molecule level can be caused by a variety of other photophysical processes, such as the singlet-to-triplet transition and the energy transfer, but these processes generally occur in

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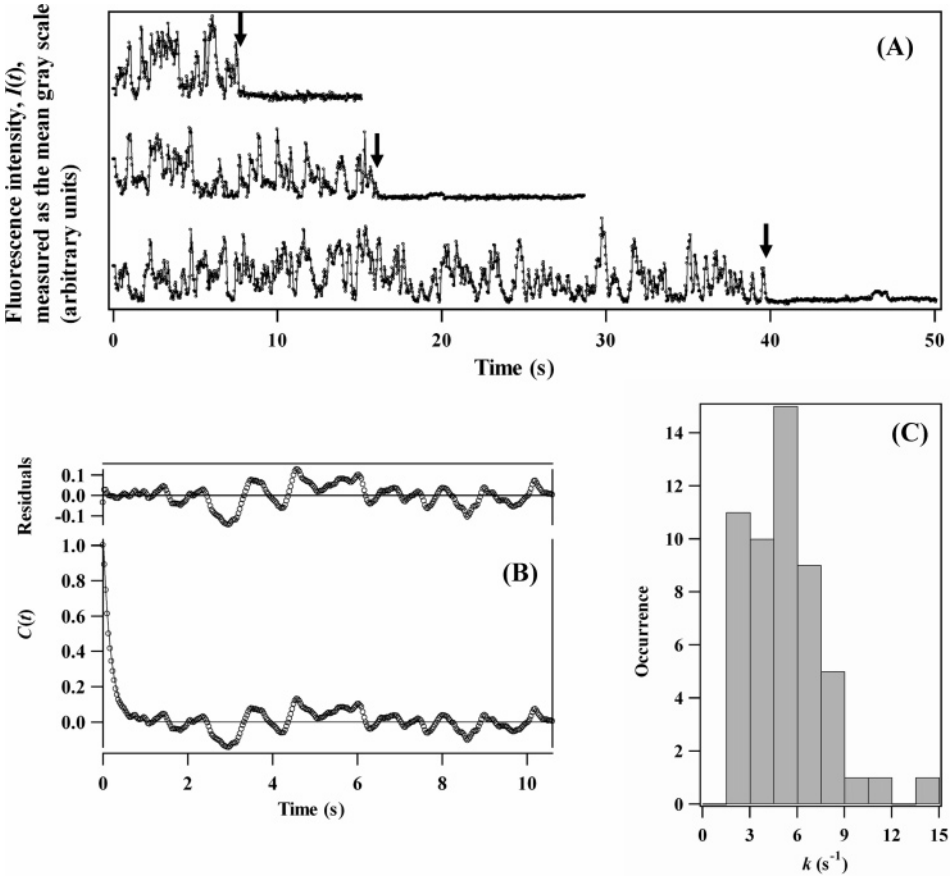

Figure 3. (A) Three typical trajectories for changes in the fluorescence intensity, each of which is emitted from a distinct Alexa-HCytb_5 molecule fixed on the glass surface. Intensity of the fluorescence from a single-molecule spot is analyzed by using ImageJ software and shown as the mean gray scale in the region of interest. (B) Autocorrelation function, $C(t)$, calculated from one fluorescence trajectory of a single Alexa-HCytb_5 molecule. The single-exponential curve to fit the autocorrelation function is shown as a solid curve, and the residuals are also shown on top of the graph. (C) Distribution of the decay rate constant, k, of the autocorrelation function (eqs 3 and 4).

the nanosecond to microsecond time scale, which cannot illustrate the observed blinking in a time scale of hundreds of milliseconds. In contrast, as implicated by Wennmalm et al.,[11] the ET reaction causes blinking of the fluorescence in a relatively slow time scale (∼seconds). Besides, theoretical estimation of the time scale of blinking supports the ET reaction between the Alexa dye and heme. The time scale, that is, the rate constant (k_{ET}), of ET reactions in proteins can be estimated by the Marcus equation (eq 2):[33]

$$k_{ET} = A \times \exp[-\beta(d-3)] \times \exp\left[-\frac{(\Delta G^\circ + \lambda)^2}{4\lambda RT}\right] \quad (2)$$

where A, R, and T are the constant, gas constant, and temperature, respectively, and β is the distance decay factor, 1.2 Å^{-1};[34] d is the distance between the donor and acceptor molecule (i.e., D−A distance) and falls between 20 and 30 Å in the Alexa-HCytb_5 molecule (22, 20, 17, and 31 Å for Lys5, Lys34, Lys72, and Lys86, respectively). ΔG° is the redox potential difference between the donor and acceptor molecules, and λ is the reorganization energy, 0.8−1.2 eV.[34] Given that the long-lived fluorescent probes usually have sufficiently negative ΔG° (−0.5 to −1.0 eV)[35] in their excited states for the reduction of heme in Cytb_5 (0.005 eV),[26] the rate constant in Alexa-HCytb_5 will fall between 10^{-2} and 10^5 s^{-1}, corresponding to seconds in the ET time scale. Fluorescent intensity from a single-molecule spot blinks in the millisecond to second time scale (Figure 3A), and therefore the theoretical ET rate constant in Alexa-HCytb_5 is consistent with the idea that the quenching and recovery of the fluorescence in the single-molecule trajectories can be ascribed to the ET reaction between the Alexa dye and heme (Scheme 1).

The estimated time-scale of the ET reaction is corroborated by the ET rate that can be obtained from the trajectories of the single-molecule fluorescence. For this purpose, the autocorrelation function of the trajectories computes the correlation in the fluorescence fluctuations recorded at two separate times and has helped the understanding of several single-molecule systems; the decay time of the autocorrelation function reflects the average period of fluorescent on/off behavior in the single-molecule experiments.[36,37] Changes in the fluorescence intensity occur on the basis of the electron transfer in the Alexa-HCytb_5 molecule (Scheme 1), and we can also assume that the laser-excitation of the dye occurs in the picosecond time scale, which cannot be resolved in the current time resolution. Continuous excitation by the laser light will further decrease the possibility for the Alexa-HCytb_5(Fe^{3+}) state to survive in Scheme 1. The

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following two-state transition is hence considered to be enough to explain the observed blinking of the fluorescence.

$$\underset{\textit{fluorescent}}{\text{Alexa*HCyt}b_5(\text{Fe}^{3+})} \underset{k_b}{\overset{k_{ET}}{\rightleftharpoons}} \underset{\textit{nonfluorescent}}{\text{Alexa}^{+}\text{-HCyt}b_5(\text{Fe}^{2+})}$$

In other words, the fluorescence intensity of "on" and "off" periods can be interpreted as single redox state turnover events, and the on/off blinking time constants correspond to the ET time constants.[38] If two states with discrete fluorescent lifetimes are in equilibrium with the interconversion rates, k_{12} and k_{21}, the autocorrelation function will decay in a monoexponential way, and its decay rate amounts to the sum of k_{21} and k_{12}.[36,37] By analogy, it can be considered that the decay rate of the autocorrelation function in the above two-state system also corresponds to the sum of the forward and back ET rate constants, $k_{ET} + k_b$, which has been implicated in recent studies on the single-molecule ET reactions.[36,37]

By using DATAPLOT software provided by NIST (http://www.itl.nist.gov/div898/software/dataplot/), the autocorrelation function, $C(t)$, was calculated from changes in the fluorescence intensity until the photobleaching (Figure 3A) based upon the following equations:[39]

$$C(t) = \frac{\langle \Delta I(t) \times \Delta I(0) \rangle}{\langle \Delta I(0)^2 \rangle} \tag{3}$$

$$\Delta I(t) = I(t) - \langle I \rangle \tag{4}$$

where $\langle I \rangle$ is the average intensity of the fluorescence until the photobleaching occurs. Figure 3B shows a typical example of the autocorrelation function of the fluorescence intensity change. By using Igor Pro ver π software from WaveMetrics, Inc., the autocorrelation function, $C(t)$, can be fit to a single-exponential function with the decay rate constant, k, which is equivalent to the rate constant for the fluorescent on/off blinking and the ET reactions between the two states (Figure 3B). It should be noted that the decay rates of the autocorrelation function are distributed approximately from 1 to 10 s^{-1} (Figure 3C), indicating that the ET reaction at the single-molecule level can be characterized by the distribution of the rate constants. The ET rate at the single-molecule level could be further populated to the region faster than 10 s^{-1}, where characterization of the reaction is difficult or impossible due to the limitation of the time resolution in this study. While the most possible site for the Alexa modification is Lys34, possible heterogeneity in the Alexa-HCytb_5 sample, in which the dye modifies the different Lys residues on Cytb_5, might also contribute to the distribution of the observed ET rate. Among the possible heterogeneous modifications (17, 20, 22, and 31 Å of D−A distances for Lys72, Lys34, Lys5, and Lys86, respectively), eq 2 predicts that ca. 10 Å difference in the D−A distance leads to a 1.6 × 10^5-fold difference in the ET rate constant. Alternatively, a 10-fold difference in the single-molecule ET rate observed here (Figure 3A) is reminiscent of the mechanism of the *inter*-protein ET reaction, in which the rate constant also shows the distribution due to the difference in the D−A distance among a variety of encounter complexes.[40] Recent studies on the single-molecule ET reaction in flavin reductase have also suggested that the fluctuation in the D−A distance can affect the ET rate constant at the single-molecule level.[41] The D−A distance thus seems to be a major factor in determining the distribution, and we analyze the distribution of the ET rate constant in a single Alexa-Cytb_5 molecule by using the Marcus theory.

As seen in eq 2, the Marcus theory indicates that the ET rate constant is largely determined by the distance, d, and the redox potential difference, $\Delta G°$, between the donor and acceptor sites. To induce ca. 10-fold variation in the ET rate constants (Figure 3C), either 2 Å change in the D−A distance or 0.2−0.5 eV change of $\Delta G°$ would be enough. While previous studies[26,27] have shown that 0.2−0.5 eV change of $\Delta G°$ seems to be too large to be realized in Cytb_5 or the Alexa dye, the dynamical behavior of the Cytb_5 structure that can exert several angstroms of the distance change has been suggested in NMR[15,16] and MD simulation studies.[17] MD simulations on Cytb_5 have estimated that the RMS deviations of the C^α atoms from the crystal structure are between 1 and 3 Å, while 0.58 Å of the backbone RMS deviations from the averaged structure has been reported in the NMR studies on the oxidized Cytb_5. Compared to the protein backbone, the side chain of the amino acid residues has, in general, larger flexibility in the protein conformation; that is, the side chain can easily flip its position with the thermal energy. The ET pathway includes the atoms of the side chains, and the ET reaction is hence considered to be affected by the structural fluctuation through its associated change in the D−A distance.

Although further studies are critical for evaluating effects of the structural fluctuation on the ET reaction, the current model system using the microscope enables us to directly see the ET reaction in one protein molecule. Besides, a metalloprotein attached with a fluorescent molecule is one good model system for examining an ET reaction at the single-molecule level. As also suggested here, ET reactions can be affected by the D−A distance change associated with the structural fluctuation. Structural fluctuation is affected by many kinds of physiological processes, such as ligand−substrate binding,[42,43] protein−protein interaction,[44] and protein folding;[45] the distance change upon the thermal fluctuation would be one of the important factors to regulate the protein ET reactions.

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