Probing the Role of Hydration in the Unfolding Transitions of Carbonmonoxy Myoglobin and Apomyoglobin

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We show that the equilibrium unfolding transition of horse carbonmonoxy myoglobin monitored by the stretching vibration of the CO ligand, a local environmental probe, is very sharp and, thus, quite different from those measured by global conformational reporters. In addition, the denatured protein exhibits an A_0-like CO band. We hypothesize that this sharp transition reports penetration of water into the heme pocket of the protein. Parallel experiments on horse apomyoglobin, wherein an environment-sensitive fluorescent probe, nile red, was used, also reveals a similar putative hydration event. Given the importance of dehydration in protein folding and also the recent debate over the interpretation of probe-dependent unfolding transitions, these results have strong implications on the mechanism of protein folding.

Introduction

Folding of globular proteins involves the burial of hydrophobic side chains. Thus, a series of dehydration events are expected to occur during the course of folding, especially in the formation of the hydrophobic core. For example, the search toward the native fold of the SH3 domain has been shown to involve an obligatory desolvation/water exclusion step occurring late in the respective folding process.[1–3] Similarly, a dehydration step following the formation of the transition state was observed in the folding simulations of protein A.[4] Despite the apparent importance of hydration/dehydration events in protein folding/unfolding,[5–7] it has been difficult to tease out such processes from thermally or chaotropically induced protein denaturation transitions measured by the commonly used spectroscopic methods, even though the resulting signal of the spectroscopic probe thus employed depends on the degree of hydration. Here, we take advantage of the sensitivity of the CO stretching vibration to its immediate environment and use it as an infrared (IR) marker to probe the role of water in the unfolding transition of carbonmonoxy myoglobin (MbCO).

As a model system for understanding the structure–function relationship, the photophysics of MbCO has been studied extensively.[8–19] This is because the CO ligand, which is photodissociable, constitutes a remarkable IR probe of the conformational substates of MbCO.[11,13,20–22] For example, the heme-bound CO exhibits four stretching bands in the spectral range of 1930–1970 cm^{-1}, which have been shown to depend on many factors, including temperature,[11,21] pH,[23,24] solvent,[25,26] and mutations of amino acids surrounding the distal side[27–31] of the heme pocket. In addition, the folding thermodynamics and kinetics of MbCO have also been studied using various spectroscopic methods.[32] Our results show that the unfolding transition of MbCO reported by the CO stretching vibration is

much sharper than that reported by circular dichroism (CD) as well as transient grating (TG),[33] suggesting that this IR probe reports a local unfolding event that to the best of our knowledge has not been reported in the literature, which most likely corresponds to hydration of the heme pocket of MbCO.

To substantiate the role of hydration, we have further studied the unfolding transition of apomyglobin (apoMb) by monitoring the fluorescence of a hydrophobic dye, nile red (NR), bound to the protein. It has been shown that NR binds to the heme cavity of apoMb with a 1:1 stoichiometry and a moderate affinity (i.e., $K_d = 25~\mu$M) and that the quantum yield of protein-bound NR is significantly increased compared to that of free NR in aqueous solution.[34] Thus, akin to the stretching vibration of the CO ligand in MbCO, the fluorescence emission of NR provides an excellent measure of the degree of hydration of the heme binding pocket of apoMb.

Experimental Method

Materials. Guanidine hydrochloride (GdnHCl), nile red, horse heart Mb, horse skeleton Mb, and sodium dithionite were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. Horse heart apoMb was prepared according to published protocols.[35] First, the heme was removed by acid–acetone extraction at −20 °C. The resultant colorless precipitate was then resuspended in cold distilled water and dialyzed against 0.1% sodium bicarbonate at 4 °C, followed by extensive dialysis against distilled water. The suspension was then centrifuged to remove precipitates, and the supernatant was lyophilized and stored at −20 °C for further use. 99.5% of the apoMb sample thus prepared was found to be heme free, as estimated by the absorbance of the apoMb solution at 408 nm using a molar extinction coefficient (ε) of 179 000 cm^{-1} M^{-1}.

CD Measurement. The CD spectra were collected on either a JASCO-715 CD spectrometer (for MbCO) or an AVIV 62DS spectropolarimeter (for apoMb). Mean residue ellipticity was calculated using the equation $[\theta] = (\theta_{obs}/10lc)/N$, where θ_{obs} is the measured ellipticity in millidegree, l is the optical path length in cm, N is the number of residues, and c is the molar

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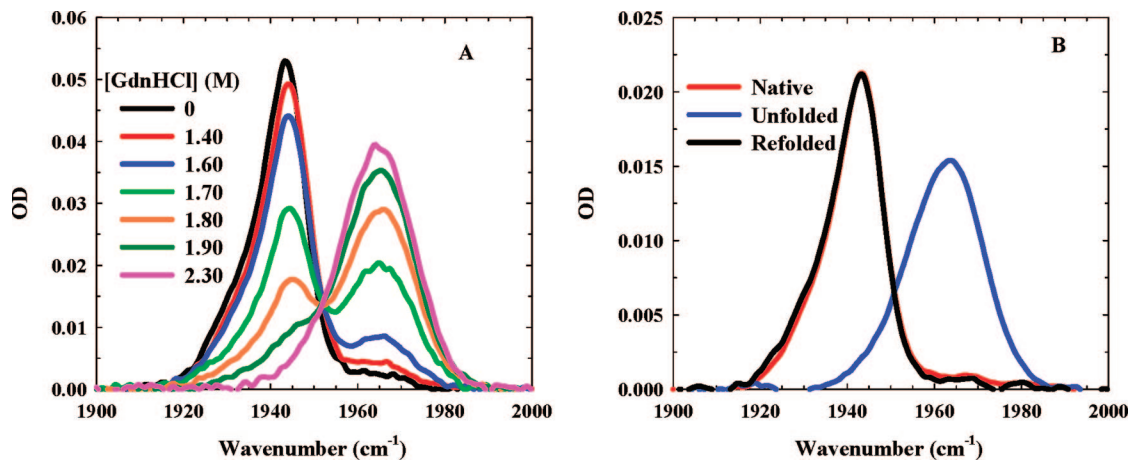
Figure 1. (A) Representative FTIR spectra of horse skeleton MbCO in the CO stretching vibration region measured at different GdnHCl concentrations, as indicated. (B) The CO stretching bands of the native (black) and a refolded MbCO sample (red) after denaturation using 3 M GdnHCl. For better comparison, the refolded curve (red) has been normalized to that of the native state spectrum (black).

concentration of the protein. For MbCO, the CD data were collected at 18 °C using a 20 μm gastight cell with CaF$_2$ windows, and the sample is identical to that used in the IR experiments. For apoMb, the CD data were collected at 25 °C using a 1 mm quartz cell and the protein concentration was 25 μM (in 10 mM PBS buffer−10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7). The apoMb concentration was determined optically using the absorbance at 280 nm and a molar extinction coefficient of 15 800 cm^{-1} M^{-1}, whereas the GdnHCl concentration was determined using the solution refractive index measured on an Abbe refractometer (Bausch & Lomb).

Fluorescence Measurement. The GdnHCl-induced unfolding transition, as well as dissociation of the apoMb−NR complex at 25 °C, was assessed via NR fluorescence. All fluorescence spectra were measured on a Fluorolog 3 spectrofluorometer (Jobin Yvon-Spex, Edison, NJ) using an excitation wavelength of 520 nm and a spectral resolution of 1 nm. For unfolding measurements, all samples were prepared from a stock solution containing 132 μM apoMb and 6 μM NR (in 10 mM PBS buffer, pH 7), which had been equilibrated at 4 °C for at least 12 h before use. Before each measurement, this apoMb−NR stock solution was further diluted with the addition of an appropriate volume of a GdnHCl solution (0.6−6.2 M) to achieve the desired GdnHCl concentration. The final apoMb and NR concentrations were 25 and 1 μM, respectively. For the dissociation constant measurements, the samples were prepared by mixing an apoMb−NR stock solution, similar to that used in the unfolding measurements, with a NR stock solution (with or without GdnHCl), in a predetermined ratio.

FTIR Measurement. The GdnHCl-induced unfolding transition of MbCO was probed by monitoring the CO stretching vibration on an Equinox 55 FTIR spectrometer (Bruker). All experiments were carried out in deoxygenated 0.1 M potassium phosphate buffer in D$_2$O (pD 7.4) at room temperature (18 °C). A 2.4 mM MbCO stock solution was prepared by dissolving lyophilized horse skeleton Mb in buffer, in the presence of a reducing atmosphere achieved by dissolving excess freshly prepared sodium dithionite and subsequently stirring under 1 atm of CO for about 10 min. To remove light-scattering dust particles and protein aggregates, the sample was further passed through a 0.45-μm membrane filter under nitrogen environment. An 8 M GdnHCl stock solution was also prepared in the same buffer. In each unfolding experiment, appropriate volumes of these stock solutions and buffer were mixed to achieve the desired GdnHCl concentration and a final MbCO concentration

of 1.2 mM. The resultant solution was then stirred for a few seconds and then allowed to stand for 4−6 h to ensure complete equilibration.[36] The equilibrated sample was then loaded into a 175 μm gastight sample cell with CaF$_2$ windows for further FTIR measurements. Control experiments showed that the FTIR spectra reached equilibrium in about 1.5 h after addition of GdnHCl. In all cases, the magnitude of the CO band did not change over a period of several hours, indicating that negligible oxidation of the protein has occurred during the experiment.

For reversibility measurements, denatured MbCO samples (1.1 mM) at [GdnHCl] = 3 M were first diluted 50-fold by adding CO-saturated buffer and were then dialyzed to recover the initial MbCO concentration and also to reach a GdnHCl concentration of less than 0.06 M. The FTIR spectrum of the refolded protein sample was then collected using a 98 μm gastight sample-cell.

UV−Vis Measurement. The Soret band of MbCO under various denaturation conditions was measured on a UV-3101PC spectrometer (Shimadzu) using a gastight sample cell with CaF$_2$ windows. The protein samples are identical to those used for CD and IR experiments.

Fluorescence Correlation Spectroscopy (FCS). The confocal setup used for FCS measurements has been described previously in detail.[37] In the current study, PEG−silane (Gelest Inc., Morrisville, PA) modified glass slips were used and the concentrations of apoMb and NR were 25 and 100 nM, respectively. Excitation of NR was accomplished by the 514 nm line of an Ar$^+$ ion laser ($\sim$100 μW before entering the microscope), and the resultant fluorescence was equally split by a polarizing beamsplitter (Newport, CA) and detected by two avalanche photodiodes (Perkin-Elmer, NJ) using an integration time of 1 μs. Correlation of the fluorescence signals in cross-correlation mode was accomplished by a Flex 03-LQ-01 correlator card (Correlator.com) for a duration of 600 s.

Results and Discussion

Carbonmonoxy Myoglobin. As shown (Figure 1A), the FTIR spectrum of horse skeleton MbCO in aqueous solution exhibits two well-resolved bands centered at about 1965 and 1944 cm^{-1}, respectively, corresponding to the well-known A$_0$ and A$_1$ states of carbonmonoxy myoglobins.[30,31,38−43] In the past, many studies have been devoted to establish the origin of these vibrational transitions, and the most prevailing belief is that these states are primarily determined by interactions of the distal

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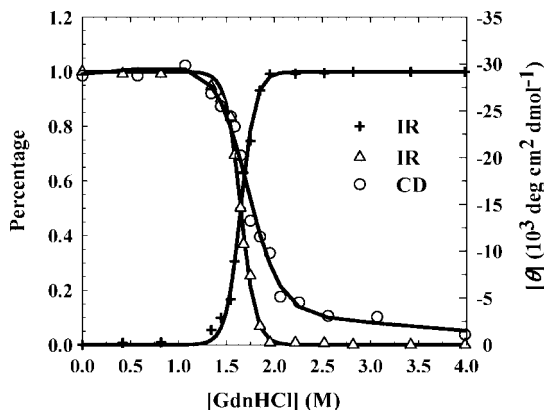
Figure 2. Relative fractions of $S_N(\nu)$ (+) and $S_U(\nu)$ (Δ) as a function of [GdnHCl]. Also shown is the CD unfolding transition ($\bigcirc$) of MbCO. The smooth lines are meant to guide the eyes.

histidine residue (H64) with CO. For example, the A_1 band, which shows a dominant intensity over the other bands under native conditions, has been attributed to a conformational ensemble wherein H64 interacts relatively strongly with the CO via either H-bonding and/or through associated electrostatics.[31,38−40] On the other hand, the A_0 state has been typically assigned to a protein conformation wherein the imidazole ring of H64 is not only protonated but also swings out of the heme pocket.[41−43] Interestingly, our data show that upon addition of GdnHCl these vibrational bands do not show any appreciable changes in their respective frequencies. Instead, the integrated area of the 1965 cm^{-1} band increases at the expense of the 1944 cm^{-1} band with increasing GdnHCl concentration ([GdnHCl]) and becomes dominant at [GdnHCl] > 1.65 M. This result is rather intriguing. While a similar result has also been observed in a pH-induced unfolding study of MbCO, wherein the increase in A_0 intensity with decreasing pH was attributed to protonation of H64,[23,24] it is unlikely that the same argument applies in the current case, since the pK_a of histidine has been shown to upshift by only 0.3−0.5 pH units even under very high concentrations (e.g., 6 M) of GdnHCl.[44] Furthermore, and perhaps more importantly, the GdnHCl-induced denaturation of MbCO under the current experimental conditions was found to be reversible. As shown (Figure 1B), the CO stretching band of the refolded MbCO (after denaturation) overlaps with that of the native protein, indicating that the aforementioned unfolding transition is also unlikely due to protein and/or heme aggregations (see below).

Moreover, we found that the IR spectrum measured at each [GdnHCl], $S(\nu,[GdnHCl])$, can be expressed as a linear superposition of the spectra measured at 0 and 2.9 M GdnHCl solutions, i.e.,

$$S(\nu, [GdnHCl]) = f_{[GdnHCl]}S_N(\nu) + (1 - f_{[GdnHCl]})S_U(\nu) \quad (1)$$

wherein $S_N(\nu)$ represents the CO spectrum of MbCO in buffer, $S_U(\nu)$ represents that in 2.9 M GdnHCl solution, and $f_{[GdnHCl]}$ is the percentage of $S_N(\nu)$, which ranges from 0 to 1 and was allowed to vary in the fitting. In addition, in the fitting, a slight shift (<1.5 cm^{-1}) in the basis spectra was allowed for providing a better fit. A similar spectral shift (0.2−2.1 cm^{-1}) has also been observed in pH-dependent measurements.[23] As shown (Figure 2), the resultant plot of $f_{[GdnHCl]}$ versus [GdnHCl] provides a more quantitative description of the GdnHCl-induced conformational changes of MbCO as probed by the CO stretching vibration. Interestingly, the unfolding transition so obtained is strikingly different, that is, much sharper than those monitored by other spectroscopic methods, such as CD (Figure

2) and TG.[33] For example, the width of the unfolding transition (i.e., the denaturant concentration interval over which the signal changes from 10% to 90% of its initial value) measured by this IR probe is only ~0.3 M of [GdnHCl], whereas those measured by CD and TG are about 0.75 and 1.5 M, respectively. Thus, these differences indicate that the GdnHCl-induced MbCO unfolding is probe-dependent, and thus, describing the denaturation process in a two-state framework probably is not a very accurate representation. However, if we were to assume that the IR signals report a two-state unfolding transition of MbCO, fitting the data to a simple two-state model recovered an m value of 12.4 kcal mol^{-1} M^{-1}, which is significantly higher than that observed for single domain proteins.[45] Additionally, the CD results are qualitatively consistent with the previous reports that the stability of both holo and apo forms of myoglobin is species dependent.[36,46−48]

The heme-bound CO has often been referred to as a molecular probe of the electrostatic potential inside the distal heme pocket of myoglobin (and other carbonmonoxy heme proteins), due to the sensitivity of its stretching vibration to mutations in this pocket (e.g., the distal histidine, H64).[29,31,49−54] For example, the H64G and H64A mutants of sperm whale myoglobin show an increase in the width to 15−20 cm^{-1} of the CO band, which has been attributed to the presence of water molecules in the heme pocket.[29] On the other hand, mutation of H64 to either Leu or Val narrows the CO band, which has been attributed to the heme core being anhydrous.[29,55−58] The A_0 band that we observe in the current study is very similar to that observed for H64G and H64A (in both width and position). Thus, the transition as monitored by the CO stretching vibration most likely reports a hydration event of the hydrophobic core of MbCO, where the CO ligand is located, en route to the denatured state. The sharpness of the transition therefore presumably arises from the local nature of this IR probe, which is sensitive only to its immediate environment. In other words, this transition might result from penetration of only a few water molecules into the hydrophobic pocket of MbCO. This is consistent with the fact that the width of the 1965 cm^{-1} band at 2.9 M GdnHCl is only about 19.4 cm^{-1}, also in accord with a recent time-resolved study of Lim and co-workers on Mb^{13}CO (note that the use of ^{13}CO shifts the heme-bound CO stretching vibration to ~1920 cm^{-1} for the denatured protein)[59] wherein the pocket sequestering the heme group has been shown to remain nativelike, even under strong denaturing conditions. Interestingly, the relatively broad (i.e., fwhm is about 35 cm^{-1}) CO vibrational band observed for substrate free cytochrome P450cam-CO was also attributed to the presence of a water cluster in the heme pocket.[60] While we cannot entirely rule out other possibilities, the current interpretation is consistent with the notion that exclusion of water molecules from the hydrophobic core occurs late in protein folding.[1−4] Furthermore, our results underscore the importance of taking a broader perspective when explaining probe-dependent unfolding transitions as well as other hydration-dependent processes.[61,62]

In order to further confirm that the aforesaid sharp transition does not arise from heme aggregation or heme loss, we further measured the Soret band absorbance of MbCO as a function of [GdnHCl]. It has been shown that the Soret band is sensitive to the heme iron coordination and also to the heme environment.[50,55,56,63,64] For example, for MbCO the peak of the Soret band is at ~422 nm, whereas for met-Mb (iron in the ferric state) with water being the sixth ligand, the Soret band is centered at ~410 nm.[55] As shown (Figure 3), the Soret band of the MbCO samples used in the IR experiments does not show

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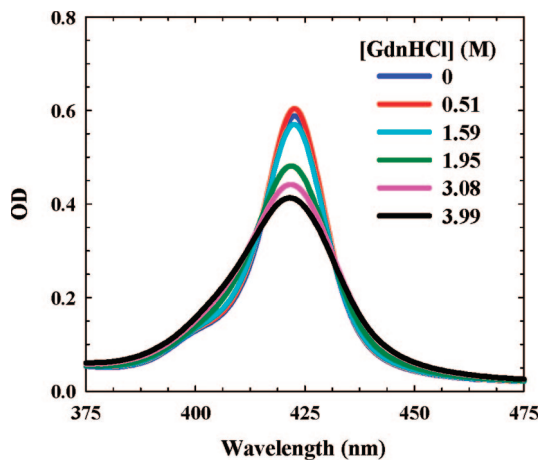
Figure 3. The Soret band of MbCO measured at different GdnHCl concentrations, as indicated.
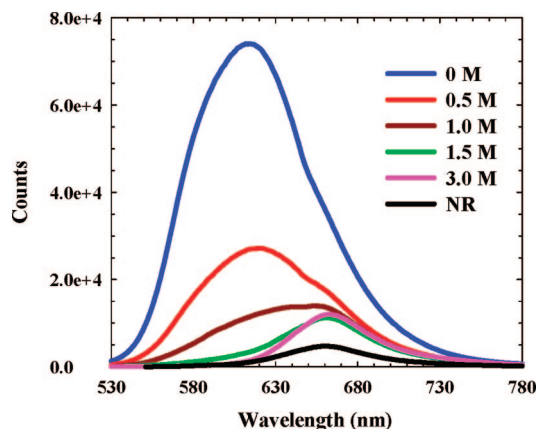
Figure 4. Fluorescence spectra of an apoMb (25 μM) and NR (1 μM) mixture at different GdnHCl concentrations, as indicated. Also shown is the fluorescence spectrum of 1 μM NR in buffer (black).

Figure 5. Integrated area of the NR fluorescence versus apoMb concentration with ($\bigcirc$) and without ($\triangle$) the presence of GdnHCl (0.5 M). Lines represent the best fits of these data to the model described in the text.

a significant change in width and position with increasing GdnHCl concentration, suggesting that upon protein unfolding the CO−heme complex remains intact[33,59,65] and the iron is hexacoordinated. Further experiments employing the Soret band as a probe also showed that the GdnHCl-induced MbCO denaturation is reversible (data not shown). Thus, taken together these results corroborate the notion that the sharp unfolding transition reported by the CO stretching vibration does not result from heme loss and/or aggregation upon MbCO unfolding.

Apomyoglobin. To further substantiate the role of hydration, we have also studied how the fluorescence intensity of apoMb-bound NR varies with [GdnHCl]. The folding/unfolding properties of apoMb have been extensively studied.[66−77] Of particular interest are recent studies indicating that expulsion of water occurs quite late/early in the folding/unfolding kinetics of apoMb.[78,79] The fluorescence quantum yield of NR is exceedingly sensitive to the polarity of surrounding environment,[80] making it an excellent reporter of the hydration status of the hydrophobic heme cavity (now devoid of heme) of apoMb, where the dye has been shown to bind.[34] As expected (Figure 4), the fluorescence spectrum of NR in the presence of apoMb is quite sensitive to [GdnHCl]. For example, compared to that obtained in buffer, the fluorescence intensity obtained in 0.5 M GdnHCl solution is decreased by almost a factor of 3. A similar trend has also been observed in GdnHCl-induced denaturation of apoMb complexed with the dye ANS,[81] wherein the authors attributed the decrease in the fluorescence solely to penetration of solvent. However, providing a more quantitative interpretation
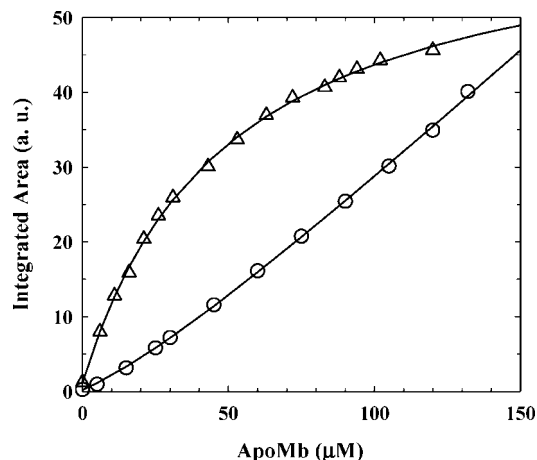
of these data is not straightforward, because the binding constant of NR to apoMb is also expected to be modulated by the change in denaturant concentration. Thus, in order to make a quantitative comparison of these data, we have further determined the dissociation constants (K_d) of the apoMb−NR complex in both buffer and 0.5 M GdnHCl solutions, respectively. This was achieved by measuring the fluorescence intensities of a series of solutions with a fixed concentration of NR (450 nM) but different apoMb concentrations. As shown (Figure 5), the binding curves so obtained are quite different. To extract K_d, we further fit the binding curve obtained in buffer to the following equation

$$F_{\text{int}} = Q_b[\text{NR}]_b + Q_f[\text{NR}]_f \qquad (2)$$

with

$$[\text{NR}]_b = \Big\{ K_d + [\text{NR}]_0 + [\text{apoMb}] - \\ \sqrt{(K_d + [\text{NR}]_0 + [\text{apoMb}])^2 - 4[\text{NR}]_0[\text{apoMb}]} \Big\}/2 \quad (3)$$

where F_{int} is the integrated area of the fluorescence spectrum, and the subscripts b and f represent the protein-bound and free NR molecules, respectively. In addition, Q represents the relative brightness of the NR, brackets denote molar concentrations, [apoMb] is the total concentration of apomyoglobin, and $[\text{NR}]_0$ is the total concentration of NR, which was kept constant during the course of the experiment. While fitting, K_d, Q_b, and Q_f were allowed to vary, and the best fit yielded a K_d of 49 $\pm$ 3 μM, which is somewhat larger than that reported in a previous study[34] wherein a slightly different approach was used to analyze the fluorescence binding data. Moreover, the ratio of Q_b/Q_f obtained from the best fit is $\sim$55, consistent with an early study.[34]

However, the binding data obtained at 0.5 M GdnHCl could not be adequately modeled by eq 2, indicating that other factors need to be considered. Under denaturation conditions, globular proteins are known to expose hydrophobic patches, and transient binding of NR molecules to such exposed patches via molecular collisions would result in an overall increase in the NR quantum yield, similar to that observed in studies where NR is used as a fluorescent reporter of the hydrophobic surface of lipid bilayers.[82] In order to further substantiate this effect, we carried out FCS experiments. As shown (Figure 6A), when both apoMb (25 μM) and GdnHCl (1 M) are present, the autocorrelation curve [i.e., $G(\tau)$] of NR overlaps almost completely with that

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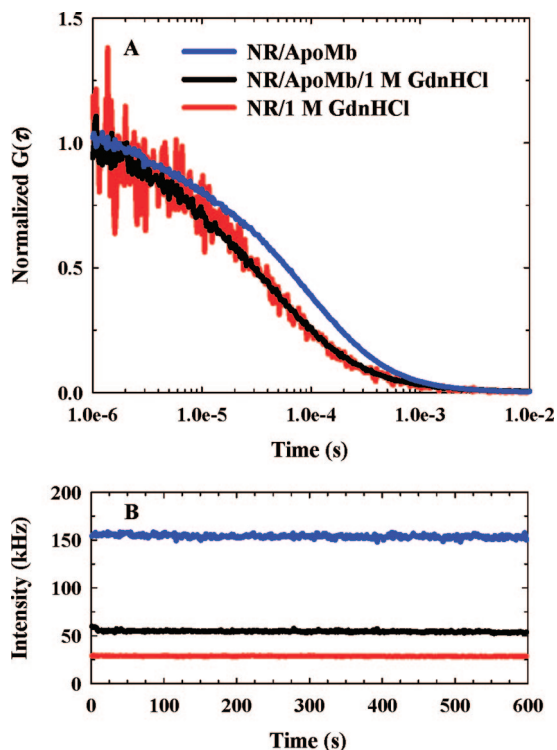
Figure 6. (A) Normalized autocorrelation traces of 100 nM NR in buffer (red), in 25 μM apoMb solution (blue), and in 25 μM apoMb and 1 M GdnHCl solution (black). (B) The corresponding fluorescence intensity profiles used to generate the FCS traces in part A. The color coding is the same as that used in A.
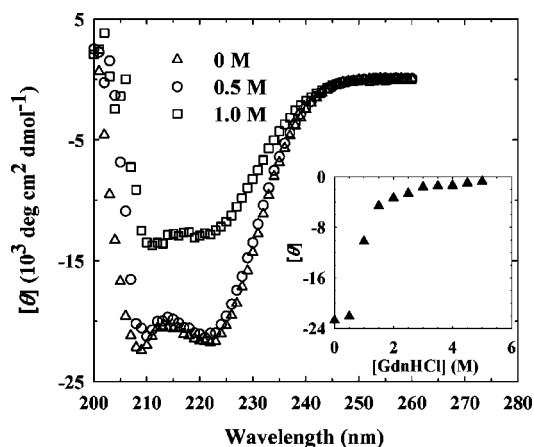
Figure 7. CD spectra of apoMb-NR measured at different GdnHCl concentrations, as indicated. Also shown in the inset is the entire CD unfolding transition.

of the free dye. On the other hand, when only apoMb (25 μM) is present, the autocorrelation curve of NR is distinctly slower. Since $G(\tau)$ reports the time course of the fluorescent molecules moving through the confocal volume and thus the size of the diffusing species,[83] our results therefore indicate that in 1 M GdnHCl, NR remains essentially dissociated from the protein. On the other hand, in buffer, an appreciable amount of the dye is bound to apoMb. However, comparison of the fluorescence counts (Figure 6B) reveals that when apoMb is present the brightness of NR is significantly increased compared to that of the free dye, even under denaturation conditions. Taken together, these FCS results thus suggest that the NR fluorescence can be transiently enhanced by forming short-lived apoMb-NR complexes arising from weak, nonspecific binding when the protein is partially or fully unfolded. Indeed, we found that in order to

quantitatively describe the data obtained at 0.5 M GdnHCl such transient effects need to be considered. Specifically, we have modified eq 2 to include an enhancement term arising from such effects, as follows

$$F_{int} = Q_b[NR]_b + Q_f[NR]_f + \alpha([apoMb] - [NR]_b)^2[NR]_f$$

(4)

where α represents the collision-induced enhancement factor. As indicated (Figure 5), eq 4 describes the experimental data well and the best fit yielded a K_d of 63.5 ± 4 μM. This result in conjunction with the K_d determined for apoMb in buffer indicate that the change in the binding affinity of NR to apoMb upon addition of 0.5 M GdnHCl alone cannot account for the aforementioned drastic decrease in the NR fluorescence intensity (i.e., the NR fluorescence intensity shows a ~70% decrease, whereas the population of the protein-bound NR is only decreased by ~16%). On the other hand, the CD measurements (Figure 7), which are consistent with previous studies,[46] indicate that apoMb shows no appreciable decrease in its helicity at 0.5 M GdnHCl. Thus, taken together, these results argue that hydration of the hydrophobic cavity wherein NR resides when bound to the protein occurs prior to the global unfolding of apoMb. While granted that any kinetic implication from the current thermodynamic study is a mere speculation, our results are consistent with previous kinetic measurements,[78,79,84] which indicate that water penetration into the core of apoMb takes place in the early stages of the unfolding process. Similarly, small-angle X-ray scattering measurements have revealed that dehydration occurs at the later stages of the folding of α-lactalbumin and protein L.[85,86]

Conclusion

In summary, we have employed two environmentally sensitive optical probes, namely, the stretching vibration of CO ligated to the heme and the fluorescence of NR, to probe the role of hydration in the GdnHCl-induced unfolding transitions of horse MbCO and apoMb. Our results show that both probes report a conformational event that is strikingly sharper than those measured by other spectroscopic methods. We tentatively attribute this sharp "unfolding" transition to either partial or full hydration of the hydrophobic core of the protein. These findings are interesting not only from the point of view of providing evidence with regard to the role of water in protein folding/unfolding but also in that they carry strong implications for the interpretation of probe-dependent folding—unfolding transitions.

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