Rebinding Dynamics of CO Following Photodissociation of 4.0 M Guanidine HCl-Denatured Carbonmonoxyhemoglobin

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Femtosecond vibrational spectroscopy was used to probe the dynamics of CO rebinding to hemoglobin (Hb), denatured by 4.0 M GdnHCl in D_2O at 283 K, after photolysis of HbCO. The stretching mode of ^{13}CO bound to the denatured $Hb^{13}CO$ showed a single band centered at 1922 cm^{-1}, indistinguishable from that of denatured $Mb^{13}CO$. Geminate rebinding of CO to the denatured Hb was accelerated more than 1000 times, suggesting that the native structure of the Hb is required to suppress efficient geminate rebinding of CO, as is the case in Mb. The geminate yield and rate for CO rebinding are almost the same in both the denatured Hb and Mb. Similarity in the equilibrium spectrum and rebinding dynamics of CO indicates that the state of the denatured Hb is very similar to that of the denatured Mb. In the denatured Hb, quaternary contact of the protein is likely severed, with the denatured protein existing as an independent subunit much like Mb.

Key Words: CO rebinding, Hemoglobin, Guanidine HCl, Femtosecond vibrational spectroscopy, Denaturation

Introduction

One of the fundamental problems in biophysics is understanding the relationships between protein structure, function, and dynamics at a molecular level of detail. Heme proteins such as myoglobin (Mb) and hemoglobin (Hb) have long served as model systems for studying these relations.[1] Mb, an oxygen storage protein, is a monomeric protein that contains a single heme group as the active ligand-binding site of the protein. Hb, an oxygen transport protein, is a tetrameric protein, each unit of which contains a heme group, with the global tertiary structure of each unit similar to that of Mb. Hb exhibits cooperative binding of ligands essential to efficient transport of O_2.[2] The cooperativity of Hb is accompanied by a substantial change in its quaternary structure. Whereas the peptide sequence of Mb is different from that of Hb, amino acid residues near the heme in both proteins are highly conserved. The residues surrounding the bound ligand are invariant in both proteins, except for isoleucine 107 in Mb that is replaced with leucine 107 in Hb. The rebinding of CO to Mb and Hb after photolysis are most often used to study the relationships between protein structure, function, and dynamics. Though the rebinding dynamics of the ligands to both proteins are similar in general, they are different in detail. Whereas cooperative binding of the ligand to Hb makes its behavior difficult to compare directly to the binding of Mb, the difference is often attributed to arise from the quaternary structure of Hb.

The dynamics and structural characterization of denatured proteins can be useful in understanding protein folding as well as various biological phenomena such as regulation of the cellular activity and onset of neurodegenerative disease.[3] Although function-related dynamics of native proteins have been extensively characterized, those of denatured proteins have rarely been investigated.[4,5] According to transient grating spectroscopy, circular dichroism (CD), and IR spectroscopy, MbCO is completely denatured by 4.0 M GdnHCl, with the heme coordinated to the denatured Mb.[5-8] The rebinding of CO to the GdnHCl-denatured Mb is more than 1000 times faster and efficient than that of native Mb.[5] Time-resolved vibrational spectra of photodissociated CO from the denatured Mb showed the ultrafast appearance of the CO interior of the protein matrix, without exhibiting any solvated CO within 1 ns.[5] From these observations, the distal side of the heme of the denatured Mb was suggested to be buried within the denatured protein matrix.[5]

Since the vibrational spectrum of a molecule is very sensitive to its environment, a vibrational spectrum of the heme-bound CO is an excellent probe for the surroundings of the ligand.[9-12] In the case of Mb, the position and width of the CO band was found to change dramatically with mutation of even a single amino acid residue near the distal site.[13] Different band positions of the CO in the MbCO have been attributed to different conformers of the protein.[9-12] When denatured, the global native structure of Hb, including its quaternary structure, would be destroyed. The vibrational spectrum of CO in the denatured HbCO can be used to probe the influence of the denatured quaternary structure on the environments of the CO and its rebinding dynamics in the denatured protein.

Herein, as an extension to our previous experiments on denatured Mb, we denatured Hb with 4 M GdnHCl and investigated its rebinding dynamics to CO using femtosecond vibrational spectroscopy. The vibrational spectrum of the CO bound to the denatured Hb and the rebinding dynamics of CO to the protein were compared with those of the denatured Mb as well as native Hb.

Experimental Section

$Hb^{13}CO$ was prepared by dissolving lyophilized Hb (Sigma) in deoxygenated D_2O (Aldrich) buffered with 0.1 M

potassium phosphate (pD 7.4). The solution was equilibrated with 1 atm of ^{13}CO (Aldrich) and reduced with an excess of freshly prepared sodium dithionate (Aldrich). The reduced Mb was stirred under 1 atm of ^{13}CO for more than 20 min to yield native Hb^{13}CO. To remove the light scattering dust particles and protein aggregates, the Hb^{13}CO solution was passed through a 5-μm membrane filter under N_2 gas. An 8.0 M GdnHCl solution was prepared in the same buffer. The appropriate amount of the GdnHCl solution was added to the native Hb^{13}CO with stirring to obtain denatured 2.0 mM Hb^{13}CO in 4.0 M GdnHCl. The denatured Hb^{13}CO was loaded in a gas-tight rotating sample cell bearing CaF_2 windows with a 50-μm path length. The temperature of the sample cell was kept at 283 ± 1 K. The integrity and concentration of the sample was checked by UV-Vis and FT-IR spectroscopy throughout the experiment. The sample was prepared in D_2O using ^{13}CO to avoid strong water bands by isotopically shifting the spectrum of interest into a region with greater IR transmission.

The femtosecond mid-IR spectrometer has been previously described.[14] Briefly, two home-built optical parametric amplifiers (OPA), pumped by a Ti:sapphire amplifier at a repetition rate of 1 kHz, generate a visible pump pulse and a mid-IR probe pulse. Frequency doubling of the signal pulse of one OPA produces a 575 nm pump pulse[15] while difference frequency mixing of the signal and idler pulse of the other OPA generates a mid-IR probe pulse centered at 1910 cm^{-1} with a 150 cm^{-1} full width at half maximum (FWHM).[16,17] To avoid multiphoton excitation and continuum generation at the sample cell, the pump energy was adjusted to approximately 3.0 μJ at the sample using a reflective neutral density filter. 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. A 64-element $N_2(l)$-cooled HgCdTe array detected the broadband transmitted and then dispersed the probe pulse by a 320 mm monochromator with a 150 *l*/mm grating (spectral resolution of *ca.* 0.9 cm^{-1}/pixel at 1910 cm^{-1}). The pump-induced change in the absorbance of the sample (ΔA) was determined by chopping the pump pulse to half the repetition frequency of the laser and calculating the difference between the pumped (A_p) and unpumped (A_u) absorbance ($\Delta A = A_p - A_u$). The excellent short-term stability of the IR light source resulted in 0.1 mOD rms after 0.5 s of signal averaging. The instrument response function was typically 150 fs FWHM.

Results and Discussion

As shown in Fig 1, the stretching mode of the ^{13}CO of native Hb^{13}CO in D_2O exhibited two well-resolved bands centered at 1906 and 1924 cm^{-1}, assigned A_1 and A_0, respectively.[18,19] It is believed that these bands are mainly due to interactions of the distal histidine with CO. Whereas the histidine directly interacts with the CO in a protein conformation resulting in the A_1 band,[20-22] it is protonated and swings out of the heme pocket in a conformation for the A_0 band.[23-25] Hb^{13}CO in 4.0 M GdnHCl revealed a single CO band centered at 1922 cm^{-1}. The new band is similar to the A_0 band, but not identical since the histidine cannot be protonated in a 4.0 M GdnHCl solution.[26] Therefore, it is assigned to an A_0-like band. Denaturation of the protein by GdnHCl can disturb the interaction between the distal histidine and CO, with elimination of the interaction resulting in an A_0-like band. The integrated area of the new band is almost identical to the total integrated area of the CO band in native HbCO, suggesting that the oscillator strength of the CO band is not affected by the change in protein conformation. MbCO is completely denatured by 4.0 M GdnHCl and, when denatured, shows an A_0-like band almost identical to the band in HbCO (see Fig. 1), suggesting that HbCO is also completely denatured by 4.0 M GdnHCl and that the environments of CO in the denatured state are very similar to those of MbCO. Differences in the CO band of native HbCO from that of native MbCO suggest that the characteristics of the band results from the native structure of the protein, and of the denatured HbCO, at least surrounding the bound CO, must be similar to that of the denatured MbCO.

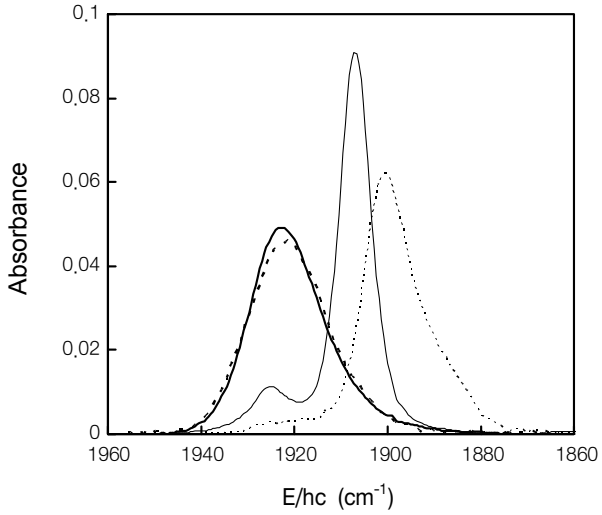

Figure 1. Equilibrium vibrational spectra of ^{13}CO bound to Hb^{13}CO (the solid lines) and Mb^{13}CO (the dotted lines) in native form (the thin lines) and denatured by 4.0 M GdnHCl (the thick lines).

Fig. 2 shows the time-resolved vibrational spectra of bound ^{13}CO after 575-nm photolysis of Hb^{13}CO denatured by 4.0 M GdnHCl in D_2O at 283 K. Since interest lies in neither coherent signals nor the truncated free induction decay of bound CO occurring when pump-probe pulses are overlapped,[27,28] but rather the incoherent population dynamics, data were collected for pump-probe delay times longer than 0.3 ps. The negative features (denoted bleach) arise from the loss of Hb^{13}CO, the magnitude of which representing the population of the deligated Hb resulting from the photodissociation of ^{13}CO from the denatured Hb^{13}CO. The bleach appears faster than the instrument response time, 150 fs, suggesting that photodissociation of CO from the denatured HbCO is ultrafast. According to ultrafast optical spectroscopy of native heme proteins and model systems, the photodissociation of CO from heme occurs in < 50 fs.[29] The ultrafast bleach of the vibrational band for the bound ligand has also been observed in CO-bound as well as NO-bound

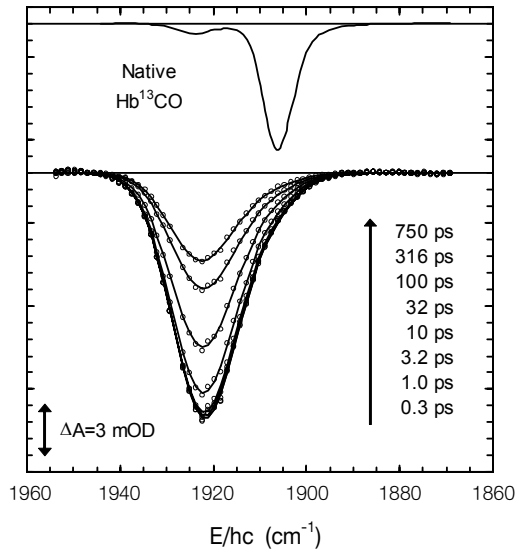

Figure 2. Representative time-resolved vibrational spectra of ^{13}CO bound to GdnHCl-denatured Hb^{13}CO in D_2O after photolysis. Data (symbols) were fitted to a sum of three Gaussians, plus a quadratic polynomial function (the solid lines). Quadratic polynomial models change in weak solvent absorption due to temperature changes induced by thermal relaxation of the excited heme. The quadratic background was subtracted for clarity. The spectrum of native Hb^{13}CO at 0.3 ps is shown for comparison.

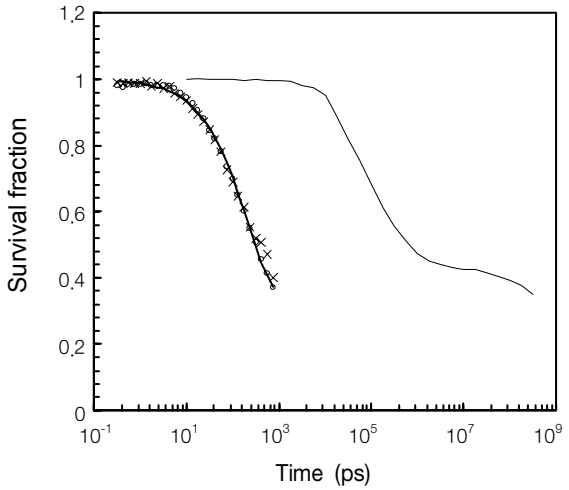

Figure 3. Change in the survival fraction of the unbound heme in the denatured Hb^{13}CO (○) and Mb^{13}CO (×) in D_2O at 283 K. The data (symbols) were fitted to the 3-state kinetic model (the thick solid lines) described in the text. The thin solid line is the native Hb^{13}CO data from the reference 33.

heme proteins.[14,30,31] The shape of the bleach spectrum is identical to the equilibrium spectrum of bound CO. Whereas a sum of two Gaussians resulted in a good fit for individual bleach, a sum of three Gaussians was required to fit the whole spectra globally. The amplitude of the Gaussians in the global fit is proportional to the population of deligated Hb. The sum of the Gaussians for the CO band in the denatured HbCO likely reflects the fact that CO is exposed to inhomogeneous environments in an ensemble of various denatured random structures of the protein. The bleach does not evolve with time, suggesting that various surroundings of the ligand do not alter the rebinding kinetics of CO to the denatured Hb. At early times after photolysis, a portion of the dissociated CO can rebind to the parent molecule (geminate rebinding, GR), causing the magnitude of the bleach to decrease. Diffused CO without GR will eventually rebinds to the deligated Hb in a bimolecular fashion under a much slower time scale than the current experimental time window.[32] The efficiency and the rate of the GR were obtained by characterizing the normalized bleach signal as a function of time.

Fig. 3 shows the change in the survival fraction of the deligated Hb denatured by 4.0 M GdnHCl in D_2O at 283 K after photolysis of CO. The rebinding of CO to the denatured Hb is about 1000 times faster than that to native Hb and is nearly identical to the denatured Mb. The CO rebinding kinetics of the denatured Hb was fitted to a equation describing a 3-state kinetic model,[32] resulting in: (0.68 ± 0.07) exp $(-(t/(310 \pm 80\ \text{ps}))^{(0.75 \pm 0.05)} + (0.32 \pm 0.07)$. The recovered parameters are very similar to those obtained in the denatured Mb.[5] At 284.3 K, about 60% of dissociated CO geminately recombines to native Hb in ~ 200 ns.[33] The time constant of CO GR for native Hb is quite similar to that of native Mb; however, the yield of CO GR for native Hb is quite higher than that for native Mb.[32,33] The difference in the geminate yield has been attributed to the quaternary structure in Hb. The similarity in the dynamics of CO rebinding and the equilibrium spectrum of CO suggests that the denatured HbCO is structurally indistinguishable from that of Mb. The quaternary structure of Hb is likely destroyed in the denatured protein and thus, each denatured subunit may liken the denatured Mb.

In conclusion, we have found that, when denatured by 4.0 M GdnHCl, the vibrational spectra of ^{13}CO in Hb^{13}CO and Mb^{13}CO are indistinguishable. The spectrum showed a single band centered at 1922 cm^{-1} with 19 cm^{-1} FWHM, similar to, but different in detail from, the A_0 band in native Hb. It was well-described by a sum of Gaussians, indicating that the environments of the CO are inhomogeneous as expected in the denatured protein. We also found that the GR of CO to the denatured Hb is approximately 1000 times faster than that to native Hb and almost identical to the denatured Mb. Clearly, the native structure is required to suppress CO GR in Hb and Mb. Similarity of the equilibrium spectrum and the rebinding dynamics of CO in both denatured proteins suggests that the state of the denatured Hb is very similar to that of the denatured Mb. It is likely that, in the denatured Hb, its quaternary contact is broken and the protein exists as an independent subunit.

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