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ARTICLE

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From metmyoglobin to deoxy myoglobin: relaxations of an intermediate state

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Abstract Metmyoglobin has been reduced at low temperature (below 100 K) using x-rays or by excitation of tris(2,2′,bipyridine)ruthenium(II) chloride with visible light. Upon reduction, an intermediate state is formed where the structure of the protein is very similar to that of metmyoglobin with the water molecule still bound to the heme iron, but the iron is II low spin. The nature of the intermediate state has been investigated with optical spectroscopy. The Q_o and Q_v bands of the intermediate state are split, suggesting that the protoporphyrin is distorted. The intermediate state undergoes a relaxation observed by a shifting of the Soret band at temperatures above 80 K. Above 140 K, the protein begins to relax to the deoxy conformation. The relaxation kinetics of the protein have been monitored optically as a function of time and temperature from minutes to several hours and from 150 K to 190 K. By measuring the entire visible spectrum, we are able to distinguish between electron transfer processes and the protein relaxation from the intermediate state to deoxy myoglobin. The relaxation has been measured in both horse myoglobin and sperm whale myoglobin with the relaxation occurring on faster time scales in horse myoglobin. Both the reduction kinetics and the relaxation show non-exponential behavior. The reduction kinetics can be fit well to a stretched exponential. The structural relaxation from the intermediate state to the deoxy conformation shows a more complex, dynamical behavior and the reaction is most likely affected by the relaxation of the protein within the intermediate state.

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Introduction

Many enzymes or respiratory proteins perform their function by switching between two or more conformations. As the protein undergoes a change in conformation, transient, intermediate states may be formed. From the intermediate state, the protein can return to the old conformation or relax to a new conformation, depending on the perturbation. Proteins in the same conformation may have small differences in the structure, referred to as conformational substates (CS). This intrinsic structural inhomogeneity of proteins within a conformation was determined by measuring the rebinding kinetics of carbon monoxide (CO) to myoglobin (Mb) in the photodissociated state (Mb*CO) below 160 K (Austin et al. 1975). Proteins in different CS have similar structures and perform the same function, but with different rates. The existence of CS has been confirmed by x-ray diffraction (Frauenfelder et al. 1979; Hartmann et al. 1982; Parak et al. 1987; Frauenfelder et al. 1988), optical spectroscopy (Šrajer et al. 1986; Ormos et al. 1990; Leone et al. 1994), molecular dynamics simulations (Elber and Karplus 1987), hole-burning experiments (Köhler et al. 1988; Zollfrank et al. 1992), and EPR spectroscopy (Bizzari and Cannistraro 1993). The conformational change is often initiated at the active site of the protein. The global, structural rearrangement that follows involves the disruption and formation of many weak, secondary bonds and, therefore, occurs on a slower time scale. For a time, the active site is in a new state, but the overall structure of the protein is still in the old conformation. The idea of stress being generated at the active center and propagating through the protein, i. e. a protein quake, was discussed in detail by Frauenfelder and coworkers (Ansari et al. 1985). Detailed studies of intermediate states and their relaxations provide insight into the dynamics of proteins and how they function.

The dissociation and rebinding of small ligands with myoglobin is an example of a well studied system that has enhanced our understanding of protein dynamics. After photodissociation of small ligands from Mb, an intermediate state is formed. The structure of the intermediate state in Mb*CO has been determined by x-ray diffraction (Schlichting et al. 1994; Hartmann et al. 1996; Šrajer et al. 1996). The photodissociated intermediate state undergoes a functionally important relaxation. The enthalpy barrier for internal rebinding in the intermediate state at low temperature is ~10 kJ/mol in Mb*CO and Mb*O_2 (Steinbach et al. 1991). After photodissociation at physiological temperatures, the protein structure relaxes from the ligand bound configuration to the deoxy structure (Mbdeoxy) before the majority of ligands can rebind. During the relaxation, the internal barrier for rebinding increases to ~20 kJ/mol. This increase in the internal enthalpy barrier for rebinding is what allows the ligand to escape the protein. The unrelaxed barrier is so small that the ligand would rebind to the protein before it could escape to the solvent. Without the relaxation, Mb would be a poor storage and transport protein. Different possible interpretations of the relaxation are discussed by Goldanskii and Parak (1994).

The rebinding kinetics of small ligands to metal containing proteins has proved to be an important tool in the study of protein dynamics (e. g. Austin et al. 1975; Doster et al. 1982; Ansari et al. 1985; Ansari et al. 1987; Šrajer et al. 1988; Steinbach et al. 1991; Ehrenstein and Nienhaus 1992; Tian et al. 1992; Balasubramanian et al. 1993; Post et al. 1993; Ansari et al. 1994; Johnson et al. 1996). A different kinetic process that can be studied is the relaxation that occurs after the reduction of proteins at low temperature. Initial experiments performed by Blumenfeld and coworkers (Magonov et al. 1978 a; Magonov et al. 1978 b; Blumenfeld 1983; Blumenfeld et al. 1986) used γ-rays to reduce proteins at low temperature and the intermediate states were recorded with optical spectroscopy and magnetic circular dichroism. More recently, we have used x-rays to reduce high spin Fe(III) aquometmyoglobin at 80 K and monitored the sample using Mössbauer spectroscopy (Parak and Prusakov, 1994; Prusakov et al. 1995; Prusakov et al. 1996). Radiolysis of the solvent with γ-rays or x-rays produces electrons that are able to reduce variable valence metals in the active centers of proteins. If these experiments are performed at low temperature, the overall protein structure will be frozen in the oxidized conformation, but the active center will be reduced. When there is enough thermal energy available for the protein to undergo structural changes, the protein will relax to the reduced conformation. The temperature region where this occurs depends on the size of the structural relaxation, the flexibility of the protein, and the solvent viscosity.

To improve the quality of the optical spectra, decrease the time necessary for reduction, and avoid the possible deleterious effects of x-ray radiation, we use tris(2,2′-bipyridine) ruthenium(II) chloride ($[Ru(bpy)_3]^{2+}$) to photochemically reduce proteins at low temperature. The intermediate state formed in Mb has been characterized with optical spectroscopy and the relaxation to the deoxy conformation has been followed as a function of time and temperature. To determine the structure of the intermediate state, x-ray diffraction experiments at 115 K have been performed (Ostermann et al., results to be published). The structure of the intermediate state is very similar to that of aquometmyoglobin (Mbmet) with a ligand still bound in the sixth position, but the iron center is II low spin (Fe(II)MbH_2O). Above 80 K, the protein undergoes a relaxation within the intermediate state as the active center adjusts to the new charge state of the heme iron. This relaxation is observed by a shifting of the Soret band. The intermediate state is metastable up to ~140 K. At higher temperatures, a further structural relaxation occurs as the intermediate state relaxes to Mbdeoxy.

By photochemically reducing proteins at low temperature, it is possible to optically trigger a conformational change within the protein. The resulting relaxation occurs in only one direction, Mbmet $\rightarrow$ Fe(II)MbH_2O $\rightarrow$Mbdeoxy. Thus, the full kinetics of the relaxation can be followed without being limited to the ligand rebinding kinetics, as in flash photolysis experiments.

In these experiments, additional reduction occurs on the same time scale as the structural relaxation to Mbdeoxy. By measuring the absorption spectrum in the entire visible region it is possible to distinguish between the electron transfer kinetics and protein relaxations. Electron transfer rates determined by measurements at a single wavelength may contain errors due to unresolved protein relaxations.

Materials and methods

Reduction with *$[Ru(bpy)_3]^{2+}$ at low temperature

To improve the time resolution of the low temperature reduction experiments, it was advantageous to develop a means of photochemically inducing the reduction or oxidation of proteins at low temperature. The feasibility of photochemically initiated reactions is known from the experiments of Chance and collaborators. They observed laser-induced oxidation of cytochrome c at temperatures as low as 4.4 K in the photosynthetic system of purple sulfur bacterium, *Chromatium* (Chance and Nishimura 1960; DeVault and Chance 1966; DeVault et al. 1967). Long range intramolecular electron transfer in hemoglobin was observed at cryogenic temperatures by Hoffman and coworkers (Peterson-Kennedy et al. 1986). In these experiments, the hemes from either the α or β subunits were substituted with zinc porphyrins (ZnP). The ZnP was photoexcited and the excited triplet state, ^{3}(ZnP), transfers an electron to the Fe^{3+} containing hemes. Experimental data and theoretical calculations show that electron tunneling can occur over a few tens of Angstroms (Zamaraev et al. 1971; DeVault 1979; Goldanskii 1979; Markus and Sutin 1985; Axup et al. 1988; Khairutdinov et al. 1989; Goldanskii et al. 1989; Cusanovich 1991; Winkler

and Gray 1992; Zamaraev and Khairutdinov 1992; Langen et al. 1995).

The requirements for a photochemically active reagent are: 1) chemical stability in the excited state and different oxidation ground states, 2) high quantum yield for the excited state capable of electron transfer, 3) suitable redox potentials, 4) a long excited state lifetime, and 5) a high efficiency of electron transfer to the protein (Balzani et al. 1990). The canonical agents used for photochemical reduction have been polypyridine ruthenium(II) complexes (Paris and Brandt 1959; Sutin and Creutz 1978; Watts et al. 1978; Balzani et al. 1990; Horvath and Stevenson 1993). We have chosen $[Ru(bpy)_3]^{2+}$ because of its chemical stability in the ground state and its highly reactive photoexcited triplet state which can be readily produced using 450 nm light with a near-unity quantum yield (Demas and Crosby 1971).

$$[Ru(bpy)_3]^{2+} \xrightarrow{h\nu} {}^*[Ru(bpy)_3]^{2+} \quad (1)$$

$[Ru(bpy)_3]^{2+}$ has been widely used to investigate electron transfer in cytochrome c (Sutin 1977; Winkler et al. 1982; Nocera et al. 1984), myoglobin (Crutchley et al. 1985; Lieber et al. 1987; Hamachi et al. 1993), and blue copper proteins (English et al. 1982). The excited state has an extremely high potential for oxidation or reduction: $E°(*Ru^{2+}/Ru^{3+}) \approx -0.86$ V and $E°(*Ru^{2+}/Ru^{+}) \approx 0.84$ V versus a normal hydrogen electrode (Balzani et al. 1990). Therefore, it can be either an effective reductant or oxidant depending on the redox properties of the other reactants.

$$*[Ru(bpy)_3]^{2+} + A \rightarrow [Ru(bpy)_3]^{3+} + A^- \quad (2)$$

$$*[Ru(bpy)_3]^{2+} + D \rightarrow [Ru(bpy)_3]^{+} + D^+ \quad (3)$$

The tri- and monovalent ruthenium complexes that are formed are also powerful oxidizing and reducing agents. Therefore, to prevent the back reaction from occurring, an additional sacrificial electron donor/acceptor needs to be included to re-reduce/re-oxidize the tri/monovalent ruthenium. In solutions with $[Ru(bpy)_3]^{2+}$, ethylenediaminetetraacetic acid (EDTA) is often added to act as a sacrificial donor (Keller et al. 1980; Harriman and Mills 1981; Miller and McLendon 1981; Prasad et al. 1985; Kennely et al. 1986). The yield of the photogenerated ferroheme from Mbmet has been shown to be strongly dependent on the initial concentration of EDTA, and 100% reduction was achieved at room temperature with 100 mM EDTA (Hamachi et al. 1993).

Until now, experiments on inter- and intramolecular electron transfer in proteins using ruthenium polypyridine complexes (Ru) were performed almost exclusively above 0 °C. McLendon and coworkers measured electron transfer from Ru complexes to methyl viologen (MV^{2+}) down to 248 K (Strauch et al. 1983; Guarr et al. 1983). Milosavljevic and Thomas (1986) have measured electron transfer from $*[Ru(bpy)_3]^{2+}$ to MV^{2+} in a cellophane matrix at 77 K. In the low temperature work, high concentrations of MV^{2+} were necessary to provide a close approach of the acceptor and the photoexcited electron donor.

Samples

Water free glycerol (Fluka Chemie AG, Buchs, Switzerland) was added to 100 mM solutions of monobasic and dibasic potassium phosphate buffer (Merck KGaA, Darmstadt, Germany) and then mixed together to create a 50% glycerol (v/v), 50 mM buffer solvent with a pH of 6.6. EDTA (Sigma Chemical Co., St. Louis, MO) and $[Ru(bpy)_3]^{2+}$ (Fluka) were added to the solvent to a final concentration of ~100 mM (or as much as was soluble) and 60 mM respectively. The solvent was centrifuged at 25,000 *g* for 10 minutes to remove any insoluble components. Sperm whale Mb (Sigma) or horse heart Mb (Fluka) were added to a portion of the solvent and allowed to stir overnight. The sample was centrifuged again for 10–15 minutes at 25,000 *g* to remove impurities. The protein concentrations was ≈5 mM. The final pH of the sample was 7.0 for sperm whale Mb and 7.2 for horse Mb. A high concentration of $[Ru(bpy)_3]^{2+}$ was needed to minimize the separation of the heme iron and the photoexcited $[Ru(bpy)_3]^{2+}$ at low temperature. Because of the absorption of $[Ru(bpy)_3]^{2+}$ in the Soret region of Mb, a high concentration of Mb was also used.

Optical absorption measurements

For the optical absorption measurements, a drop of ~2 µl of protein solution was loaded between 2 fused silica windows (1 mm thick) separated by a 12 µm spacer and placed in a sample cell constructed from high conductivity, oxygen free copper. An approximately 5 mm×2 mm region of the sample, without bubbles, was selected and the rest of the sample masked with copper tape. The sample cell was loaded in a Leybold closed-cycle helium refrigerator (RDK 10-320, Hanau, Germany) equipped with a top loading cell. The sample chamber was filled with helium to act as a thermal contact gas. The temperature was measured to ±1 K using a silicon temperature diode (DT-471, Lake Shore Cryogenics, Westerville, Ohio) mounted on the sample cell near to the sample and a Leybold LTC 60 temperature controller. A Perkin Elmer (Lambda 19, Überlingen, Germany) spectrometer has been modified to allow contact-free mounting of the cryostat within the spectrometer and was used for measuring the spectra. Spectra were collected with a 1 nm slit width, 120 nm/min scan speed, and 2 nm smoothing.

For reduction experiments, samples were cooled to various temperatures and the spectrum was collected as a function of illumination time and laser energy. The samples were illuminated at 1 Hz with 1.2 µs pulses from a flashlamp pumped dye laser (Candela EDL-3, Wayland, Massachusetts) using 2×10^{-4} mM coumarin 47 (Lambda Physik, Göttingen, Germany) in ethanol (460 nm, 6 nm FWHM). Samples were always rotated by 180° so that they were illuminated from both sides.

The samples for kinetic measurements were cooled to 20 K for illumination. The laser was run at 1 Hz and the samples were illuminated for a total of 45 to 60 minutes. The absorption of a typical sample was 1 OD at the wavelength of illumination. Most of the reduction occurs at the beginning of the illumination time. However, even after 1 hr of illumination, ~45% of sperm whale Mb samples and 20%–40% of horse Mb samples were still in the met conformation. Both the EDTA and the glycerol function as electron donors. One reaction that occurs upon illumination is:

$$\begin{aligned} {}^{*}[\mathrm{Ru(bpy)_3}]^{2+} + \text{heme Fe}^{3+} + \mathrm{D} \;&\rightarrow \\ [\mathrm{Ru(bpy)_3}]^{3+} + \text{heme Fe}^{2+} + \mathrm{D} \;&\rightarrow \\ [\mathrm{Ru(bpy)_3}]^{2+} + \text{heme Fe}^{2+} + \mathrm{D}^{+} & \end{aligned} \qquad (4)$$

where D is either EDTA or glycerol. After illumination at low temperature, $[\mathrm{Ru(bpy)_3}]^{+}$ is observed. Either $[\mathrm{Ru(bpy)_3}]^{2+}$ is acting as an electron acceptor from $^{*}[\mathrm{Ru(bpy)_3}]^{2+}$ or the $^{*}[\mathrm{Ru(bpy)_3}]^{2+}$ is accepting an electron from some other molecule in the solvent.

After reducing the sample at 20 K, the temperature of the sample was increased to 130 K. At 130 K, the protein does not relax to the deoxy structure, but there is some additional reduction that occurs. After 30 minutes, a spectrum was taken and the sample warmed to a temperature between 150 K and 190 K. Upon reaching the temperature of the experiment, spectra were collected at 2.1, 6.3, 10.5, 15, 30 minutes and every 30 minutes thereafter. Warming took between 7 and 12 minutes depending on the temperature.

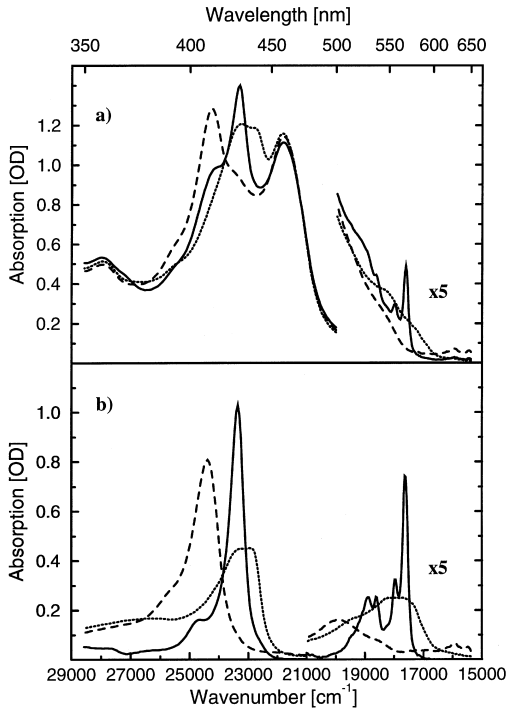

Fig. 1 a The visible absorption spectra of sperm whale Mb with $[\mathrm{Ru(bpy)_3}]^{2+}$ at 20 K before illumination (oxidized state, *dashed line*), immediately after illumination (mixture of oxidized and intermediate states, *solid line*), and after the sample has been cycled to room temperature (reduced state, *dotted line*). **b** The visible absorption spectra of Mbmet (*dashed line*), Fe(II)MbH_2O (*solid line*), and Mbdeoxy (*dotted line*) at 20 K after correcting Fig. 1 a for the other absorptions

Data evaluation

Determination of the intermediate optical spectrum

The optical spectra at 20 K before illumination, after illumination, and after the protein has relaxed to the deoxy structure are shown in Fig. 1 a. Figure 1 b shows the spectra after correcting for absorption from $[\mathrm{Ru(bpy)_3}]^{+/2+}$ and from other conformations of the protein. To estimate the accuracy of the determined optical absorption spectra, we have compared the spectra of the intermediate state generated by x-rays and from $^{*}[\mathrm{Ru(bpy)_3}]^{2+}$. Although the difficulties are not the same for the different experiments, the spectra are very similar, verifying that the intermediate state absorption spectra are accurate.

When reducing by x-rays, it is important to minimize the absorption of the x-rays before the sample. Therefore, a 115 μm mylar window was used on the front of the sample. The thin window did not lay flat on the sample and ringing was observed in the optical spectra. X-ray irradiation causes the mylar window to turn brown and with the large, broad absorption band around 560 nm from radicals in the solvent, distorts the absorption spectrum. Absorption from the radicals could be removed by warming the sample above 150 K. In addition, the clarity of the sample after irradiation is often diminished, decreasing the quality of the spectrum.

To determine the spectrum in Fig. 1 b of the intermediate state after reducing with $^{*}[\mathrm{Ru(bpy)_3}]^{2+}$ from the spectrum in Fig. 1 a, one has to 1) correct for the absorption of Ru, 2) correct for the absorption from Mbmet, and 3) correct for the absorption of any part of the sample that has relaxed to Mbdeoxy.

Subtraction of the Ru absorption from the reference spectra, though not necessary for fitting the relaxation kinetics, made visible inspection of the reference spectra possible and made it easier to hold the total Mb and Ru concentrations constant while fitting. Because of the difficulties in measuring the intermediate state spectrum above 180 K, the 180 K spectrum (170 K for horse Mb) was used when fitting higher temperatures. The quality of the fit to the kinetics would be reduced if the Ru absorption were not previously subtracted from the intermediate state spectrum at 180 K.

The total concentration of Ru can be determined by fitting the spectrum before illumination to a linear combination of Ru and Mbmet spectra. At low temperature, a shift in the Soret band of Mbmet is observed when $[\mathrm{Ru(bpy)_3}]^{2+}$ has been added to the solvent. This may be due to a direct interaction between the Ru with Mb molecules at low tem-

perature, or may be an indirect effect arising from changes in the properties of the solvent. All of the Mb reference spectra were collected with Ru and then the Ru contribution to the spectrum was subtracted. Upon illumination, a portion of the $[Ru(bpy)_3]^{2+}$ is converted into $[Ru(bpy)_3]^{+}$, which is metastable at low temperature. To account for the change in the Ru spectrum, an identical sample without Mb was illuminated under the same conditions. We make the assumption that there is no change in the visible absorption spectrum from the uptake of electrons by the solvent. The spectrum of Ru after illumination was normalized to the Ru concentration of the Fe(II)MbH_2O sample and subtracted. Occasionally, the resulting absorption spectrum went negative at 21,740 cm^{-1} (the peak of the Ru spectrum), implying too much $[Ru(bpy)_3]^{2+}$ contribution was subtracted. This can result from the photobleaching of $[Ru(bpy)_3]^{2+}$. When this occurred, absorption from $[Ru(bpy)_3]^{2+}$ was added to keep the absorption spectrum positive. This correction, only necessary for horse Mb, was approximately 2% of the total Ru concentration or less.

To determine how much Mbmet was not reduced during illumination, the charge transfer band at 15,800 cm^{-1} was used. The main absorption of the intermediate state between 14,285 cm^{-1} and 16,670 cm^{-1} comes from the tail of the Q_0 band. The absorption of the intermediate state in this region was verified by fully reducing a 200 μm thick sample with x-rays. Therefore, the spectrum between 14,925 cm^{-1} (670 nm) and 16,130 cm^{-1} (620 nm) could be fit to a linear baseline plus the absorption spectrum from Mbmet measured at the same temperature and the concentration of unreduced protein determined.

To avoid contamination of the spectrum from Mbdeoxy, the sample was reduced at 20 K. Above 140 K, the protein begins to relax on the time scale of a measurement. To obtain an intermediate state spectrum above 140 K, it is necessary to subtract out the fraction of the sample that has already relaxed to Mbdeoxy. The absorption spectrum of the intermediate state at a temperature T_{exp} was fit to a linear combination of the intermediate state at T_{exp}–10 K, the difference spectrum between the intermediate at T_{exp}–10 K and T_{exp}–20 K, and Mbdeoxy measured at T_{exp}. The total protein concentration was held constant. The temperature dependence of the corrected spectra was checked to verify that the intensity and width of the intermediate state spectral bands varied with temperature in a consistent manner.

Determination of the relaxation kinetics

A spectrum of the solvent taken at the same or a similar temperature was subtracted from the measured spectra. The relaxation kinetics were determined by fitting the measured spectra between 14,286 cm^{-1} (700 nm) and 25,641 cm^{-1} (390 nm) to a linear combination of spectra from Mbmet, Fe(II)MbH_2O, Mbdeoxy, Ru^{2+}, the Ru^{+}– Ru^{2+} difference spectrum at the corresponding temperature, and a quadratic baseline (in frequency) using a Marquardt nonlinear least-squares-fitting routine. The Mb and Ru reference spectra were taken every 10 K with the exception of the intermediate state spectra, and the other temperatures were interpolated from these spectra. Because of the short lifetime of the intermediate state at higher temperatures, the intermediate state spectra were only determined to 180 K and 170 K in sperm whale Mb and horse Mb respectively. These temperatures were used to fit the kinetics at higher temperature. The baseline was necessary to correct for scattering within the sample. The entire time series at one temperature was fit to determine the average Mb and Ru concentrations. The data were then fit a second time with the total Mb and Ru concentrations held constant. A typical fit as well as the individual components of the fit are shown in Fig. 2.

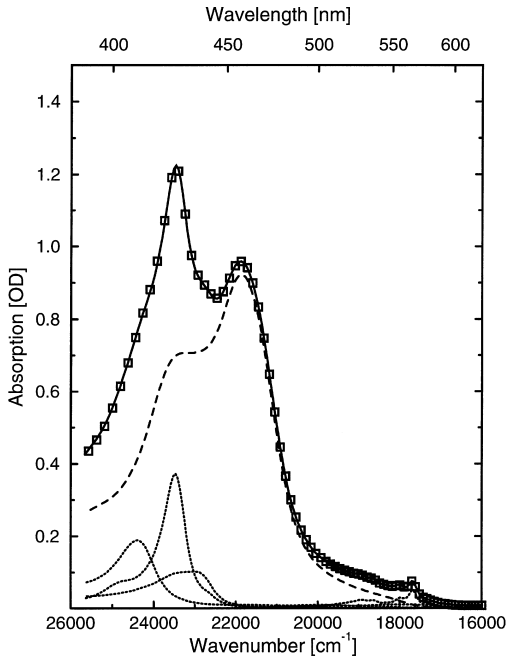

Fig. 2 A spectrum measured during the relaxtion kinetics of sperm whale Mb at 175 K (□). Only one-third of the data points are shown for clarity. The fit to a linear combination of spectra from Mbmet, Fe(II)MbH_2O, Mbdeoxy, and Ru is shown as a *solid line*. The contribution from Ru is shown as a *dashed line* while the individual Mb spectra are shown as *dotted lines*

Results

The reduction of myoglobin

The efficiency of reduction as a function of temperature in sperm whale Mb is shown in Fig. 3. The sample was illuminated at 20 K, 100 K, 160 K, 180 K, and 295 K. At room temperature, it is possible to completely reduce the sample with a single laser flash (1.2 μs, 33 mJ). As the temperature is lowered, it becomes more difficult to reduce the sample. It was observed upon illumination above 80 K that part of the sample was already in the Mbdeoxy conformation even when no thermal relaxation was observable. The

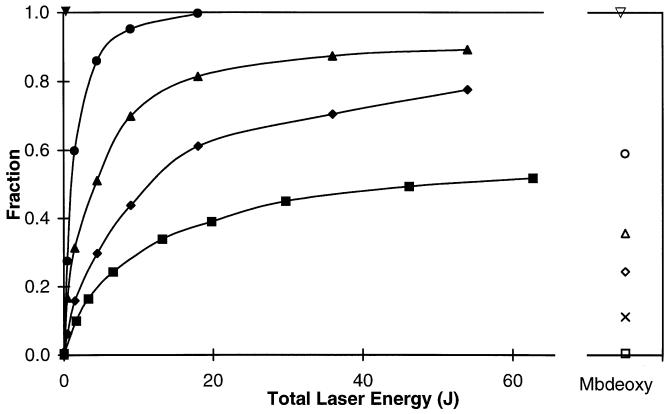

Fig. 3 The fraction of reduced sperm whale myoglobin as a function of laser power multiplied by illumination time is shown for 20 K (■), 100 K (◆), 160 K (▲), 180 K (●), and 295 K (▼). The lines serve as a guide to the eye. The fraction of the sample that is in the Mbdeoxy conformation after illumination is shown with open symbols on the right side of the figure. For comparison, an × marks how much of the sample is in the Mbdeoxy state after the sample is completely reduced with x-rays at 80 K

fraction of the sample that is in the Mbdeoxy state after illumination is shown with the open symbols on the right side of the figure. For comparison, the fraction of Mbdeoxy after illumination with x-rays at 80 K is labeled with an ×. Samples without Ru that were reduced with x-rays showed no increase in Mbdeoxy after illumination. The amount of Mbdeoxy that is generated has a non-linear intensity dependence. To avoid the formation of this light-induced Mbdeoxy, samples for kinetics measurements were reduced at 20 K.

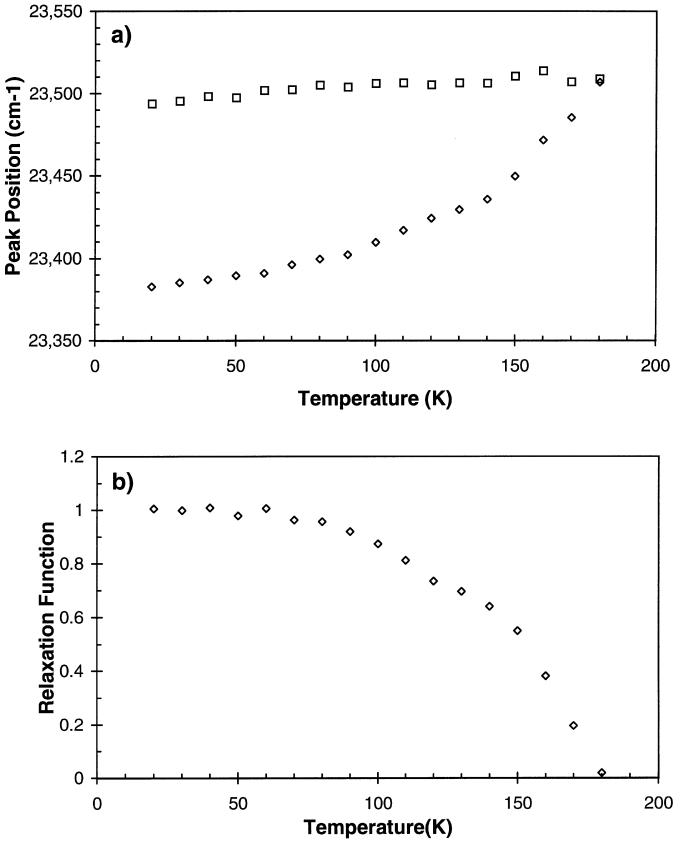

Fig. 4 a The peak position of the Soret band of sperm whale Mb in the intermediate conformation as a function of temperature. The sample was initially reduced at 20 K and spectra collected every 10 K up to 180 K (◇), then cooled to 20 K and the spectra collected a second time (□). **b** The relaxation function as a function of temperature determined by subtracting the temperature dependence of the peak position of the second temperature cycle from the first and normalizing the function to 1 for T ≤ 60 K

Relaxation within the intermediate state

Comparison of the intermediate state spectrum at 20 K before and after warming to 180 K shows a shift in the peak position of the Soret band of 110 cm^{-1}. To investigate this shift in more detail, the temperature dependence of the intermediate state spectra was followed from 20 K to 180 K. The sample was then cooled a second time to 20 K, and the temperature dependence of the spectrum followed again. The peak position of the Soret band as a function of temperature is shown in Fig. 4 a. Spectra collected after cooling to 20 K a third time showed no difference from the second temperature cycle. Assuming that the unrelaxed intermediate state has the same temperature dependence, only shifted by 110 cm^{-1}, as the relaxed intermediate state, provided no relaxation occurred, the relaxation function, $\Phi(t, T)$, can be calculated:

$$\Phi(t, T) = \frac{\nu(t, T) - \nu(\infty, T)}{\nu(0^+, T) - \nu(\infty, T)} \quad (5)$$

where $\nu(t,T)$ is the peak position as a function of time and temperature, $\nu(\infty, T)$ is the peak position of the fully relaxed intermedite state, and $\nu(0^+, T)$ is the peak position of the intermediate state immediately after reduction. The value of $\nu(0^+, T) - \nu(\infty, T)$ is taken as the difference between the unrelaxed and relaxed intermediate state at low temperature, 110 cm^{-1}. The function $\Phi(t,T)$ is shown in Fig. 4 b as a function of temperature. The spectra were taken every 40 minutes. Because of the temperature dependence of the relaxation, the thermal history of the sample at lower temperatures can be ignored.

Kinetics of the Fe(II)MbH_2O → Mbdeoxy relaxation

The populations of Mbmet, Fe(II)MbH_2O, and Mbdeoxy during the relaxation of sperm whale Mb and horse heart Mb at 175 K are shown in Fig. 5. The relaxation in horse heart Mb occurs on a faster time scale than in sperm whale Mb. In both proteins, there is a significant portion (≥18%) of the sample that relaxes between the initial spectrum collected at 130 K and the first spectrum taken ~12 minutes later at 175 K. This fast process occurs too quickly to be resolved at 170 K and above, but is observable at lower temperatures.

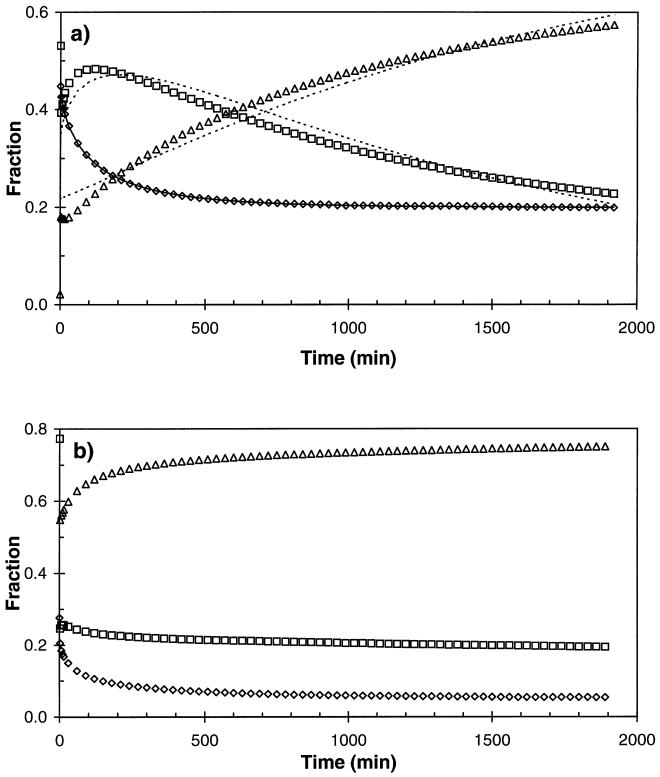

Fig. 5 a, b The fraction of Mbmet (◇), the intermediate state (□), and Mbdeoxy (△) as a function of time at 175 K for sperm whale Mb (**a**) and horse Mb (**b**). For sperm whale Mb, the Mbmet concentration is fit to a stretched exponential (Eq. (7), *solid line*) while the intermediate state and Mbdeoxy concentrations are fit to a single structural relaxation rate (Eq. (9), *dotted line*)

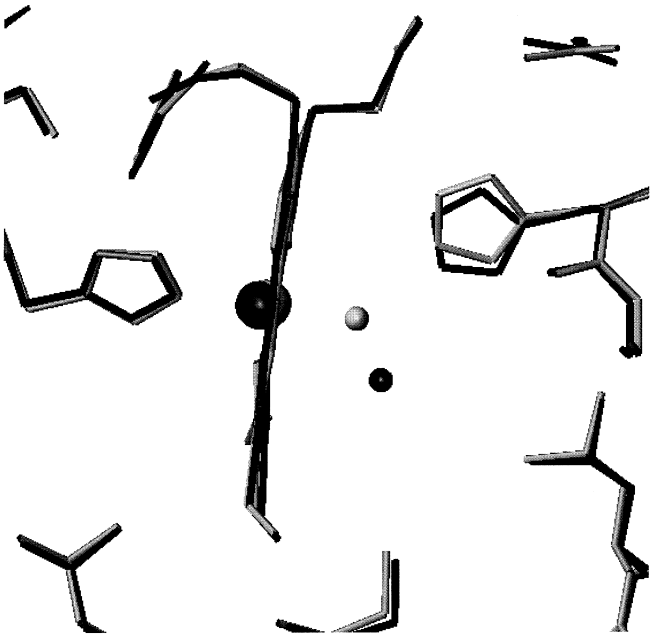

Fig. 6 A comparison between the Mbmet structure (*grey*) and the deoxy structure (*black*) of sperm whale Mb at room temperature. The difference in the structures reflect the movement that occurs as the protein relaxes from the intermediate state to the deoxy conformation

Discussion

The intermediate state

In our experiments, we start from myoglobin in the met structure with the iron displaced 0.15 Å from the mean heme plane and an iron – N_ε distance to the proximal histidine of 2.2 Å. The 6th coordination position is occupied by a water molecule. The structural relaxation ends at Mbdeoxy where the iron is about 0.4 Å out of the mean heme plane, the Fe–N_ε distance is unchanged within the resolution of structural determination, and the 6th coordination position is unoccupied. However, a water molecule is found about 3.6 Å away from the iron within the distal heme pocket. The proximal histidine and the F helix move to accommodate the change in the iron position. With no ligand bound to the heme iron, the distal histidine swings deeper into the heme pocket. The intermediate state should have a structure somewhere in between. Figure 6 shows the x-ray structure around the heme pocket for Mbmet and Mbdeoxy. At low temperatures, the global structure of the protein is frozen. After reduction, the structure of the intermediate state is found to be very similar to the met conformation (Ostermann et al. to be published). Mössbauer spectroscopy shows that the active center is in the Fe(II) low spin configuration (Prusakov et al. 1995, 1996). The water molecule is still bound to the heme iron, even though there is enough room in the intermediate state for the ligand to move away.

The optical spectrum of the intermediate state (Fig. 1 b) has the large absorption coefficient and narrow width of the Soret band that is typical for Fe(II) low spin myoglobin complexes (MbCO in particular). The splitting of the Q_0 and Q_v bands implies that the protoporphyrin is distorted. The structure of the Q_0 and Q_v bands is similar to the spectrum of Mb(Fe(II))–CN (Bellelli et al. 1990; Saigo et al. 1993; Reddy et al. 1996). The peaks are separated by 344 cm^{-1}, 985 cm^{-1}, and 1264 cm^{-1} from the lowest frequency band of Q_0 at 17,620 cm^{-1}. Distortion of the porphyrin is not unexpected, while the overall structure of the active site is in non-equilibrium.

At first glance, it is astonishing that the water molecule does not dissociate after reduction even though there is enough room in the heme pocket of the frozen Mbmet structure. It seems reasonable to assume that it is the iron position with respect to the heme plane and the proximal histidine, which favors the binding of the water. From EPR (Braunitzer et al. 1974) and Mössbauer (Parak and Kalvius 1983) investigations, it has been concluded that the oxygen affinity in hemoglobins is partly regulated by the so called "trans effect". The binding of a ligand with σ donor and π acceptor properties at the 6th coordination position is always in competition with the binding of the iron to the imidazole group which also has σ donor and π acceptor properties. A more in-plane position of the iron with a larger Fe – N_ε distance favors ligation at the 6th coordination site.

The reduction of myoglobin

Figure 3 shows the percentage of the sample reduced as a function of time and temperature. When reducing with $*[Ru(bpy)_3]^{2+}$ at room temperature, it is possible in one laser flash to effectively reduce the entire sample. As the temperature is lowered, it becomes more difficult to reduce the sample. At 20 K, we have only been able to reduce 55% of the protein in the sperm whale Mb samples and 80% of the protein in the horse heart Mb samples. The solvent is the same in these two cases, so the difference should be due to differences in the redox potential of the two proteins. The fact that we are unable to reduce the entire sample using Ru, implies that only proteins in CS with a high redox potential are initially reduced. In contrast, it is possible to reduce 100% of the sample using x-rays. This is a fundamental difference between the kinetics measured with optical spectroscopy after reduction with Ru and those measured previously with Mössbauer spectroscopy after x-ray reduction.

The reduction kinetics

For the kinetic measurements, the sample is illuminated at 20 K and then warmed to 130 K. After 30 minutes at 130 K, the sample was warmed to the temperature of the experiment. Upon warming, additional reduction of the remaining Mbmet occurs from the $EDTA^{\bullet}$ and/or glycerol ($Glyc^{\bullet}$) radicals, $[Ru(bpy)_3]^{+}$, and electrons partially trapped in the frozen glassy solvent that were created by illumination at 20 K. Examination of the Mbmet concentration in Fig. 5 reveals that the additional reduction of Mbmet without illumination is non-exponential. There is a subpopulation of the protein that remains in the met state throughout the experiment. This subpopulation can be ignored since the molecules are not involved in the reaction. If the reaction is limited by the diffusion of the electron donor and the myoglobin to a certain reaction radius, the rate constant for electron transfer is no longer time independent, but is given by

$$k_{et}(t) = \beta\, k^{\beta}\, t^{\beta-1}\,, \tag{6}$$

where k is a pseudo rate constant of electron transfer and β is a constant between 0 and 1 (Berlin et al. 1991). The kinetics of the Mbmet population would then follow a stretched exponential,

$$\text{Mbmet}(t) = \text{Mbmet}(0)\, \exp\{-(kt)^{\beta}\}\,. \tag{7}$$

Figure 7 a shows fits for 160 K, 170 K, and 180 K and k and β are given in Table 1. The variation of β with temperature is similar to what has been measured in Mössbauer experiments of iron in glycerol (Nienhaus et al. 1991).

The non-exponential kinetics could also be explained by a distribution of the electron transfer rates. The electronic coupling drops off exponentially with separation, and would be affected by a distribution of donor-acceptor distances. The Franck-Condon factor depends on the reorganizational energy and the free energy of the electron transfer reaction. Mb is known to exist in many CS and has a large reorganizational energy (Crutchley et al. 1985; Siddarth and Marcus 1993). Both the driving force and the reorganizational energy may depend on the protein CS, and thus, be distributed. However, since in our samples there is no fixed distance between the electron acceptor and donor, the non-exponential electron transfer kinetics are certainly influenced by the diffusion of the electron through the solvent.

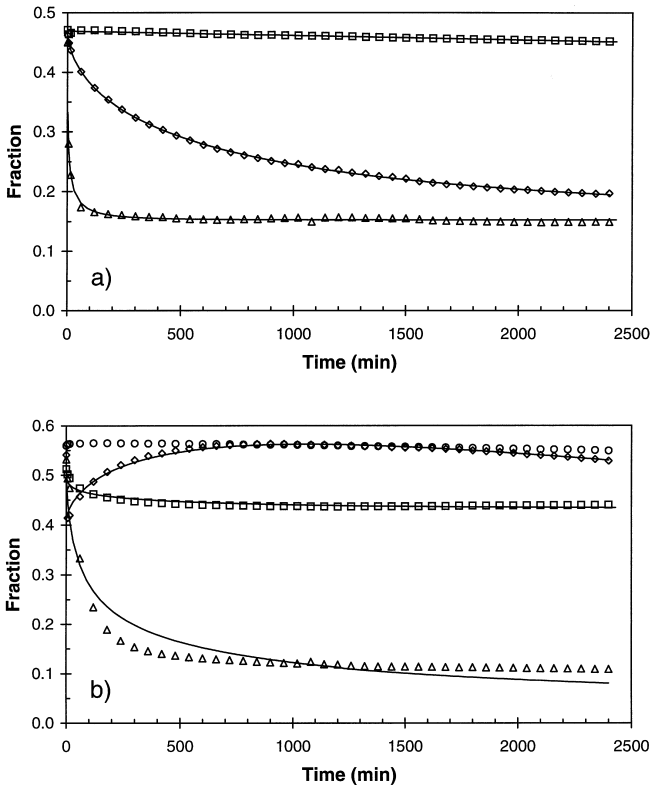

Fig. 7 a The concentration of sperm whale Mbmet as a function of time for 160 K (□), 170 K (◇), and 180 K (△) kinetics. The corresponding fits to a stretched exponential are shown as *solid lines*. The parameters are given in Table 1. **b** The concentration of the sperm whale intermediate state is shown as a function of time for 150 K (○), 160 K (□), 170 K (◇), and 180 K (△). Fits to a Gaussian $f(\text{Log}\,[k_r])$ distribution are shown as *solid lines* for 160 K – 180 K and the parameters are listed in Table 2

Table 1 Parameters for the stretched exponential fits to the decay of [Mbmet]

Temperature (K)	Sperm Whale Mb		Horse Mb	
	β	Log (k)	β	Log (k)
160 K	1.00 [a]	–6.3	1.00 [a]	–6.2
165 K	0.94	–6.4	0.71	–4.9
170 K	0.62	–4.6	0.56	–4.4
175 K	0.66	–3.9	0.36	–3.5
180 K	0.43	–2.6	0.41	–2.5
185 K			0.41	–2.3

[a] Beta was fixed at 1.0

Relaxation within the intermediate state

The intermediate state that is formed upon reduction at low temperature undergoes a structural relaxation at higher temperatures as seen by the shift in the Soret band. Initially, the protein is in the met conformation at 20 K with the heme iron displaced by 0.15 Å from the mean heme plane. An electron is transferred to the heme iron, making it less positively charged, but with the water ligand still bound. In sperm whale Mb, this relaxation results in a 110 cm^{-1} blue-shift of the Soret band from 23,380 cm^{-1} to 23,490 cm^{-1}. The Soret band in Fe(II), six-coordinated structures (23,700 cm^{-1} in MbCO) are, in general, blue shifted from its position in the intermediate state, whereas the Soret band in Mbdeoxy is red-shifted (23,120 cm^{-1}). The blue shift suggests that during the relaxation, the active center relaxes towards a Fe(II), six-coordinated structure with the iron closer to the heme plane than in the Mbmet conformation.

A similar relaxation is found in the Mb*CO → Mbdeoxy relaxation observed in the charge transfer band III after the photodissociation of MbCO. The band, only observable when the heme iron is 5 coordinate, is sensitive to the displacement of the iron from the mean heme plane. Upon photodissociation of MbCO at low temperature, this band is red-shifted from its position in Mbdeoxy by 110 cm^{-1} to 240 cm^{-1} depending on the solvent, pH, and protein concentration of the sample (Iizuka et al. 1974; Steinbach et al. 1991; Nienhaus et al. 1992). As the structure of the protein changes from the photodissociated structure to the deoxy conformation, the iron moves from about 0.3 Å to about 0.4 Å from the mean heme plane and the position of band III in Mb*CO shifts to the value observed in Mbdeoxy (Schlichting et al. 1994; Hartmann et al. 1996; Nienhaus et al. 1992; Lim et al. 1993).

The function for the relaxation within the sperm whale intermediate state is shown in Fig. 4b. The relaxation occurs between 100 K and 170 K (Φ between 0.9 and 0.1). The 1/e point corresponds to 160 K. The relaxation has a strong temperature dependence, so that the duration of the measurements at lower temperatures can be ignored. Thus, the rate for Φ at 160 K in Fig. 4b corresponds to 4×10^{-4} s^{-1}. Taking the parameters from Steinbach et al. (1991) for comparison, the rate for the Mb*CO at 160 K would be 3×10^{-7} s^{-1}, 3 orders of magnitude slower. Since the kinetics are non-exponential in time, non-Arrhenius in temperature, and measured on different time scales, a direct comparison is difficult. However, it is clear that the relaxation we observe within the intermediate state is faster than the Mb*CO → Mbdeoxy relaxation (at least below 170 K). This suggests that there is less structural rearrangement between the relaxed and unrelaxed intermediate state than between Mb*CO and Mbdeoxy. The relaxation within the intermediate state demonstrates that structural rearrangements can occur in proteins even at temperatures of 100 K.

The difference in time scales that we observe between the relaxation within the intermediate state and the Mb*CO → Mbdeoxy relaxation is consistent with the motions occurring along different tiers. The idea of a hierarchical arrangement of CS was put forth by Frauenfelder and collaborators (Ansari et al. 1985; Ansari et al. 1987; Frauenfelder et al. 1991; Steinbach et al. 1991). In a hierarchy, the energy barriers between CS of higher tiers becomes successively smaller. Fluctuations among CS of different tiers occur at different energies and thus are observable at different temperatures.

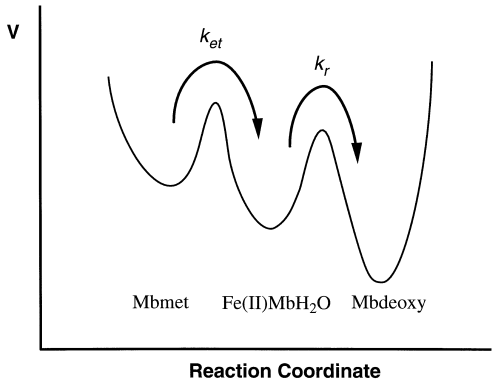

Fig. 8 A schematic of the three well model used to describe the structural relaxation kinetics

For the current discussion, we have correlated the structural changes occurring during the relaxation within the intermediate with the distance of the Fe from the mean heme plane. This correlation has been made to facilitate the discussion. However, other structural parameters are also relevant to the relaxation, such as the Fe – N_ε bond distance, or the orientation of the proximal histidine.

Kinetics of the Fe(II)MbH$_2$O → Mbdeoxy relaxation

Above 140 K, the structural relaxation from Fe(II)MbH$_2$O to Mbdeoxy is observed. The population of the intermediate state in sperm whale Mb as a function of time and temperature is shown in Fig. 7b. Because of the additional reduction that occurs during the experiment, a three well model (shown schematically in Fig. 8) is the minimum necessary to describe the kinetics. Before illumination, the sample is in the Mbmet state. After illumination, at t = 0, the population is split between the Mbmet and intermediate state. The rate equations are given by:

$$\begin{aligned} d\,\mathrm{Mbmet}(t)/dt &= -k_{et}(t)\,\mathrm{Mbmet}(t) \\ d\,\mathrm{Fe(II)MbH_2O}(t)/dt &= -k_r\,\mathrm{Fe(II)MbH_2O}(t) \\ &\quad + k_{et}(t)\,\mathrm{Mbmet}(t) \\ d\,\mathrm{Mbdeoxy}(t)/dt &= k_r\,\mathrm{Fe(II)MbH_2O}(t) \end{aligned} \tag{8}$$

where k_{et} is given by Eq. (6) to provide the stretched exponential dependence of the Mbmet population [Eq. (7)]. Assuming a single rate for the relaxation, k_r, the concentration of the intermediate state is given by:

$$\begin{aligned} \mathrm{Fe(II)MbH_2O}(k_r,t) &= \mathrm{Fe(II)MbH_2O}(0)\exp(-k_r t) \\ &+ \mathrm{Mbmet}(0)\,\beta k^\beta \int_0^t dt'\,t'^{\beta-1}\exp\left\{-(kt')^\beta + k_r(t-t')\right\}. \end{aligned} \tag{9}$$

Table 2 Parameters of the Gaussian f (Log [k_r]) distribution used to fit the concentration of the intermediate state (Eqs. (9)–(11))

Temperature (K)	Sperm Whale Mb		Horse Mb	
	Log [k_{pk}]	σ	Log [k_{pk}]	σ
160 K	–8.4	8.3	–6.1	2.6
170 K	–5.8	0.3	–2.2	4.5
180 K	–3.4	1.6	–0.8	4.1

A fit to a single exponential rate at 175 K is shown in Fig. 5 a. The quality of the fit suggests that the kinetics are non-exponential. If k_r is distributed for the relaxation from Fe(II)MbH_2O to Mbdeoxy, the population of Fe(II)MbH_2O is given by:

$$\mathrm{Fe(II)MbH_2O}(t) = \int d\{f(\mathrm{Log}[k_r])\} \cdot f(\mathrm{Log}[k_r])\,\mathrm{Fe(II)MbH_2O}(k_r, t), \quad (10)$$

where f (Log [k_r]) is the probability that a protein has a logarithm of the rate between Log [k_r] and Log [k_r] + d Log [k_r]. Figure 7 b shows the fits of the kinetics to a Gaussian f(Log [k_r]) distribution,

$$f(\mathrm{Log}[k_r]) = \frac{\exp\left\{-(\mathrm{Log}[k_r] - \mathrm{Log}[k_{pk}])^2/2\sigma^2\right\}}{\sqrt{2\pi}\,\sigma}, \quad (11)$$

where Log [k_{pk}] is the peak of the distribution and σ is the width. The fit parameters are listed in Table 2. We have not yet succeeded in fitting the kinetics between 160 K and 190 K to a static distribution with an Arrhenius temperature dependence. Each temperature except for 180 K could be fit individually with a Gaussian distribution. From MbCO flash photolysis kinetics, it is known that structural relaxations in Mb begin between 160 K and 180 K (Steinbach et al. 1991). Since our measurements are conducted on longer time scales, it is not surprising that the relaxation kinetics from Fe(II)MbH_2O to Mbdeoxy cannot be fit with a static distribution.

There is a fast component of approximately 18% at 175 K that relaxes to deoxy before the first measurement could be made (Fig. 5). We have not included the fast component of the relaxation in the fits above. The fast component arises from CS that have a low enthalpy barrier between the intermediate state and the deoxy conformation. It is possible to reduce 100% of a sample with x-rays at 80 K. When fully reduced, approximately 10% of the sample is in the deoxy conformation, even at 80 K, suggesting that some CS have even faster relaxation times. Since no deoxy was observed at 130 K after reduction with *$[Ru(bpy)_3]^{2+}$ at 20 K, the fraction of proteins that relaxed at 80 K in the x-ray reduced samples was unreduced by the *$[Ru(bpy)_3]^{2+}$ at low temperature. This suggests that there may be a correlation between the redox potential for reduction and the barrier height for the relaxation to Mbdeoxy and that they may be dependent on the same conformational coordinate. As the temperature is raised, the proteins in these low barrier CS are reduced and quickly relax. This also implies that partial reduction only selects the high barrier part of the distribution of relaxation rates.

In trying to fit the structural relaxation from Fe(II)MbH_2O to Mbdeoxy, we have ignored the relaxation that occurs within the intermediate state. Analogous to the Mb*CO → Mbdeoxy relaxation, we would expect that the relaxation within the intermediate state influences the Fe(II)MbH_2O → Mbdeoxy relaxation. As the structure of the protein rearranges to that of the Fe(II) six-coordinate conformation, the barrier for relaxation between the intermediate state and Mbdeoxy will be affected. More investigations will be necessary to understand the fast and slow components of the Fe(II)MbH_2O → Mbdeoxy relaxation and how it is influenced by the relaxation occurring within the intermediate state.

The intermediate state population at 150 K is shown in Fig. 7 b as a function of time. Approximately 1% of the sample relaxes to Mbdeoxy in 40 hours. This is considerably less than the relaxation of 5% observed in 5 hours at 147 K with Mössbauer spectroscopy. It was also possible to fit the relaxation kinetics measured with Mössbauer spectroscopy at 180 K and below to a static distribution. This discrepancy may arise from the partial reduction at low temperature. The entire sample can be reduced using x-rays, so the full distribution of relaxation rates is measured. When reducing with *$[Ru(bpy)_3]^{2+}$, full reduction of the sample is not possible. As discussed earlier, a relationship between the redox potential and relaxation rate is observed. Thus, the CS with low barriers for relaxation to Mbdeoxy are mostly unreduced at 20 K and hence are missing in the structural relaxation measurements below 170 K. The fast component that is observed above 170 K comes from the additional reduction of these CS during warming. The Fe(II)MbH_2O → Mbdeoxy relaxation measured with Ru reduction begins at the same temperature (160 K) where the relaxation within the intermediate state is still observable.

Conclusions

We have demonstrated a new technique for investigating structural relaxations in proteins. By using $[Ru(bpy)_3]^{2+}$ to reduce proteins at low temperature rather than x-rays, we can investigate dynamics on a faster time scale than was possible with Mössbauer spectroscopy (Prusakov et al. 1995, 1996). At room temperature, we can completely reduce the sample with a single laser flash. Therefore, dynamics on a faster time scale could be measured with faster equipment.

Reduction relaxation experiments have an advantage over relaxations measured from photodissociation kinetics. When monitoring rebinding kinetics, the signal disappears as the ligand rebinds while reduced Mb is stable for time scales considerably longer than those of experimental interest. At 180 K, the rebinding of CO occurs in about 1 s. However, the structural relaxation after reduction occurs only in a single direction, Mbmet → Interme-

diate State → Mbdeoxy. Therefore, the kinetics can be measured on much longer time scales.

One result is very clear from these studies. Intermediate states can be created upon reduction. When measuring electron transfer rates, it is important to choose the monitoring wavelength carefully. Typically, electron transfer rates are determined by measuring fluorescence life times at the Fe^{3+}/Fe^{2+} isosbestic point. However, the Fe^{3+}/Fe^{2+} isosbestic point is not the same as the isosbestic point between the oxidized and the intermediate states. Furthermore, there are changes in the intermediate state spectrum due to a relaxation of the protein. Therefore, it is possible that measured electron transfer rates in the literature are a mixture of electron transfer rates and protein relaxations. By monitoring and fitting the complete visible spectrum, we are able to distinguish between the electron transfer kinetics and protein relaxation.

On similar time scales as the structural relaxation from Fe(II)MbH_2O to Mbdeoxy, we have observed a relaxation within the intermediate state. It appears that the relaxation within the intermediate state influences the reaction coordinate for the relaxation of Fe(II)MbH_2O → Mbdeoxy, although the mechanism is not yet clear. This coupling of the reaction and conformational coordinates is also observable in the Mb*CO → Mbdeoxy relaxation and appears to be a common phenomena in proteins.

Both the electron transfer kinetics and protein relaxation that we have measured show non-exponential behavior. Non-exponential kinetics appear to be typical for myoglobin, if not for proteins in general.

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