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Photodissociation and Rebinding of H_2O to Ferrous Sperm Whale Myoglobin

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The investigation of the binding of small ligands has provided valuable insights into the relations among structure, dynamics, and function of proteins.[1] In particular, the binding of carbon monoxide (CO) and dioxygen (O_2) to myoglobin (Mb) has been studied in great detail and lead to the notion of conformational substates[1a] (CS) and protein relaxations.[1b,d,2] The changes in ligand affinity for CO and O_2 imposed by the polypeptide chain prevents the endogenously produced levels of CO from being toxic.[3] Water, while being abundant in the physiological environment and even present in the heme pocket, does not bind to ferrous Mb at physiological temperatures. Here, we provide evidence that water binds to ferrous Mb at cryogenic temperatures in a photodissociable complex. Through reduction of aquometmyoglobin (Mbmet) at 20 K, we succeeded in producing an Fe(II) low-spin configuration at the active center with the water molecule still bound. The water ligand was photodissociated with a short laser pulse, and the nonexponential rebinding kinetics were monitored as a function of time and temperature.

Mbmet can be reduced at low temperature using γ-rays,[4] X-rays,[5] visible light,[6] or photochemical methods.[7] Here, we used either X-rays to reduce samples that were optically thick or tris-(2,2′,bipyridine)ruthenium(II) ($[Ru(bpy)_3]^{2+}$) as a photoactivated reducing agent. The metastable intermediate state formed is structurally similar to Mbmet, with the water molecule still bound, but the heme iron is in the Fe(II) low-spin configuration. The absorption spectrum of the intermediate state has been characterized[4b,7] as well as the relaxation of the intermediate state to the equilibrium deoxy Mb structure at temperatures above 160 K.[5b,c,7]

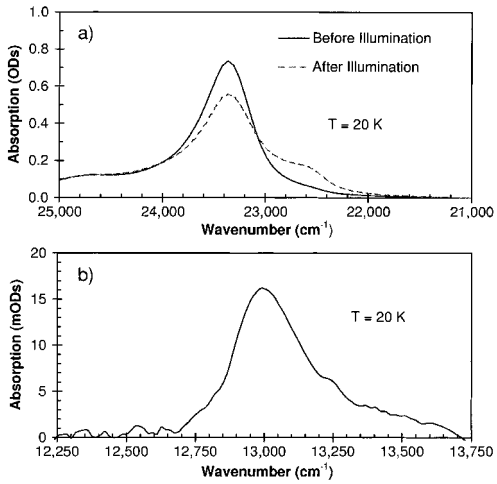

Figure 1. (a) The Soret spectrum of the intermediate state at 20 K before and after illumination with a laser diode. Absorption from $[Ru(bpy)_3]^{2+}$ has been subtracted to more clearly demonstrate the changes in the Soret region. The sperm whale Mb concentration was 5 mM. (b) The difference spectrum from before and after illumination measured in the near-IR using a 17 mM sperm whale Mb sample.

Upon illumination of the low-spin intermediate species (Fe^{II}-MbH_2O) with low intensity light at 20 K, there is a decrease in the amplitude of the Soret band at 23 360 cm^{-1} and a new band at 22 600 cm^{-1} appears, indicative of a high-spin-like state (Figure 1a). After the sample was warmed above 60 K and cooled back to 20 K, the new band essentially disappeared while the Soret band of the $Fe^{II}MbH_2O$ state returned to its original value, implying that this deoxy-like state reverted back to the Fe(II) low-spin intermediate state. Photodissociation of a water ligand from the low-spin iron with concomitant conversion to high-spin was considered as a sensible explanation of the spectral changes. To obtain further support for this scenario, we investigated band III near 13 200 cm^{-1} which has been assigned to a porphyrin-to-iron $a_{2u} \rightarrow d_{yz}$ charge-transfer transition.[8] It is only observed in Fe(II) high-spin, five-coordinate hemes. Because of the approximately 1000-fold weaker absorption of this band compared with the Soret, a concentrated protein solution (17 mM) with a sample thickness of 2 mm was used. It was mounted between thin Mylar windows and irradiated with X-rays for 17.5 h at 80 K. Subsequently, the sample was transferred at low temperature to an optical cryostat mounted within the optical spectrometer. Spectra were taken at 20 K before and after illumination with a 20 mW laser diode at 14 600 cm^{-1} for 30 min. As shown in Figure 1b, the charge-transfer band III, indicative of a high-spin deligated species, indeed appears with a peak position of 13 000 cm^{-1} upon illumination. Consequently, we conclude that the

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water ligand can indeed be photodissociated from the Fe(II) low spin intermediate. Below 100 K, deoxy Mb in glycerol−water solvents shows band III near 13 200 cm^{-1}, whereas in the low-temperature photoproducts of Mb*CO and Mb*O_2, the band peaks around 13 070 cm^{-1}.[1d,9] This red shift with respect to the equilibrium deoxy form has been attributed to the inability of the protein to relax to its equilibrium conformation, which is characterized by a considerable shift of the heme iron out of the mean heme plane. The position of band III in the photoproduct Fe^{II}Mb*H_2O is shifted similarly (even a bit further) to the red than in Mb*CO and Mb*O_2. This suggests that the local structure at the heme is similar in the CO bound and H_2O bound forms of Mb and that the ability to relax toward the deligated structure is even more restricted in Fe^{II}Mb*H_2O than in Mb*CO or Mb*O_2.

A 3.5 mM sperm whale Mb sample with 60 mM $\{Ru(bpy)_3\}^{2+}$, ~100 mM ethylenediaminetetraacetic acid (saturated), 50 mM potassium phosphate buffer (pH 6.25), and 75% glycerol (v/v) was prepared to investigate the rebinding kinetics of the water ligand. The sample was loaded between two 30 × 10 mm microscope slides with a 10 μm spacer. By illumination with the third harmonic of a Nd:YAG laser ($\lambda = 28\,170$ cm^{-1}) run at 10 Hz with 25 mJ/pulse (6 ns fwhm) for a total of 25.5 min, the sample was photoreduced at 20 K and subsequently warmed to 180 K for 40 min to allow additional reduction to occur. Because of the additional reduction, the intermediate state population is maximized even though a fraction of the intermediate state relaxes completely to deoxy Mb. Moreover, there is a structural relaxation at the active center within the intermediate state that can be observed through a shift of the Soret band.[7] By warming to 180 K, this relaxation is allowed to complete, and hence, it does not interfere with the rebinding kinetics.

Geminate rebinding of water ligands was monitored using transient absorption spectroscopy at 22 730 cm^{-1}.[10] The ligands were photodissociated with using the frequency-doubled pulse of a Nd:YAG laser at 18 800 cm^{-1} (100 mJ, 6 ns fwhm). Due to fluorescence from excited $[Ru(bpy)_3]^{2+}$, we were only able to measure the rebinding kinetics of H_2O for times longer than 100 μs. The kinetics between 60 and 150 K are shown in Figure 2a. Although the sample is a mixture of Mbmet, $Fe^{II}MbH_2O$, and deoxy Mb, the signal is only due to rebinding of Fe^{II}Mb*H_2O, as there is no contribution from deoxy Mb or Mbmet. The amplitude of the observed signal was less than was expected for complete photolysis. We have only succeeded in photolyzing about 50% of the $Fe^{II}MbH_2O$, which may be analogous to MbO_2 where the microsecond quantum yield is less than 1.[11] The kinetics are nonexponential in time and can be fit to a temperature-independent distribution of enthalpy barriers and an Arrhenius relationship

$$\Delta\mathrm{Abs}\,(t,T) = \Delta\mathrm{Abs}_{\max}\int_0^\infty \mathrm{d}H\, g(H)\, \exp\{-k(H,T)t\} \quad (1)$$

$$k(H,T) = \frac{AT}{T_0}\exp\left\{-\frac{H}{RT}\right\} \quad (2)$$

$$g(H) = \frac{(H - H_{\min})^{\alpha(H_{\mathrm{pk}}-H_{\min})}}{\Gamma[\alpha(H_{\mathrm{pk}} - H_{\min}) + 1]} \times \exp\{-\alpha(H - H_{\min})\}\alpha^{\alpha(H_{\mathrm{pk}}-H_{\min})+1} \quad (3)$$

where A is the preexponential in the Arrhenius equation, T_0 is a reference temperature (100 K), R is the molar gas constant, and

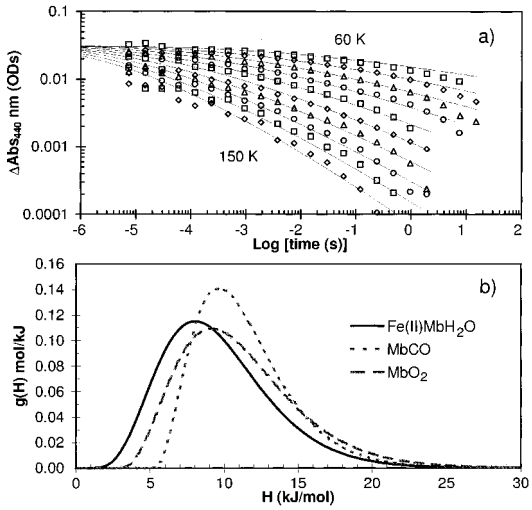

Figure 2. (a) The rebinding kinetics of H_2O to Fe^{II}Mb*H_2O measured from 60 to 150 K in 10 K intervals. The kinetics are shown on a log−log scale. The fit to a Γ $g(H)$ distribution for $t > 100$ μs is shown as solid lines and the parameters are listed in Table 1. (b) The $g(H)$ distribution is shown for Fe^{II}Mb*H_2O, Mb*CO, and Mb*O_2.

Table 1. Parameters of the Γ g(H) Distribution Used To Fit the Rebinding Kinetics to Fe^{II}Mb*H_2O, Mb*CO, and Mb*O_2

system	temp range (K)	log(A/s^{-1})	$H_{\min}$ (kJ/mol)	H_{pk} (kJ/mol)	α (kJ/mol)	ref
Fe^{II}Mb*H_2O	60−150	8.1 ± 1.1	0.7[a]	8.0 ± 1.5	0.6 ± 0.2	*b*
Mb*CO	60−150	8.8 ± 0.2	5.2 ± 1.5	9.7 ± 0.4	0.6 ± 0.1	1d
Mb*O_2	60−160	8.9 ± 0.3	2.7[a]	9.2 ± 0.6	0.5 ± 0.1	1d

[a] Errors are in excess of 50% of the best value. [b] This work.

$H_{\min}$, H_{pk}, and α are the parameters that specify the gamma distribution of enthalpy barriers, g(H). The fit is shown as solid lines in Figure 2a, and the $g(H)$ distribution is shown in Figure 2b along with the $g(H)$ distributions for Mb*CO and Mb*O_2.[1d] The parameters are given in Table 1. The enthalpy distributions arise from structural heterogeneity of the proteins (CS). The similar shapes of the distributions suggest a similar degree of heterogeneity in the photoproduct Fe^{II}Mb*H_2O (and also of Mbmet, as this is the structure from which the intermediate was produced).

The $g(H)$ distributions of Mb*CO, Mb*O_2, and Fe^{II}Mb*H_2O are similar in shape, with Fe^{II}Mb*H_2O shifted by about 2 kJ/mol toward lower barriers. It is interesting to note that this is also the species with the most red-shifted band III. This is consistent with the interpretation of band III as a marker that measures the extent of heme relaxation.[1d,9] For Mb*H_2O, when the protein relaxes fully to the deoxy structure, the heme iron moves too far away from the heme plane to form a sufficiently strong bond with the water ligand and the affinity for H_2O drops dramatically.

The flash photolysis experiments on $Fe^{II}MbH_2O$ presented here have shown that the water ligand, which cannot bind to the heme iron in the deoxy conformation, binds in the intermediate state in a photolabile complex. This gave us the opportunity to investigate the properties of H_2O as a ligand and the effect of structural changes on ligand affinity.

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