THE JOURNAL OF PHYSICAL CHEMISTRY B

Article

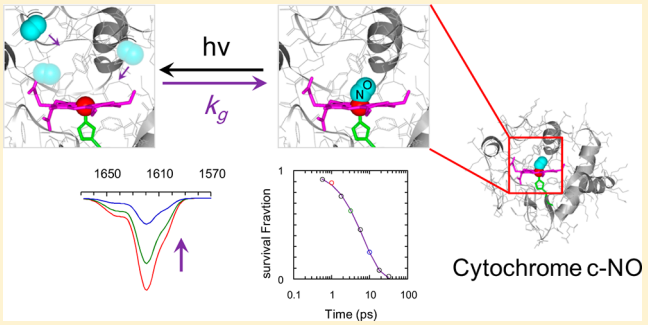
pubs.acs.org/JPCB

Dynamics of Geminate Rebinding of NO with Cytochrome c in Aqueous Solution Using Femtosecond Vibrational Spectroscopy

Jooyoung Kim, Jaeheung Park, Taegon Lee, and Manho Lim*

Department of Chemistry and Chemistry Institute for Functional Materials, Pusan National University, Busan 609-735, Korea

ABSTRACT: Using femtosecond vibrational spectroscopy, we investigated the rebinding dynamics of NO to cytochrome c (Cytc) and a model heme, microperoxidase-8 (Mp), after photodeligation of CytcNO in D_2O solution and MpNO in an 81% glycerol/water (v/v) mixture at room temperature. Whereas the stretching mode of the NO band in MpNO was described by a Gaussian centered at 1653 cm^{-1} with a full width at half-maximum (fwhm) of 41 cm^{-1}, that in CytcNO revealed an asymmetric structured band that peaked at 1619 cm^{-1} with an fwhm of about 27 cm^{-1}. The structured NO band in CytcNO was well described by the sum of three Gaussians, and its shape did not evolve with time but its amplitude decayed exponentially with a time constant of 7 ± 1 ps. The transient NO band in MpNO also decayed exponentially with a time constant of 8 ± 1 ps. Rebinding of NO to Cytc was slightly faster than that of NO to Mp and was almost complete by 30 ps, which was much faster than the rebinding of NO to myoglobin (Mb). When the deligated NO was constrained near the Fe atom either by a viscous solvent or by the protein matrix, it rebound to heme Fe much faster than CO, suggesting that NO has a higher propensity for binding to heme Fe and the high reactivity governed the rebinding kinetics. Moreover, the faster ligand rebinding in Cytc than in Mb suggests that Cytc does not have a primary docking site (PDS)-like structure found in Mb that suppresses rebinding by restraining ligand motion and the PDS can also hold the deligated NO in a manner that impedes NO rebinding; however, due to higher NO reactivity with heme Fe, the impediment is not as efficient as for CO.



I. INTRODUCTION

Knowledge of how a protein's function is related to its structure and dynamics is essential to fully understanding its functioning mechanism. The rebinding dynamics of a deligated ligand to a heme protein after the photodeligation of ligated proteins has been widely studied to ascertain the ways in which protein dynamics and structure control ligand binding, a key function for ligand-binding heme proteins.[1,2] In particular, the rebinding of diatomic ligands (i.e., CO, NO, and O_2) to ligand-binding heme proteins such as myoglobin (Mb) and hemoglobin (Hb) after photodeligation of the ligated proteins has been extensively studied.[1−8] About 4% of photodeligated CO from MbCO under physiological conditions geminately rebinds, even though CO is located in close proximity to heme Fe immediately after deligation and remains there for 180 ns.[4,9,10] In contrast to the slow and inefficient rebinding of CO to Mb,[4,10] deligated NO from MbNO and HbNO under physiological conditions rebinds on the picosecond time scale.[3,7,11−20] Thus, the kinetics of NO rebinding to Mb and Hb was explored to unveil the diffusion pathway of the deligated NO within the protein and to understand how protein function is related to its dynamics and structure.[7,11−20]

Ligand rebinding to heme proteins with activities other than the binding of diatomic ligands was also investigated to reveal functional structure in the ligand-binding protein, by comparatively studying the rebinding kinetics.[21−23] One example was a study of CO rebinding to cytochrome c (Cytc), an electron transfer protein with a heme cofactor.[23,24] The heme iron in native Cytc is axially coordinated by two internal ligands, a proximal histidine (His-18) and a distal methionine (Met-80).[25,26] Thus, CO cannot bind to native Cytc in neutral solution, and extreme pH or denaturant was required to make Cytc bind to CO.[27−29] On the other hand, Cytc in a neutral solution could be chemically modified to bind external diatomic ligands.[24,30] In chemically modified Cytc, the bond between Met-80 and heme Fe was broken and Met-80 was carboxymethylated to disable its rebinding.[23,24,30] The carboxymethylated Cytc (cCytc) can bind external ligands such as CO and NO in its sixth coordination site; thus, the rebinding of CO to cCytc in a neutral solution without the addition of any denaturant was investigated. The rebinding of CO to cCytc is nonexponential with the mean time constant for geminate rebinding (GR) of 107 ps.[23] More than 1000 times faster GR of CO to cCytc than Mb is consistent with the suggestion that Mb has heme pocket(s) holding deligated CO in a way that restrains CO from rebinding, while keeping it near the Fe atom.[9,23]

NO is a unique ligand that can bind to ferric (Fe^{3+}) and ferrous (Fe^{2+}) hemes.[31,32] When NO is introduced into native ferric Cytc in neutral solution, ferric CytcNO is readily

Received: August 26, 2012
Revised: October 26, 2012
Published: October 31, 2012

formed.[33,34] Ferric CytcNO can be reduced to NO-bound ferrous Cytc in a neutral solution without any chemical modification, as required for CO ligation.[35] Recently, the rebinding dynamics of NO to native ferrous Cytc under physiological conditions was investigated by time-resolved visible spectroscopy.[36,37] According to the transient optical spectra, most of NO photodeligated from ferrous CytcNO geminately rebinds with a time constant of 8 ps,[36,37] much faster than CO rebinding to cCytc. Much faster NO rebinding to Cytc and Mb than CO rebinding to the corresponding protein has been attributed to the fact that NO can bind to the heme in a domed configuration.[36,38,39] Dissociation of diatomic ligand such as NO or CO from a ligated heme protein is known to induce quasi-instantaneous out-of-plane displacement of the Fe atom and heme doming.[36,40,41] Whereas NO can bind to the displaced Fe atom of heme and, thus, the NO rebinding to heme is almost barrierless, CO binding requires the heme Fe atom to be driven into the heme plane by thermal fluctuations.[38] Because in-plane displacement of the Fe atom requires energy, CO rebinding is slower than NO. Most of NO was also found to geminately rebind to ferrous M80A Cytc mutant with a time constant of 7 ps.[42] Equally efficient and similar rebinding rate of NO to ferrous M80A Cytc mutant was ascribed to insensitivity of the deligated NO to changes in the heme environment and its close stay to the heme.[42]

Another example for the ligand binding study for heme proteins with activities other than the binding of diatomic ligands was a study of NO rebinding to horseradish peroxidase (HRP), a heme protein with peroxidase activity.[21] About 95% of NO from ferrous HRPNO was found to rebind with a time constant of 6.3 ps. The ultrafast NO rebinding to HRP can be attributed to the high reactivity of NO to the heme that it binds to the heme in a domed configuration. Although NO rebinds to Cytc and HRP exponentially with a time constant of 6−8 ps, NO rebinding to Mb is nonexponential and slower than that to Cytc and HRP. The nonexponential GR of NO to Mb has been described by a biexponential function with time constants of 5−30 ps and 100−220 ps.[7,12,16,17,43,44] The exponential kinetics of NO rebinding to HRP was used to refute the conformational relaxation (CR) of Mb as a source of the nonexponential NO rebinding to Mb.[21] Diffusive motion among heme pockets in the distal pocket of Mb was suggested to be the main cause for the nonexponential rebinding kinetics of NO to Mb.[21]

Mb has a small vacant site on top of the heme group, about 3 Å from heme Fe, where CO is trapped immediately after the photodeligation of MbCO.[9,10,45−47] The vacant site surrounding amino acids that are highly conserved in mammalian Mb[48] is named the primary docking site (PDS). The PDS was suggested to have a specific structure that can suppress CO rebinding by constraining the orientation of CO such that it is unfavorable to the transition state for the rebinding, resulting in inefficient and slow rebinding of CO to Mb.[9,10,23] Close inspection of the 3D structure of Cytc does not show any PDS-like structure,[23] which is consistent with the faster CO rebinding to cCytc. Indeed, a comparative study on the dynamics of CO rebinding to cCytc and Mb supported the functional role of the PDS in Mb for the CO ligand. However, because NO rebinding to Mb occurs on the picosecond time scale, further exploration is required to assess the functional role of the PDS for the NO ligand. A comparative study on NO rebinding to Cytc and Mb may unveil any functional role of the PDS for NO. Because chemically modified Cytc can also bind NO, the kinetics of NO rebinding to cCytc is also informative for a comparative study with cCytcCO.

Microperoxidase-8 (Mp) is a heme octapeptide prepared by enzymatic digestion of Cytc.[49] Because the peptide is covalently linked to the heme through a histidine, it imitates the active site of the heme protein in the absence of distal structure while preserving the proximal histidine and its structure (see Figure 1). Consequently, Mp has served as an excellent model system for heme proteins, capturing the intrinsic reactivity of the active heme free from conformational change and any distal protein structure.[23,49,50]

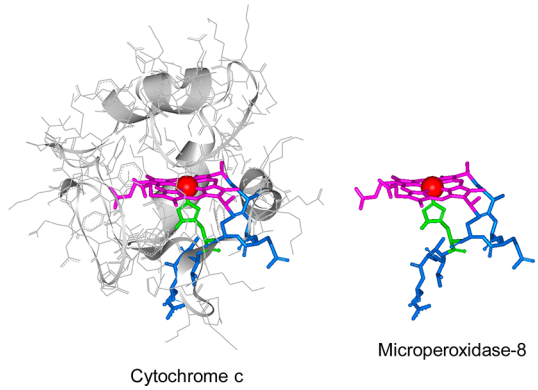


Figure 1. 3D structures of cytochrome c (Protein Data Bank entry 1ATT) and microperoxidase-8. For better comparison, the part corresponding to Mp in Cytc is shown in the colored stick model and the remaining polypeptide in gray ribbon and line. The heme is depicted in purple, the Fe atom as a red ball, the proximal histidine in green, and the remaining peptide residue of Mp in blue.

In this study, using femtosecond mid-IR spectroscopy, we measured the dynamics of NO rebinding to ferrous Cytc in D_2O and Mp in a glycerol/water (G/W) mixture after photodeligation of the corresponding molecule at room temperature. We found that the GR of NO to Cytc was exponential with a time constant of 7 ± 1 ps and slightly faster than that of NO to Mp in a viscous solution, where NO rebinds with a time constant of 8 ± 1 ps. We attributed the efficient and ultrafast NO rebinding to Cytc to the efficient trapping of the deligated NO by the protein matrix and the high reactivity between NO and the heme. We suggest that the Cytc does not have any heme pocket that can suppress ligand rebinding by restraining the spatial motion of the ligand.

II. MATERIALS AND METHODS

The femtosecond time-resolved IR spectrometer used in this study is described elsewhere.[17,23] In short, a visible pump pulse and a mid-IR probe pulse were generated by two home-built optical parametric amplifiers (OPA), pumped by a Ti:sapphire amplified pulse. One OPA generated a pump pulse at 570 nm with 3 μJ of energy by frequency doubling of its signal pulse[51] and the other OPA generated a tunable mid-IR probe pulse by difference frequency mixing of its signal and idler pulses.[52,53] The probe pulse was sent through the sample for the time-resolved mid-IR absorbance after the optically delayed pump pulse irradiated the sample. The broadband-transmitted probe pulse was detected by a 64-element N_2(l)-cooled HgCdTe array detector that was mounted on the focal plane of a 320 mm IR monochromator with a 150 *l*/mm grating. The spectral resolution of this configuration resulted in about 1.2 cm^{-1}/pixel

dx.doi.org/10.1021/jp308468j | J. Phys. Chem. B 2012, 116, 13663−13671

at 1630 cm^{-1}. Spectra spanning more than 80 cm^{-1} were superposition of two 64-point spectra that overlapped by several elements. The isotropic absorption spectrum was obtained by setting the polarization of the pump pulse at the magic angle (54.7°) relative to the probe pulse. The instrument response function was typically 160 fs.

Because native Cytc is in a ferric form that can bind the NO ligand, both ferric and ferrous Cytc can form nitrosylated Cytc. A solution of 2.5 mM ferrous CytcNO was prepared as follows. First, lyophilized horse heart Cytc (Sigma) was dissolved in D_2O (Aldrich, 99.9% D) buffered with 0.2 M potassium phosphate (pD = 7.4); then, NO gas was added into the solution to prepare ferric CytcNO. NO gas was prepared by adding $FeSO_4$ to $NaNO_2$ solution. Second, the ferric form was reduced to ferrous CytcNO with 3 equivalents of freshly prepared sodium ascorbate (Aldrich).[35] The Soret peak at 411 nm and the Q-band confirmed NO ligation to ferrous Cytc.[35,54] Because we investigated only ferrous CytcNO in the present study, we have omitted hereafter the term "ferrous" in front of CytcNO.

For cCytcNO, cCytc was first prepared as follows.[23] Lyophilized horse heart Cytc was dissolved in 0.2 M phosphate buffer (pD 7.4). For chemical modification, 0.5 M KCN and, subsequently, 0.5 M $BrCH_2CO_2H$ were added to the Cytc solution. The mixture was kept for about 6 h, the minimum incubation time needed for complete carboxymethylation of the sulfur atom of Met-80 after cleavage of the Met-80−Fe bond.[23,24,30] Then, to quench further reaction with other side groups, the excess $BrCH_2CO_2H$ and CN^- were removed from the sample solution by extensive dialyzation. The cCytcNO was prepared by reducing the 5-coordinate cCytc with three equivalents of freshly prepared $Na_2S_2O_6$ (Aldrich), and by adding an equivalent quantity of 0.1 M degassed $NaNO_2$ solution. Formation of cCytcNO was confirmed by the Soret peak at 414 nm.[24,30] The final concentration of the cCytcNO sample was 4.5 mM.

A stock solution of 1 mM Mp (Sigma) in deoxygenated 1.0 M NaOD (Aldrich) was diluted into a deoxygenated 81% G/W (v/v) mixture, reduced with excess amounts of freshly prepared $Na_2S_2O_6$ and ligated with NO by bubbling the gas for more than 10 min. The concentration of the final MpNO sample was 0.1 mM at pD = 12. Because Mp tends to aggregate,[49,55] an alkaline G/W solution was used and the concentration was kept low to minimize the aggregation.[49,55,56] Monomeric MpNO was confirmed by UV−vis and IR spectra of the sample.[57]

For NO band assignment in the protein sample, the ^{15}NO-bound protein was also prepared and its spectra were obtained. The sample was loaded in a gastight rotating or flowing sample cell with two 2 mm-thick CaF_2 windows. To minimize protein usage and background absorption from solvent, concentrated CytcNO and cCytcNO were loaded in the 25 and 12 μm path length rotating sample cells, respectively. A rather longer 100 μm path length flow cell was used for the dilute MpNO sample. The flowing sample cell, which was immune from scattering due to the moving cell window, had a better signal-to-noise ratio but required more sample. During data collection, the sample cell was rotated or the sample solution flowed sufficiently fast such that a fresh volume of the sample was excited by each photolyzing laser pulse at 0.5 kHz. The temperatures of the rotating and flowing sample cells were kept at 283 ± 1 K and 294 ± 2 K, respectively. Throughout the data collection, the integrity and concentration of the samples were checked using UV−vis and FT-IR spectroscopy. Use of D_2O solvent shifted the spectrum of interest into a region with greater IR transmission.[58]

III. RESULTS

Figure 2 shows the femtosecond time-resolved mid-IR absorption spectra in the bound NO region of CytcNO in

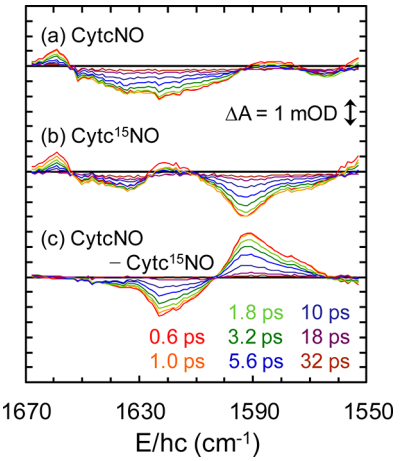


Figure 2. Time-resolved vibrational spectra of (a) CytcNO and (b) $Cytc^{15}NO$ in D_2O at 283 K after photolysis with 570 nm pump pulse. (c) Time-resolved difference spectra obtained by subtracting the transient spectra of $Cytc^{15}NO$ from those of CytcNO. The unit of the ordinate is the difference in optical density (ΔA = OD; 1 mOD = 10^{-3} OD) between the photolyzed and unphotolyzed samples. The time delays of 0.6, 1.0, 1.8, 3.2, 5.6, 10, 18, and 32 ps are color coded in the corresponding spectra.

D_2O at 283 K after photodeligation with a 570 nm pulse. The spectra overlap with the amide I (1700−1600 cm^{-1}) and amide II (1600−1500 cm^{-1}) regions of the protein, which are sensitive to its conformation and temperature.[59−61] Immediately after photodeligation, ligated heme proteins undergo conformational and thermal relaxation that can induce a rich spectral evolution in the transient spectra.[17,61] Thus, the transient spectra of NO in CytcNO (Figure 2a) have contributions from the changing conformation and temperature of the protein as well as the loss of the ligated protein by photodeligation of CytcNO. The transient spectra evolve as the protein undergoes conformational and thermal relaxation, and the deligated NO geminately rebinds, decreasing the population of the deligated protein, Cytc. The population of Cytc is proportional to the magnitude of the NO band in the transient spectra. To isolate the NO band from the spectral evolution due to the conformational and temperature change of the protein, the NO stretching band of CytcNO was assigned by collecting time-resolved spectra for $Cytc^{15}NO$ (see Figure 2b).[17,20] Because the spectra for CytcNO and $Cytc^{15}NO$ should be identical, except for the position of the NO band, difference spectra were obtained by subtracting the $Cytc^{15}NO$ spectra from the corresponding CytcNO spectra collected with identical experimental conditions. The difference spectra revealed the vibrational band to be related only to NO in the nitrosylated Cytc. As shown in Figure 2c, the time-resolved difference spectra showed a negative-going feature (bleach) that peaked near 1620 cm^{-1} and a positive-going feature that peaked near 1592 cm^{-1}. The bleach in the difference spectra comes from the loss of bound NO after photodeligation of CytcNO. The positive-going feature, arising from the ^{15}NO band in the

dx.doi.org/10.1021/jp308468j | J. Phys. Chem. B 2012, 116, 13663−13671

Cytc^{15}NO, should be a flipped replica of the bleach with an isotopic shift of ^{15}NO in Cytc^{15}NO. Obviously, the NO band of CytcNO is located near 1620 cm^{-1} and the ^{15}NO band of Cytc^{15}NO near 1592 cm^{-1}. Other spectral features in the transient spectra of CytcNO or Cytc^{15}NO can be assigned to the background signal due to change in protein conformation and/or temperature following photodeligation.

The bleach, appearing faster than the time resolution of our instrument, implies ultrafast photodeligation of NO from CytcNO; this is consistent with the photodeligation of NO (faster than 0.1 ps) from other heme proteins such as MbNO and HbNO.[7,12,15] As mentioned, the bleach in the difference spectrum results from the bleach in the transient spectrum of CytcNO and the absorption from the inverted bleach in the transient spectrum of Cytc^{15}NO. Thus, the absorption feature in the difference spectrum can be described by flipping the bleach after the isotopic shift of ^{15}NO. The difference spectra were fitted to the sum of three Gaussians modeling the bleach, and its flipped and red-shifted replica modeling the absorption. Figure 3 shows the fitted difference spectrum and the recovered

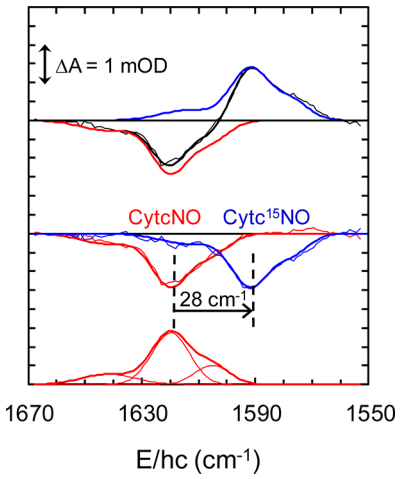


Figure 3. (a) Difference spectrum of CytcNO (CytcNO − Cytc^{15}NO) at the delay time of 1 ps (thin black line). The spectrum was described by a sum of three Gaussians modeling the negative-going feature (thick red line) and its flipped and isotope-shifted replica (thick blue line) modeling the absorption. The thick black line is made of the thick red line plus the thick blue line. (b) Isolated NO band for CytcNO peaked at 1619 cm^{-1} (red lines) and Cytc^{15}NO peaked at 1591 cm^{-1} (blue lines). The isotope shift of ^{15}NO in CytcNO (28 cm^{-1}) is indicated by the arrow. Thin lines are data and thick lines are the fits. (c) Fitted NO band for CytcNO (thick red line) and its three Gaussian components (thin red lines).

NO band at 1 ps as a representative example. The difference spectra were well described by an asymmetric structured band that peaked at 1619 cm^{-1} and its 28 cm^{-1} red-shifted flipped replica. The structured band shape indicates that the NO band in CytcNO consists of three bands that can be assigned to three conformational substates (CS) of CytcNO.[6,63,64] The shape of the asymmetric structured band does not evolve with time, indicating that each component of the band decays on the same time scale. The same decay kinetics for the constituent band implies that the CS of CytcNO has the same GR kinetics. The isotopic shift of 28 cm^{-1} is almost identical to the reported shift of NO in MbNO, about 29 cm^{-1},[59,62,65] corroborating our band assignment. Time-resolved spectra of cCytcNO in D_2O were also measured and analyzed in the same way. As shown in Figure 4, the NO band in cCytcNO, which peaked at 1600 cm^{-1}, is also asymmetrically structured but lower in energy than

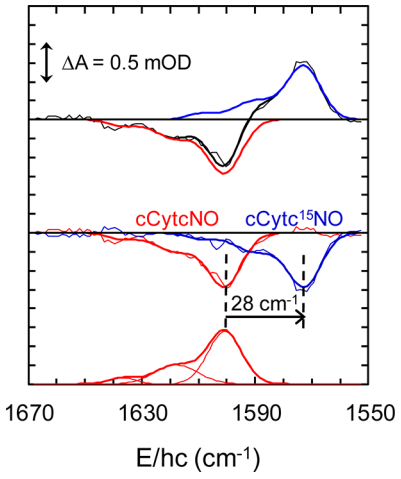


Figure 4. (a) Difference spectrum of cCytcNO (cCytcNO − cCytc^{15}NO) at 1 ps (black thin line). The spectrum was described by a sum of three Gaussians modeling the negative-going feature (thick red line), and its flipped and isotope-shifted replica (thick blue line) modeling the absorption. The thick black line is made of the thick red line plus the thick blue line. (b) Separated NO band for cCytcNO peaked at 1600 cm^{-1} (red lines) and Cytc^{15}NO peaked at 1572 cm^{-1} (blue lines). Thin lines are data and thick lines are the fits. (c) Fitted NO band for cCytcNO (thick red line) and its three Gaussian components (thin red lines).

CytcNO. The isotope shift of ^{15}NO in cCytc^{15}NO is also 28 cm^{-1}, the same as that in CytcNO. The shape of the difference spectra of cCytcNO do not evolve with time, either, and the NO band is also well modeled by the sum of three Gaussians, indicating that the cCytcNO also has at least three CSs. The vibrational band of NO in MbNO was used to assign its active site conformation.[66] The main difference in the conformation of MbNO was suggested to arise from the degree of interaction between NO and the distal histidine of MbNO.[66] Unfortunately, there is no known structure for diatomic ligand bound ferrous Cytc. Lack of structural information on CytcNO does not warrant any further assignment of the NO vibrational band to the specific structure of CytcNO.

Kinetics of the spectral features other than the NO band after photodeligation of CytcNO was sampled in a few representative spectral positions away from the NO band, such as 1658, 1622, and 1584 cm^{-1} (see Figure 2). When the background spectra were separated by subtracting the NO band from the transient spectra, they were almost the same in both CytcNO and Cytc^{15}NO (see Figure 5a), validating our band assignment. As shown in Figure 5b, the kinetic behaviors of these features are very similar in the spectra of both CytcNO and Cytc^{15}NO, confirming that they are indeed not from change in the NO band but from protein conformation and vibrational excitation. The kinetics of the background was fitted to a single exponential function with lifetimes of 6 ± 1 ps at 1658 cm^{-1}, 6 ± 1 ps at 1622 cm^{-1}, and 4 ± 1 ps at 1584 cm^{-1}. These time constants are consistent with the spectral evolution reported in the amide region with time constants of 6−12 ps after photodeligation of MbCO.[61] The spectral evolution of the amide band after photodeligation of MbCO was found to be dominated by CR of the protein with negligible thermal contribution.[61,67] The background change observed here may

dx.doi.org/10.1021/jp308468j | J. Phys. Chem. B 2012, 116, 13663−13671

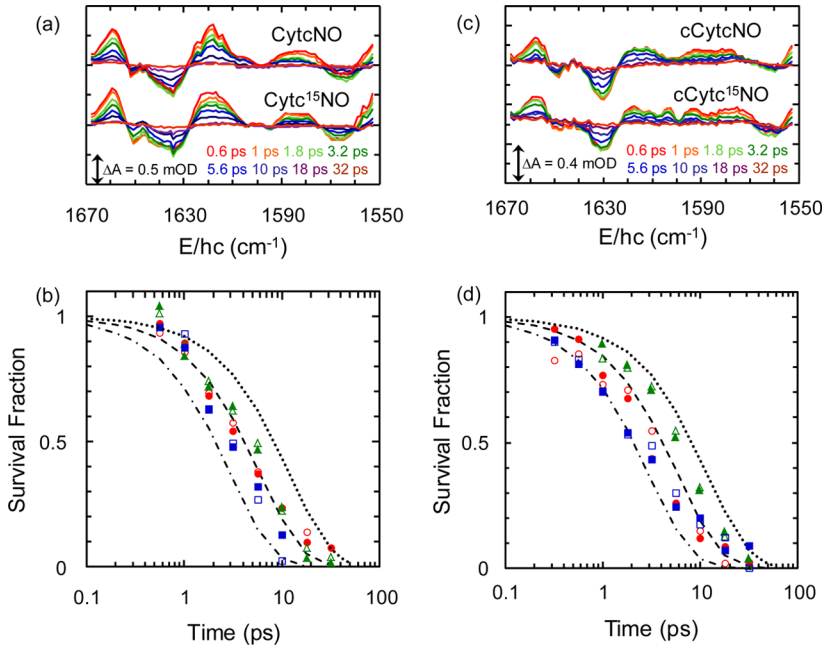


Figure 5. (a) Representative time-resolved spectra for amide band after photodeligation of NO from CytcNO (upper spectra) and Cytc^{15}NO (lower spectra). (b) Kinetics traces of CytcNO (open symbols) and Cytc^{15}NO (filled symbols) in the amide region at 1658 cm^{-1} (red circles), 1622 cm^{-1} (green triangles), and 1584 cm^{-1} (blue squares). (c) Representative time-resolved spectra for amide band after photodeligation of NO from cCytcNO (upper spectra) and cCytc^{15}NO (lower spectra). (d) Kinetics traces of cCytcNO (open symbols) and cCytc^{15}NO (filled symbols) in the amide region at 1658 cm^{-1} (red circles), 1630 cm^{-1} (green triangles), and 1592 cm^{-1} (blue squares). Transient spectra in (a) and (c) were obtained by subtracting transient NO band from the corresponding time-resolved spectra. The time delays of 0.6, 1, 1.8, 3.2, 5.6, 10, 18, and 32 ps are color coded in the corresponding spectra. When the kinetic trace overlaps with the NO band, the kinetics was obtained after subtracting the contribution from the NO band. To guide the time scale of the decay, the figure includes exponential decay functions with time constants of 3 ps (dot-dashed line), 6 ps (dashed line), and 12 ps (dotted line).

also result predominantly from CR of Cytc following deligation of CytcNO. The background spectra of cCytcNO were almost same as those of cCytc^{15}NO, too (see Figure 5c). The kinetics of the background after photodeligation of cCytcNO was also sampled at 1658, 1630, and 1592 cm^{-1}. They were described by a single exponential function with lifetimes of 5 ± 1 ps at 1658 cm^{-1}, 9 ± 1 ps at 1630 cm^{-1}, and 3 ± 1 ps at 1592 cm^{-1}. The background spectra of cCytcNO and its decay kinetics are not the same as, but very similar to, those of CytcNO (see Figure 5).

Time-resolved vibrational spectra of MpNO in 81% G/W (v/v) mixture after photodeligation were also measured and analyzed. Since a dilute sample was used to avoid molecular aggregation, the transient signal was smaller than 0.1 mOD (see Figure 6). The background in the transient spectra of MpNO, free from the amide bands causing the spectral evolution in the background signal, was described by a linear function. The NO band in the transient spectra of MpNO was well modeled with a single Gaussian function centered at 1653 cm^{-1} and the full width at half-maximum (fwhm) of 41 cm^{-1}. The broad single band in MpNO indicates that the NO band is inhomogeneously broadened, and supports the notion that the multiple bands within the protein arise from CS of the protein.

The NO band of CytcNO that peaked at 1619 cm^{-1} decays exponentially, and its decay kinetics is virtually identical to that of the ^{15}NO band in Cytc^{15}NO that peaked at 1591 cm^{-1}. Decay kinetics of the NO band was well described by a single exponential function with a time constant of 7 ± 1 ps (see Figure 7). The magnitude of the NO band in MpNO also decays exponentially, with a time constant of 8 ± 1 ps (see Figure 7). In contrast, the NO band in cCytcNO decays

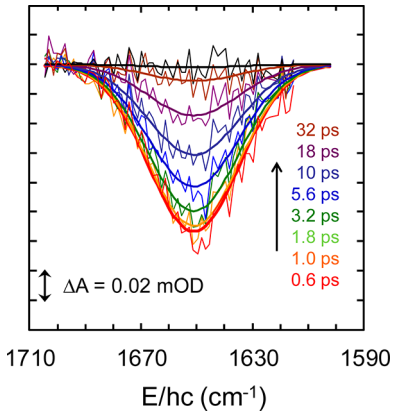


Figure 6. Time-resolved spectra of NO band after photodeligation of MpNO in 81% glycerol/water (v/v) mixture. The spectrum (thin lines) was described by a Gaussian (thick lines) peak at 1653 cm^{-1}. The time delays are 0.6, 1.0, 1.8, 3.2, 5.6, 10, 18, and 32 ps and color coded in the corresponding spectra.

nonexponentially, which could be modeled by a biexponential function of 0.52 exp(−t/2 ps) + 0.48 exp(−t/12 ps) or a stretched exponential function of 0.98 exp(−(t/4 ps)$^{0.64}$) + 0.02. Although the decay kinetics in cCytcNO is different from that in CytcNO, both decays were almost complete by 30 ps. The decay kinetics was used to represent the kinetics of GR of NO to the corresponding protein.[17]

IV. DISCUSSION

The GR of NO to Cytc proceeds with time constant of 7 ± 1 ps, very similar to the time constant for that to HRP (6.3 ps)[21]

dx.doi.org/10.1021/jp308468j | J. Phys. Chem. B 2012, 116, 13663−13671

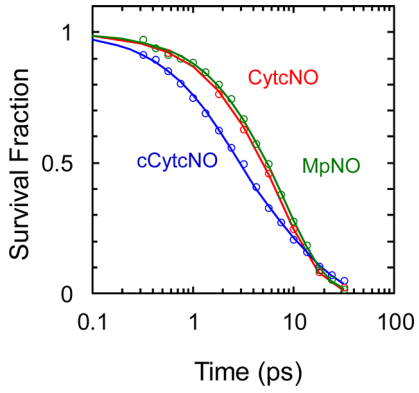


Figure 7. Normalized magnitude change of the NO band in CytcNO (red circles), cCytcNO (blue circles), and MpNO (green circles). The decay kinetics of the NO band in CytcNO and MpNO are well described by an exponential function with time constants of 7 ps (red solid line) and 8 ps (green solid line), respectively. The decay kinetics of the NO band in cCytcNO is fitted to a biexponential function of 0.52 exp(−t/2 ps) + 0.48 exp(−t/12 ps) (blue solid line) or a stretched exponential function of 0.98 exp((−t/4 ps)$^{0.64}$) + 0.02.

and consistent with the reported value of 8 ps measured by time-resolved optical spectroscopy.[36,37] The time constant for the GR of NO to Mp in 81% G/W (v/v) mixture, 8 ± 1 ps, appears to be slower than the previously reported value of 5.6 ps for the GR of NO to Mp in D_2O.[17] The short GR time constant for NO to Mp in D_2O was measured using a highly concentrated (12 mM) MpNO. Although it was argued that aggregation by the intermolecular coordination to the iron is impossible for the 6-coordinate MpNO,[17] 6-coordinate MpCO showed concentration-dependent GR rate (faster with a concentrated sample), indicating that 6-coordinate MpCO or MpNO may form aggregate.[50] There might be an aggregation mechanism other than the intermolecular coordinate to the iron, resulting in MpNO aggregate in the concentrated sample. Because we optimized the present sample condition for a monomeric Mp (high pH, low concentration, low dielectric constant for solution),[50,56] the time constant of 8 ± 1 ps obtained here likely represent an intrinsic GR of NO to monomeric Mp in 81% G/W (v/v) mixture. The ultrafast GRs of NO to Mp, Cytc, and HRP suggest that NO is highly reactive with heme Fe. The high reactivity is manifested by NO binding to the heme in a domed configuration,[36,39] which was described as a harpoon mechanism.[38]

The GR kinetics of CO to Mp in solutions with various viscosities and temperatures was well described by the theory of a diffusion-controlled reaction with no interaction potential between the deligated ligand and heme Fe.[56] Based on the observation that the GR rate of CO to Mp increased as the viscosity of the solution increased, it was suggested that the deligated ligand rebinds more efficiently as it is retained longer near heme Fe by a more viscous solution.[56] Furthermore, the faster GR of CO to cCytc than that of CO to Mp in a highly viscous solution was used to suggest that the protein matrix surrounding the heme group acts as a very efficient trap for the deligated ligand and thus accelerates the rebinding.[23] The deligated NO from CytcNO is also expected to be trapped by the surrounding protein matrix more efficiently than the viscous solution. However, the GR of NO to Cytc is slightly more efficient than or equal to that of NO to Mp in viscous solution. The negligible difference in the GR rates of NO between Cytc and Mp is consistent with the suggestion that NO is highly reactive with heme Fe and the binding is dominated by high reactivity. As shown in Figure 8, the GR of NO to Mp in a viscous solution is more than 30 times faster than that of CO to Mp in the same solution.[56] Indeed, the reactivity of heme Fe with NO is much higher than that with CO, consistent with the reactant-like transition state for NO rebinding[68] as well as a harpoon mechanism introduced for the ultrafast NO rebinding.[38]

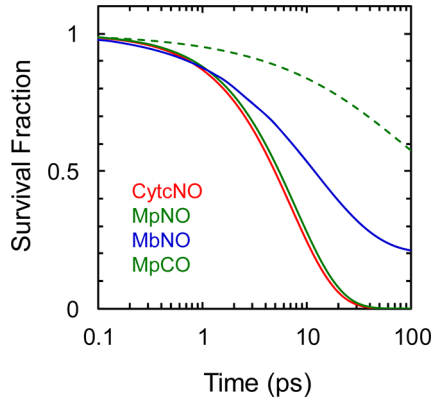


Figure 8. Kinetics of GR of NO to Cytc (red line) and Mb (blue line, data is from ref 17) in D_2O. For comparison, also shown are GR kinetics of NO (green line) and CO (dotted green line, data is from ref 56) to Mp in viscous 81% glycerol/water (v/v) mixture.

Although the deligated NO in both Cytc and Mb can be retained in the vicinity of the Fe atom by the protein matrix surrounding the heme more efficiently than a viscous solution, NO rebinding to Cytc is slightly faster than or equal to that to Mp but that to Mb is slower. CO rebinding to cCytc is faster than that to Mp and much faster than that to Mb, which was attributed to the PDS that holds the deligated CO near the Fe atom in a manner that hinders rebinding of CO.[9,23] Based on the time-resolved vibrational spectroscopy of deligated NO from MbNO, Kim et al. suggested that the deligated NO in Mb was distributed among the PDS and the center of the distal pocket.[19] NO in the PDS of Mb appears to be retained in a manner that impedes NO rebinding. As reported,[23] the electron-transfer protein, Cytc, does not have the PDS-like structure that exists in diatomic ligand-binding proteins such as Mb and Hb. Evidently, the protein matrix in Cytc holds the deligated ligand in a manner that does not obstruct the rebinding but keeps the ligand near heme Fe longer; thus, NO trapped by the protein matrix has a higher chance to rebind, resulting in faster and almost complete GR.

The time constant for the GR of NO to Mp in a viscous solution (8 ps) is almost the same as that for the GR of NO to protoheme in a glycerol solution (7.7 ps in H_2O-FePPIX-NO in an 80% G/W mixture at 290 K).[38] Because there is no distal moiety in these model hemes and the solution viscosity dictating the diffusion of the deligated NO is about the same in both samples, the deligated NO is distributed to about the same distance in both samples at a given delay time after deligation. Thus, the same time constant for these two model hemes with very different proximal ligands suggests that the GR rates of the deligated NO to the model heme is negligibly affected by the identity and structure of the proximal ligand to the heme. Since H_2O is a very weak proximal ligand, it may be displaced by NO binding to the distal heme side of the model heme. Consequently, the Fe displacement in H_2O-FePPIX after

dx.doi.org/10.1021/jp308468j | J. Phys. Chem. B 2012, 116, 13663−13671

photodeligation of NO may be very different from that in Mp. However, the GRs of NO to these molecules are almost the same, suggesting that the GR rate of NO to the heme is dominated by the reactivity between heme Fe and the NO ligand. The ultrafast GR of NO to heme could not be described by the theory of diffusion-controlled reaction in the absence of any interaction between the pair, nor by a distributed linear coupling model which was successful in describing the GR of CO to the model heme.[56,69] A harpoon mechanism was introduced to describe the GR of NO that was decoupled from the Fe distance from the heme plane. The diffusion model was also suggested to be a possible model for NO rebinding to heme if the attractive interaction between deligated NO and heme Fe was introduced.[56] Further theoretical and experimental studies are in progress to test the validity of the diffusion model with the attractive potential between NO and heme Fe.

As mentioned above, CR following photodeligation of CytcNO likely dominates the spectral evolution in the amide band region of the time-resolved spectra, as is the case for MbCO. The GR of the deligated ligand is known to be strongly coupled with the CR of heme proteins.[17−20] Under physiological conditions, the GR of CO to Mb is much slower and inefficient,[4] whereas the GR of NO to Cytc, as shown here, is very efficient, occurring on the picosecond time scale. Under physiological conditions, CO does not geminately rebind to Mb until tens of nanoseconds;[4] thus, there is a continuous CR in Mb over 100 ps, the time span in which the amide band evolves.[61] However, the deligated NO in Cytc geminately rebinds with a time constant of 7 ps. As NO rebinds, the CR that has proceeded for a few picoseconds from CytcNO toward 5-coordinate Cytc halts and the direction of the CR is reversed toward CytcNO. Clearly, the spectral change in the amide band will be influenced by this CR reversal. Since the GR of NO to Cytc that halts and reverses the CR occurs on the picosecond time scale, which is about the same time scale for the spectral evolution of the amide band due to CR, the kinetics for the spectral evolution due to the CR could be faster in CytcNO than in MbCO (which is the case). Evidently, the change in the spectral evolution in the amide band region of Cytc is strongly coupled with the GR of NO to the protein. Since the CR of a protein after photodeligation is coupled to the GR, the time scale of CR after photolysis of CytcNO could be different from that of MbCO.

Although native ferrous Cytc can bind NO, it cannot bind CO without modifying the solution pH to extreme[29] or adding a denaturant such as guanidinium HCl.[28,70,71] Therefore, chemically modified Cytc was used to study the GR dynamics of CO to this electron transfer protein after CO-binding protein under physiological conditions.[23,24] To compare the GR of CO with that of NO, we also measured the kinetics of GR of NO to cCytc. As shown in Figure 7, the GR of NO to cCytc occurs on about the same time scale as that of NO to Cytc but in a nonexponential manner. The nonexponential kinetics was described by either the biexponential function with life times of 2 (52%) and 12 ps (48%) or the stretched exponential function with a characteristic time of 4 ps with β = 0.64. In the biexponential function, the 2-ps component is unprecedently fast for GR of deligated ligand to heme protein. It might arise from thermal relaxation of photoexcited protein without photodeligation. If the 2-ps component is from thermal relaxation, the remaining 12-ps component represents GR of NO to cCytc. Because the electronic structure of heme that governs the fate of the photoexcited heme is unlikely modified by the chemical modification of the side chain of the protein, the quantum yield and reaction coordinate for the photodeligation of cCytcNO should be similar to those of CytcNO. Thus, if thermal relaxation is observed in cCytcNO, it likely presents in the kinetics of CytcNO, too. However, there is no hint of fast decay component in the kinetics of CytcNO. Furthermore, the remaining 12-ps component is too slow for GR time of NO, which ranges from 8 to 6 ps. Therefore, the 2-ps component is not from thermal relaxation but from GR of NO to Cytc. Whereas the biexponential function fits the rebinding kinetics well, the rebinding does not necessarily proceed with two physical barriers. A physically more reasonable view is that the time constants obtained by the biexponential function is mere parameters characterizing nonexponential rebinding kinetics of NO to the inhomogeneous and relaxing protein.[17] However, we cannot exclude the possibility that there is a 2-ps GR process of NO to cCytc. If the 2-ps process is present, it might arise from a particular conformation of cCytc that hampers diffusion of the deligated NO by steric constraints or hydrogen bond even after the Fe−NO bond is broken.

The stretched exponential function can be interpreted as a time-dependent rate constant due to CR of the protein after photodeligation, distributed rate constants due to a distribution of CS of the protein with different rebinding rates or diffusive motion of the deligated NO within the protein, or both. Because CR of cCytc probed by amide band was found to be similar to that of Cytc but its decay time constants may be more heterogeneous in cCytc (see Figure 5), the influence of CR to GR can be either similar in both proteins or different each other. Thus, CR of cCytc can be the main cause or no cause for the nonexponential rebinding of NO in cCtyc. Both CytcNO and cCytcNO have at least three CSs, as can be seen in three vibrational bands. The fact that CytcNO shows exponential kinetics for GR of NO suggests that CS of cCytcNO may not have different rebinding rates. Thus, we speculate that the nonexponential kinetics of NO to cCytc arises mainly from a distribution of the deligated cCytc structure or diffusive motion of the deligated NO in cCytc and/or the inhomogeneous CR of cCytc. Carboxymethylation of Met-80 in cCytc can cause enough perturbation in the distal side of the heme that the deligated NO in cCytc is distributed very differently from that in Cytc and thus it may take different rebinding path causing the nonexponential kinetics and/or cCytc has different CR from Cytc. The large structural modification in the distal side of cCytcNO is manifested by the large spectral shift (19 cm^{-1} red shift) in the NO band.

Although we assumed that the decay kinetics of the fundamental NO band represents the kinetics of GR of NO to the corresponding molecule, the assumption is valid only in the absence of the hot band for the bound NO.[20,72] Photodeligation of a ligated heme protein can generate a photodissociated ligand in the vibrationally excited state.[9,18] If any of the deligated NO molecules are vibrationally excited, some of them rebind while they are vibrationally excited, resulting in the hot band for the bound NO. When the hot band for the bound NO was present, GR kinetics was found to be always faster than the decay kinetics of the fundamental band obtained by fitting the fundamental band whether the hot band was ignored or treated as a background.[72] To extract GR kinetics of NO from the transient vibrational spectra of bound NO, one needs to know the magnitude change of both the

fundamental band and the hot band. However, our data does not warrant independent determination of hot band kinetics. When the data were fit assuming that 13% of the deligated NO is vibrationally excited (the value reported in the photodeligation of MbNO in the similar experimental conditions) and the vibrationally hot band has the same spectral parameters as those in a model heme, 1-methlyimidazole-hemin-NO (a vibrational relaxation time of 3.5 ps and an anharmonicity of 28 cm^{-1}),[72] GR kinetics of NO for CytcNO was well described by an exponential function with a time constant of 6 ps, slightly faster than the decay kinetics of the fundamental band with a time constant of 7 ps. Here, for the sake of simplicity, we assumed that the NO hot band is negligible. Although the decay kinetics of the NO fundamental band can be slower than the GR kinetics, it is likely within the error range of true GR kinetics of NO to Cytc and the main result of this work is negligibly affected by the presence of the hot band.

In conclusion, we measured the kinetics of the GR of NO to Cytc and a model heme, Mp, at room temperature. Most of the deligated NO geminately rebinds to Cytc with a time constant of 7 ± 1 ps, slightly faster than or equal to the 8 ± 1 ps rebinding to Mp. This suggests that NO has high reactivity with heme Fe and the diffusion of the deligated NO is retarded by either the viscous solution or the protein matrix. This also indicates that the Cytc does not have a heme pocket that can hold NO and suppress the GR of NO, such as the PDS in Mb. Slower NO rebinding to Mb suggests that the PDS can also hold the deligated NO in a manner that impedes NO rebinding; however, due to higher NO reactivity with heme Fe, the impediment is not as efficient as for CO.

AUTHOR INFORMATION

Corresponding Author

*E-mail: mhlim@pusan.ac.kr. Tel.: (051) 510-2243.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by Basic Science Research Program through a NRF grant funded by the Ministry of Education, Science, and Technology of Korea (No. 2010-0007469). This work was also supported by MEST and PAL, Korea.

REFERENCES

(1) Austin, R. H.; Beeson, K. W.; Eisenstein, L.; Frauenfelder, H.; Gunsalus, I. C. *Biochemistry* **1975**, *14*, 5355−5373.
(2) Springer, B. A.; Sligar, S. G.; Olson, J. S.; Phillips, G. N., Jr. *Chem. Rev.* **1994**, *94*, 699−714.
(3) Chernoff, D. A.; Hochstrasser, R. M.; Steele, A. W. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 5606−5610.
(4) Henry, E. R.; Sommer, J. H.; Hofrichter, J.; Eaton, W. A. *J. Mol. Biol.* **1983**, *166*, 443−451.
(5) Pin, S.; Valat, P.; Tourbez, H.; Alpert, B. *Chem. Phys. Lett.* **1986**, *128*, 79−83.
(6) Ansari, A.; Berendzen, J.; Braunstein, D.; Cowen, B. R.; Frauenfelder, H.; Hong, M. K.; Iben, I. E.; Johnson, J. B.; Ormos, P.; Sauke, T. B.; et al. *Biophys. Chem.* **1987**, *26*, 337−355.
(7) Petrich, J. W.; Poyart, C.; Martin, J. L. *Biochemistry* **1988**, *27*, 4049−4060.
(8) Findsen, E. W.; Ondrias, M. R. *Photochem. Photobiol.* **1990**, *51*, 741−748.
(9) Lim, M.; Jackson, T. A.; Anfinrud, P. A. *Nat. Struct. Biol.* **1997**, *4*, 209−214.
(10) Schotte, F.; Lim, M.; Jackson, T. A.; Smirnov, A. V.; Soman, J.; Olson, J. S.; Phillips, G. N., Jr.; Wulff, M.; Anfinrud, P. A. *Science* **2003**, *300*, 1944−1947.
(11) Jongeward, K. A.; Magde, D.; Taube, D. J.; Marsters, J. C.; Traylor, T. G.; Sharma, V. S. *J. Am. Chem. Soc.* **1988**, *110*, 380−387.
(12) Petrich, J. W.; Lambry, J. C.; Kuczera, K.; Karplus, M.; Poyart, C.; Martin, J. L. *Biochemistry* **1991**, *30*, 3975−3987.
(13) Gibson, Q. H.; Regan, R.; Elber, R.; Olson, J. S.; Carver, T. E. *J. Biol. Chem.* **1992**, *267*, 22022−22034.
(14) Walda, K. N.; Liu, X. Y.; Sharma, V. S.; Magde, D. *Biochemistry* **1994**, *33*, 2198−2209.
(15) Petrich, J. W.; Lambry, J. C.; Balasubramanian, S.; Lambright, D. G.; Boxer, S. G.; Martin, J. L. *J. Mol. Biol.* **1994**, *238*, 437−444.
(16) Shreve, A. P.; Franzen, S.; Simpson, M. C.; Dyer, R. B. *J. Phys. Chem. B* **1999**, *103*, 7969−7975.
(17) Kim, S.; Jin, G.; Lim, M. *J. Phys. Chem. B* **2004**, *108*, 20366−20375.
(18) Kim, S.; Lim, M. *J. Am. Chem. Soc.* **2005**, *127*, 8908−8909.
(19) Kim, S.; Lim, M. *J. Phys. Chem. B* **2012**, *116*, 5819−5830.
(20) Kim, S.; Park, J.; Lee, T.; Lim, M. *J. Phys. Chem. B* **2012**, *116*, 6346−6355.
(21) Ye, X.; Yu, A.; Champion, P. M. *J. Am. Chem. Soc.* **2006**, *128*, 1444−1445.
(22) Lee, T.; Park, J.; Kim, J.; Joo, S.; Lim, M. *Bull. Korean Chem. Soc.* **2009**, *30*, 177−182.
(23) Kim, J.; Park, J.; Lee, T.; Lim, M. *J. Phys. Chem. B* **2009**, *113*, 260−266.
(24) Silkstone, G.; Jasaitis, A.; Vos, M. H.; Wilson, M. T. *Dalton Trans.* **2005**, 3489−3494.
(25) Louie, G. V.; Brayer, G. D. *J. Mol. Biol.* **1990**, *214*, 527−555.
(26) Flynn, P. F.; Urbauer, R. J. B.; Zhang, H.; Lee, A. L.; Wand, A. J. *Biochemistry* **2001**, *40*, 6559−6569.
(27) Jones, C. M.; Henry, E. R.; Hu, Y.; Chan, C. K.; Luck, S. D.; Bhuyan, A.; Roder, H.; Hofrichter, J.; Eaton, W. A. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 11860−11864.
(28) Eaton, W. A.; Thompson, P. A.; Chan, C.-K.; Hagen, S. J.; Hofrichter, J. *Structure* **1996**, *4*, 1133−1139.
(29) Choi, J.; Jung, Y. O.; Lee, J. H.; Yang, C.; Kim, B.; Ihee, H. *ChemPhysChem* **2008**, *9*, 2708−2714.
(30) Schejter, A.; George, P. *Nature* **1965**, *206*, 1150−1151.
(31) Moller, J. K. S.; Skibsted, L. H. *Chem. Rev.* **2002**, *102*, 1167−1178.
(32) Traylor, T. G.; Sharma, V. S. *Biochemistry* **1992**, *31*, 2847−2849.
(33) Hoshino, M.; Ozawa, K.; Seki, H.; Ford, P. C. *J. Am. Chem. Soc.* **1993**, *115*, 9568−9575.
(34) Hoshino, M.; Maeda, M.; Konishi, R.; Seki, H.; Ford, P. C. *J. Am. Chem. Soc.* **1996**, *118*, 5702−5707.
(35) Ascenzi, P.; Coletta, M.; Santucci, R.; Polizio, F.; Desideri, A. *J. Inorg. Biochem.* **1994**, *53*, 273−280.
(36) Kruglik, S. G.; Yoo, B.-K.; Franzen, S.; Vos, M. H.; Martin, J.-L.; Negrerie, M. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 13678−13683.
(37) Vos, M. H. *Biochim. Biophys. Acta, Bioenerg.* **2008**, *1777*, 15−31.
(38) Ionascu, D.; Gruia, F.; Ye, X.; Yu, A.; Rosca, F.; Beck, C.; Demidov, A.; Olson, J. S.; Champion, P. M. *J. Am. Chem. Soc.* **2005**, *127*, 16921−16934.
(39) Yoo, B.-K.; Kruglik, S. G.; Lamarre, I.; Martin, J.-L.; Negrerie, M. *J. Phys. Chem. B* **2012**, *116*, 4106−4114.
(40) Kuczera, K.; Lambry, J. C.; Martin, J. L.; Karplus, M. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 5805−5807.
(41) Lim, M.; Jackson, T. A.; Anfinrud, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 5801−5804.
(42) Silkstone, G.; Jasaitis, A.; Wilson, M. T.; Vos, M. H. *J. Biol. Chem.* **2007**, *282*, 1638−1649.
(43) Kholodenko, Y.; Gooding, E. A.; Dou, Y.; Ikeda-Saito, M.; Hochstrasser, R. M. *Biochemistry* **1999**, *38*, 5918−5924.
(44) Ye, X.; Demidov, A.; Champion, P. M. *J. Am. Chem. Soc.* **2002**, *124*, 5914−5924.
(45) Teng, T. Y.; Srajer, V.; Moffat, K. *Nat. Struct. Biol.* **1994**, *1*, 701−705.

(46) Schlichting, L.; Berendzen, J.; Phillips, G. N., Jr.; Sweet, R. M. *Nature* **1994**, *371*, 808−812.
(47) Srajer, V.; Ren, Z.; Teng, T.-Y.; Schmidt, M.; Ursby, T.; Bourgeois, D.; Pradervand, C.; Schildkamp, W.; Wulff, M.; Moffat, K. *Biochemistry* **2001**, *40*, 13802−13815.
(48) *Swiss-Prot Protein Database*; National Center of Biotechnology Information: Bethesda, MD, 1994.
(49) Aron, J.; Baldwin, D. A.; Marques, H. M.; Pratt, J. M.; Adams, P. A. *J. Inorg. Biochem.* **1986**, *27*, 227−243.
(50) Cao, W.; Ye, X.; Georgiev, G. Y.; Berezhna, S.; Sjodin, T.; Demidov, A. A.; Wang, W.; Sage, J. T.; Champion, P. M. *Biochemistry* **2004**, *43*, 7017−7027.
(51) Lim, M.; Wolford, M. F.; Hamm, P.; Hochstrasser, R. M. *Chem. Phys. Lett.* **1998**, *290*, 355−362.
(52) Hamm, P.; Kaindl, R. A.; Stenger, J. *Opt. Lett.* **2000**, *25*, 1798−1800.
(53) Hamm, P.; Lim, M.; Hochstrasser, R. M. *J. Phys. Chem. B* **1998**, *102*, 6123−6138.
(54) Yoshimura, T.; Suzuki, S. *Inorg. Chim. Acta* **1988**, *152*, 241−249.
(55) Urry, D. W.; Pettegrew, J. W. *J. Am. Chem. Soc.* **1967**, *89*, 5276−5283.
(56) Park, J.; Lee, T.; Lim, M. *J. Phys. Chem. B* **2010**, *114*, 10897−10904.
(57) Ricoux, R.; Boucher, J.-L.; Mandon, D.; Frapart, Y.-M.; Henry, Y.; Mansuy, D.; Mahy, J.-P. *Eur. J. Biochem.* **2003**, *270*, 47−55.
(58) Venyaminov, S. Y.; Prendergast, F. G. *Anal. Biochem.* **1997**, *248*, 234−245.
(59) Thomas, M. R.; Brown, D.; Franzen, S.; Boxer, S. G. *Biochemistry* **2001**, *40*, 15047−15056.
(60) Causgrove, T. P.; Dyer, R. B. *J. Phys. Chem.* **1996**, *100*, 3273−3277.
(61) Kim, S.; Jin, G.; Lim, M. *Bull. Korean Chem. Soc.* **2003**, *24*, 1470−1474.
(62) Hu, S. *Inorg. Chem.* **1993**, *32*, 1081−1815.
(63) Balasubramanian, S.; Lambright, D. G.; Boxer, S. G. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 4718−4722.
(64) Muller, J. D.; McMahon, B. H.; Chien, E. Y. T.; Sligar, S. G.; Nienhaus, G. U. *Biophys. J.* **1999**, *77*, 1036−1051.
(65) Miller, L. M.; Pedraza, A. J.; Chance, M. R. *Biochemistry* **1997**, *36*, 12199−12207.
(66) Nienhaus, K.; Palladino, P.; Nienhaus, G. U. *Biochemistry* **2008**, *47*, 935−948.
(67) Causgrove, T. P.; Dyer, R. B. *Biochemistry* **1993**, *32*, 11985−11991.
(68) Szabo, A. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 2108−2111.
(69) Ye, X.; Lonascu, D.; Gruia, F.; Yu, A.; Benabbas, A.; Champion, P. M. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 14682−14687.
(70) Choi, J.; Yang, C.; Kim, J.; Ihee, H. *J. Phys. Chem. B* **2011**, *115*, 3127−3135.
(71) Kim, S.; Chung, J. K.; Kwak, K.; Bowman, S. E. J.; Bren, K. L.; Bagchi, B.; Fayer, M. D. *J. Phys. Chem. B* **2008**, *112*, 10054−10063.
(72) Park, J.; Lee, T.; Lim, M. *Chem. Phys.* **2012**, http://dx.doi.org/10.1016/j.chemphys.2012.09.030.