On the Origin of Heme Absorption Band Shifts and Associated Protein Structural Relaxation in Myoglobin following Flash Photolysis*

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The role of the protein structural change monitored by absorption band shifts following flash photolysis of CO from myoglobin is discussed in terms of structure-function relationships. Evidence is presented that the Soret band shift does not depend primarily on the covalent linkage of the heme iron to the protein by using the mutation H93G(L) in which the proximal histidine 93 is replaced by glycine and an exogenous ligand L, which coordinates the heme iron but is not covalently bound to the globin. While CO rebinding kinetics depend strongly on the nature of the exogenous ligand L in H93G(L), the magnitude and time evolution of the Soret band shift in a viscous buffer on the nanosecond time scale are hardly perturbed in all cases studied. Comparison of the Soret band and charge transfer Band III shifts demonstrates that both have a similar time dependence on the nanosecond to microsecond time scale following flash photolysis in viscous solvents. We conclude that the nonexponential kinetics of protein relaxation probed by transient absorption band position shifts involves primarily distal coordinates prior to ligand escape. This result agrees with earlier measurements of Soret band shifts in distal pocket mutants of myoglobin (1). We suggest that the band shifts are primarily a response to changes in the electrostatic field around the heme (a transient Stark shift) associated with changes in protein structure that occur following ligand photodissociation.

The nature of the protein structural changes that control the competition between ligand rebinding (geminate recombination) and ligand escape remains an unsolved problem for heme proteins such as myoglobin (Mb).[1] A widely held hypothesis asserts that the barrier to ligand rebinding and hence the geminate rate is controlled by forces that the proximal histidine, histidine 93, and associated F α-helix exert on the iron displacement from the heme plane. At the same time, the conformation of amino acid residues in the distal (or diatomic ligand binding) pocket must also affect ligand dynamics within the globin and escape from the protein, as shown by the diverse behavior of both CO and NO rebinding for a large number of distal pocket mutations (1–7). Furthermore, recent transient

IR data have been interpreted as demonstrating a stable docking site for CO in the geminate state at room temperature (8), and this requires that there be specific and rapid conformational changes that persist for hundreds of nanoseconds.

There is indirect evidence for rapid (subnanosecond), but highly nonexponential protein relaxation at physiological temperatures from several different techniques (1, 8–10). Time-dependent shifts in absorption (1, 9, 11) and Raman bands (12) have often been interpreted in terms of a constraint by proximal amino acid residues on the iron displacement out of the heme plane; however, recent time-resolved resonance Raman experiments with subpicosecond resolution indicate that iron displacement from the heme following ligand photodissociation is extremely rapid and complete within several picoseconds (13, 14). In this paper we consider band shifts in the Soret band (a_{2u}, $a_{1u} \rightarrow e_g$) (7) and charge transfer Band III ($a_{2u} \rightarrow d_{yz}$) (15) as spectroscopic markers of protein structural relaxation following flash photolysis of CO- ligated Mb.

The hypothesis that the iron atom displacement influences the Soret and Band III absorption band center frequencies rests on the idea that changes in the overlap between the d_{yz}, d_{xz} (or d_π) orbitals of the iron with e_g π^* excited state orbitals of the porphyrin affect the absorption energy. Accordingly, constraints on the out-of-plane heme iron motion would also control the ligand rebinding barrier, and this could give rise to a correlation between spectroscopic observables and rebinding rates. These ideas have been developed most extensively in low temperature studies of kinetic hole burning observed in Band III, where changes in the iron coordinate should cleanly affect the energy difference between porphyrin a_{2u} and iron d_{yz} orbitals (16, 17). The hypothesis that protein relaxation can affect ligand rebinding has been debated for NO geminate rebinding, which occurs on a time scale comparable with the rapid phase of protein relaxation at ambient temperature (5, 6). By contrast the recombination rate of the most studied ligand, CO, is several orders of magnitude slower (18), and hence protein relaxation would be expected to affect the CO rebinding rate only under conditions of very high viscosity or at lower temperatures, *e.g.* near the glass transition temperature (17). The rebinding of the physiologically important ligand O_2 is highly nonexponential even at ambient temperature with a rapid phase that is more rapid than the protein relaxation (19), but little is known about the relationship of these rebinding components to structure.

Although there are x-ray structures of the bound state (20) and a geminate state trapped at low temperatures (21, 22), to date there are no methods that allow a direct view of the protein as it undergoes conformational relaxation. Unfortunately, the hypothesis of proximal control over the rebinding barrier depends on changes in position of the iron-histidine complex on the order of 0.1 Å or less that are difficult to detect

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[1] The abbreviations used are: Mb, myoglobin; Hb, hemoglobin; Im, imidazole; 4-Me Im, 4-methyl imidazole; 1-Me Im, 1-methyl imidazole; MbCO, horse heart myoglobin; HbCO, human hemoglobin.
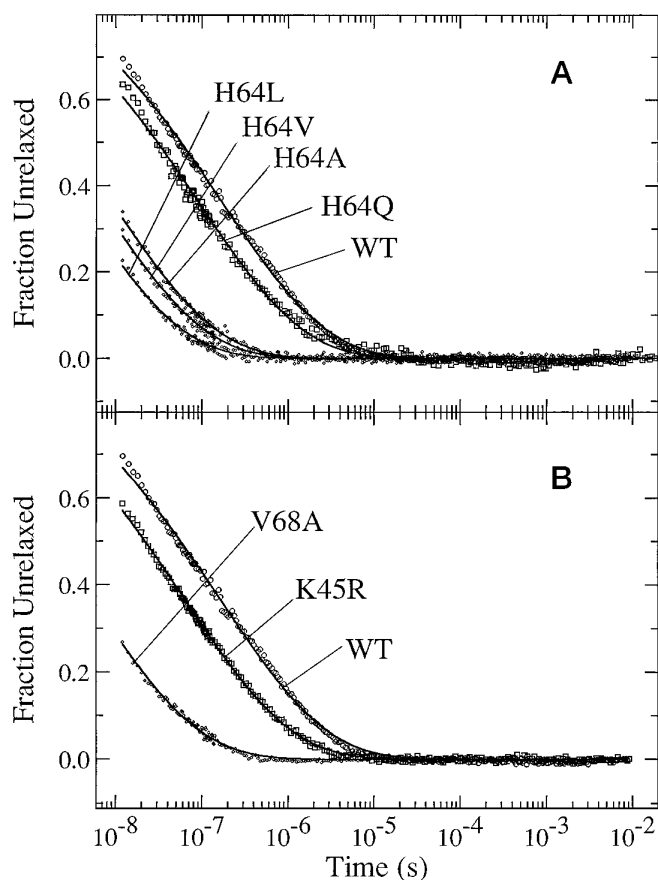
FIG. 1. **The time and temperature dependence of the Soret band frequency shift in distal side myoglobin mutants and wild type.** The data show large differences in a protein relaxation as monitored by the Soret band frequency shift as measured by Lambright *et al.* (1). A correlation between the rate of the relaxation of the Soret band frequency and the CO rebinding kinetics was also observed for the distal side mutants.

FIG. 2. **A depiction of the proximal cavity of the H93G mutant of sperm whale myoglobin demonstrating the possibility of proximal ligand exchange.** The coordinates for the figure are taken from the x-ray crystal structure of the met-aquo (Fe^{III}) form of the protein (26). The distal side of the protein is essentially identical to that found in wild-type (*WT*) sperm whale myoglobin.

these experiments leave open the question of whether the proximal histidine-iron bond also plays a significant role.

In this paper we present data obtained on the proximal cavity mutant of Mb in which the proximal histidine 93 has been replaced by glycine (Fig. 2) by means of site-directed mutagenesis (25), and exogenous proximal ligands L to the iron can be dialyzed into the proximal cavity denoted H93G(L) (26). The H93G(L) series addresses both aspects of the structural question raised above. Because the covalent connection between the heme iron and protein is severed, the possibility of conformational substates specifically linked to the F α-helix affecting the heme iron position is removed or at least greatly reduced. The substitution of different exogenous ligands allows for alteration of the barrier to ligand recombination independent of the protein conformation which has been characterized in the metCN and CO forms by NMR spectroscopy (27)[2] and for Im in the met-aquo form by x-ray crystallography (25). By comparing CO rebinding kinetics as a probe of the barrier to rebinding with the Soret band shift as a probe of protein conformational relaxations, we can determine whether constraints at the heme iron or alternatively distal pocket conformations are playing a major role in the protein relaxation. The data suggest that the former are not important, in contrast with the prevailing view that the proximal histidine is the primary determinant of protein relaxation dynamics.

even in a high-resolution x-ray crystal structure. The high resolution x-ray crystal structure of a photolyzed intermediate trapped at 20 K indicates that iron out-of-plane motion is at least 80% complete, but it also reveals relatively large changes in the conformation of the distal pocket, in particular a displacement of the distal histidine, histidine 64, away from the iron (22). The energetic barriers to protein relaxation are not known, and therefore it is not possible to relate the 20 K structure directly to the dynamics at room temperature.

An alternative approach to determining the role of proximal or distal control over ligand rebinding could emerge from correlations based on single- and multiple-site mutations on both the proximal and distal side that selectively alter the rebinding barrier and relaxation dynamics. We demonstrated several years ago that the Soret band position immediately following photolysis of CO was not identical to the equilibrium deoxy spectrum, but that it evolved to that spectrum on the time scale of tens of nanoseconds, which is considerably faster than geminate rebinding (23). This relaxation has been observed in the frequency shift of Band III as well (8), and the Soret band shift was later used to characterize the coupling between bulk solvent viscosity and internal protein dynamics (24). As shown in Fig. 1, we also demonstrated that the time scale for relaxation sensed by the Soret band shift varies over a considerable range in a series of *distal* pocket mutants and that there is a correlation between the relaxation rate and the ligand recombination rate in these mutants (1). This connection is important because it relates the relaxation process to function; however,
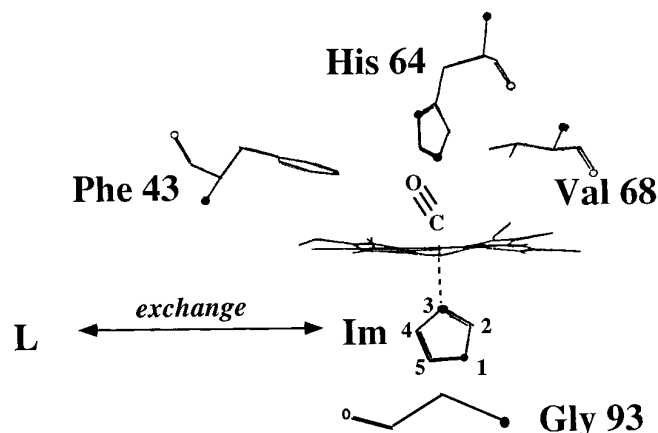
EXPERIMENTAL PROCEDURES

The sperm whale Mb mutant H93G was prepared and purified by methods described in detail elsewhere (25, 26). Samples containing different exogenous ligands, L, in the proximal cavity (L = imidazole (Im), 4-methyl imidazole (4-Me Im), or 1-methyl imidazole (1-Me Im)) were prepared by dialysis (26). The CO-containing samples were prepared by saturating a 75% glycerol, 25% phosphate buffer solution (w/v) at pH 7 with 1 atmosphere of CO and injecting a concentrated aliquot of H93G(L) or wild-type Mb. Transient absorption measurements were performed in two spectral regions, the intense Soret band that has a maximum at 432 nm in H93G deoxymyoglobin and in the region of the very weak charge transfer Band III with a maximum at 762 nm. The sample concentration used for Soret transient absorption experiments was ≈5 μM (A_{420} ≈ 0.8 at the peak of the CO-bound Soret band at 420 nm). The samples were reduced to the Fe(II) state by rinsing a 2-μl aliquot of the sample in a syringe that had been previously rinsed with 1 M sodium dithionite. Comparison of the band shifts in the Soret band and Band III were done only for commercially available horse heart myoglobin (Sigma). We have shown elsewhere by NMR spectroscopy

that the distal pocket structure of the CO complexes is essentially identical to wild-type.[2]

Transient absorption spectra were obtained using an apparatus and methodology described in detail elsewhere (23). Spectral band shifts were analyzed by fitting the peak of the deoxy-Mb band to a polynomial to accurately determine the peak position as a function of time. This method gives results comparable with the singular value decomposition results presented in earlier work. Both the protein relaxation and rebinding kinetics are nonexponential and direct quantitative comparisons are not made here. The distinction that we point out in the present work is the similarity of the nonexponential protein relaxation progress curves for various proximal ligands in contrast with CO rebinding kinetics that are significantly altered. We will present a detailed kinetic analysis elsewhere. From the observed kinetics the CO ligand recombination occurs subsequent to the protein relaxation at $T > 250$ K. By $T \approx 250$ K there is some amount of recombination in the fastest subpopulation.

RESULTS

Transient Spectra of the Soret Band—The spectral changes in the Soret band region following photolysis of MbCO by a laser flash have been presented in earlier work (1, 23). Following photodissociation of the ligand, the Soret band of the CO-bound form (six-coordinate Fe^{II}, peaked at 420 nm) bleaches, and the Soret band of the deoxy form appears (five-coordinate Fe^{II}, peaked at 430–435 nm). The peak positions of the equilibrium deoxy Soret bands differ for wild type and for each substitution in the proximal cavity of H93G(L) as shown in Table I. Prior to ligand recombination in viscous solvents, spectral shifts are observed in both the deoxy (430–435 nm) and CO-bound (419–420 nm) Soret bands. The deoxy Soret band shift is a common feature to H93G(L) and wild type and is a nonexponential relaxation that is observed to slow as the solvent viscosity is increased. The CO-bound band shift is due to kinetic hole burning and is observed only when the rebinding rate is faster than the slowest phase of protein relaxation as it arises from differential rebinding rates to different conformational states of unrelaxed myoglobin (24). In the results reported here, the CO-bound Soret band shift is significant only for H93G(1-Me Im) at 250 K, as this species has the most rapid rebinding process at the lowest temperature studied.

CO Ligand Recombination Kinetics—The ligand rebinding progress curves for wild type, H93G(Im), H93G(1-Me Im), and H93G(4-Me Im) at 250, 270, and 290 K are shown in Fig. 3. The ligand rebinding kinetics of all H93G(L) mutants are nonexpo-

nential, more rapid than wild type, and the geminate yields are larger than wild type at all temperatures studied. The order for rebinding rate (and geminate yield) at all temperatures is H93G(1-Me Im) > H93G(Im) > H93G(4-Me Im) > wild type. The geminate rate coefficient is dominated by ligand escape at the highest temperatures, and we demonstrate elsewhere that the ligand escape rates are very similar for all of the species studied here.

Soret Band Shift—For a quantitative comparison of time-dependent Soret band shifts, the peak position was determined by fitting the Soret line shape to a polynomial and finding the maximum of the polynomial (1, 8, 23). The time and temperature dependence of the deoxy Soret band frequency shift with respect to deoxy heme at equilibrium is presented in Fig. 4. There is a small systematic decrease in the magnitude of the total band shift for all of the H93G(L) mutants compared with wild type; however, the time dependence of the band shift is identical within the signal-to-noise at all temperatures in all of the H93G(L) species despite the observation that the CO rebinding rates span more than an order of magnitude for these mutants. The ligand recombination kinetics and Soret band shift relaxation were also studied in H93G(pyridine), H93G(4-Br Im), and H93G(1,2-diMe Im), all of which are as rapid as H93G(1-Me Im), but show different ligand rebinding progress curves. In all cases the Soret band relaxation time dependencies were identical to those of 1-Me Im, 4-Me Im, and Im (data not shown). The deoxy band shift time dependence in these photolyzed species is nonexponential as has been observed previously for distal mutants (1) and for shifts in Band III (9).

Relation to Band III Shift—The charge transfer Band III, assigned as a $a_{2u} \rightarrow d_{yz}$ transition (15), is present only in five-coordinate deoxy heme and hence appears in the difference spectrum of photolyzed MbCO. The largest kinetic phase of relaxation occurs on the picosecond time scale in buffer solution at room temperature, but the relaxation of Band III is slowed significantly by increasing the solvent viscosity or lowering the temperature. This is shown in Fig. 5 on the nanosecond to millisecond time scale at 250 K. The nonexponential Soret band frequency shift is correlated with the Band III frequency shift as seen in the direct comparison of their relaxation kinetics at temperatures of 250 and 290 K in a 3:1 glycerol/buffer mixture shown in Fig. 6. The protein relaxation associated with the Soret and Band III shifts ceases altogether below the glass transition temperature (17) or in the high viscosity limit (31). At cryogenic temperatures both the Soret band and Band III have the same frequency shift ($\sim$137 cm^{-1}) as determined by comparison of their photoproduct spectra at 10 K (16). As shown in Figs. 5 and 6, above the glass transition temperature the largest total Soret or Band III frequency shift recorded at 250 K on the 10–100-ns time scale is considerably less than the $\sim$137 cm^{-1} shift seen in the photoproduct spectrum at 10 K

TABLE I
Peak position of Soret band in deoxy myoglobin and H93G(L)

Species	Soret λ_{max}
	nm
Wild type	434
H93G(4-Me Im)	433
H93G(Im)	432
H93G(1-Me Im)	428

FIG. 3. **The kinetics of CO ligand rebinding following ligand photolysis of H93G(L)CO and wild type at 250, 270, and 290 K in 75% glycerol, 25% buffer solution.** The rebinding progress curves are shown in *solid lines* in order from *top* to *bottom* as wild-type (*WT*), H93G(4-Me Im), H93G(Im), and H93G-(1-Me Im) at all three temperatures.
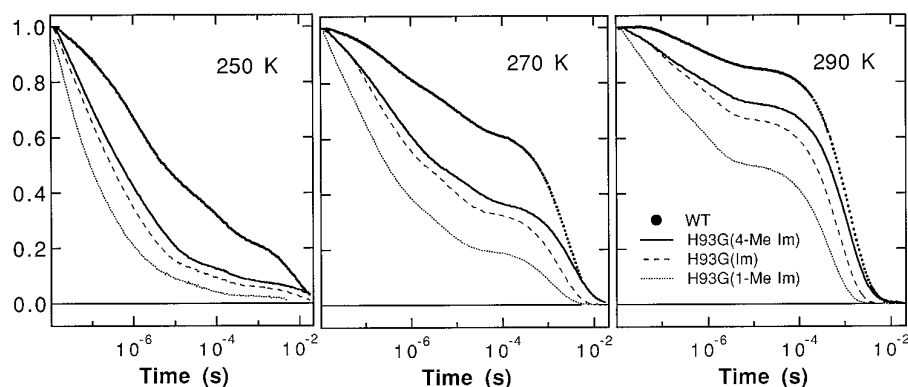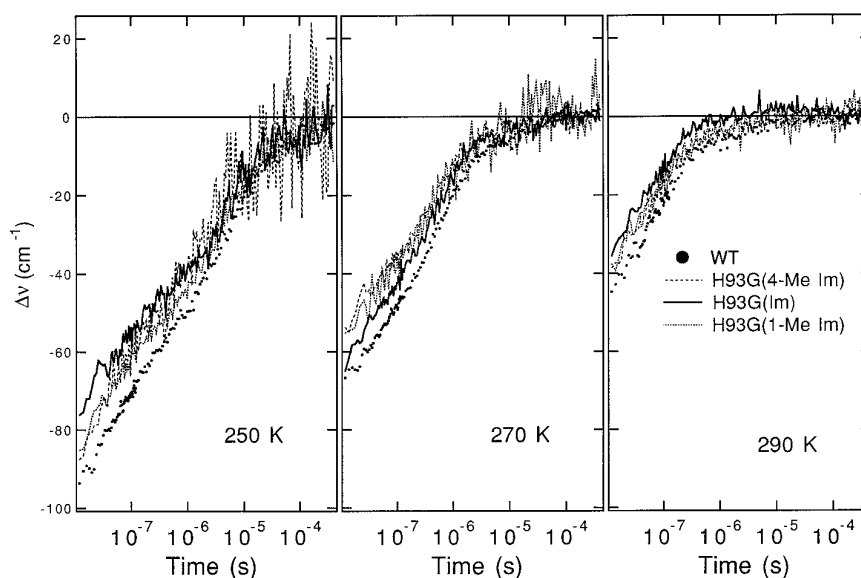
Fig. 4. **The time and temperature dependence of the Soret band frequency shift in H93G(L) and wild type.** The frequency shift was obtained from the time dependence of peak frequency of the deoxy Soret absorption band following photolysis of H93G(L)CO and wild type (*WT*) MbCO at 250, 270, and 290 K.

(16), indicating that there is a component of the relaxation that is more rapid than 10 ns even at 250 K. Data on the Band III shift for H93G(L) in nonviscous buffer will be published elsewhere.[3]

DISCUSSION

The key result is that the CO ligand rebinding kinetics vary greatly for different exogenous proximal ligands in photolyzed H93G(L)CO (Fig. 3), while the Soret band shift kinetics are identical for the same set of proximal ligands (Fig. 4). The Soret band shift is apparently a response to some significant protein relaxation that occurs following photodissociation. The comparison of protein relaxations for various proximal ligands in H93G(L) myoglobin demonstrates empirically that the structural origin of the relaxation resides in degrees of freedom that are not directly connected with the heme through the iron-proximal histidine-protein bond. The observation of identical relaxation kinetics for exogenous proximal ligands with differences in steric bulk (*e.g.* methyl substitution) and hydrogen bonding (*e.g.* *N*-Me Im cannot hydrogen bond to Ser-92) further suggests that noncovalent interactions on the proximal side are not producing relaxations of the heme iron in H93G(L). Changes in solvent viscosity that alter the dynamics of nonexponential protein dynamics are thus affecting other aspects of protein structure. This result is consistent with the observation that distal mutants of Mb do produce significant changes in the relaxation dynamics (1) as shown in Fig. 1. Moreover, this conclusion is also corroborated by subpicosecond time-resolved resonance Raman data obtained on photolyzed HbCO, MbCO, and H93G(Im) MbCO that show no heme iron dynamics on the picosecond time scale (13, 14). The absence of a change in the intensity or frequency of the structure-sensitive iron-histidine out-of-plane mode at ~ 223 cm^{-1} on the picosecond time scale can be contrasted with the Band III frequency shift indicative of (protein) relaxation on the picosecond to nanosecond time scale (30). These results provide no evidence for a linkage between protein conformational substates and heme spectra via the covalent heme iron connection to the F α-helix.

A possible explanation for the observed relaxation phenomena is a change in the distal pocket structure in response to ligand photodissociation. Evidence for distal pocket relaxation has been obtained by double flash excitation experiments that indicate that the relaxation time for the static distribution of the protein conformations that give rise to the observed geminate kinetics is of the order of 50 μs in glycerol buffer at 264 K (31). This time scale is consistent with a distribution of protein conformations (B states) measured by a Soret band shift relaxation having 50–60 μs as its slowest phase. Recent measurements of the evolution of B states using time-resolved IR spectroscopy indicate that the relaxation event associated with the Soret band shift that occurs on the 10-ps time scale in buffer solution is likely a response of the heme to an evolution from an initial B state structure to a more relaxed structure that precedes the opening of the distal pocket to allow ligand escape (9).

If a change in the overlap of the iron d_{yz} orbital with the a_{2u} orbital of the porphyrin is not the origin of the shifted Soret band, we must propose an alternative mechanism. The Soret band is sensitive to the electrostatic configuration of the protein surrounding the heme.[4] The distinction between the electrostatic field of different protein conformers and specific chemical interactions on the bound CO stretching frequency has been discussed elsewhere (32, 35). Analogous effects of the protein environment and changes in iron bonding can be formulated for heme electronic spectra (32). If the electrostatic field of the protein can affect spectral features, it is reasonable to suggest that the structural relaxation that follows photodissociation of CO could affect the electrostatic environment of the heme, and this could be the physical origin of the shifts observed in both charge transfer Band III and the Soret band, *i.e.* these would be transient Stark shifts. This would provide a unified origin for the Soret and Band III shifts and an explanation effect of distal mutants on the Soret band shift (Fig. 1).

It is also of interest to compare the temperature and viscosity dependence of the Soret band shift progress curve with the progress of ligand rebinding. The interpretation of such a comparison depends on the total magnitude of the shift at zero time. If we take this value to be ~ 140 cm^{-1}, consistent with the 10 K photoproduct spectrum, the band shift relaxation is more rapid than the geminate CO recombination kinetics of any of the species studied at 290 K. This observation is consistent with the room temperature studies of photolyzed MbCO in buffer that show picosecond protein dynamics (9) in a system where the CO recombination time is of the order of 180 ns (18, 23). As the medium viscosity is increased and the temperature

[3] M. Lim, T. A. Jackson, P. A. Anfinrud, S. M. Decatur, and S. G. Boxer, manuscript in preparation.

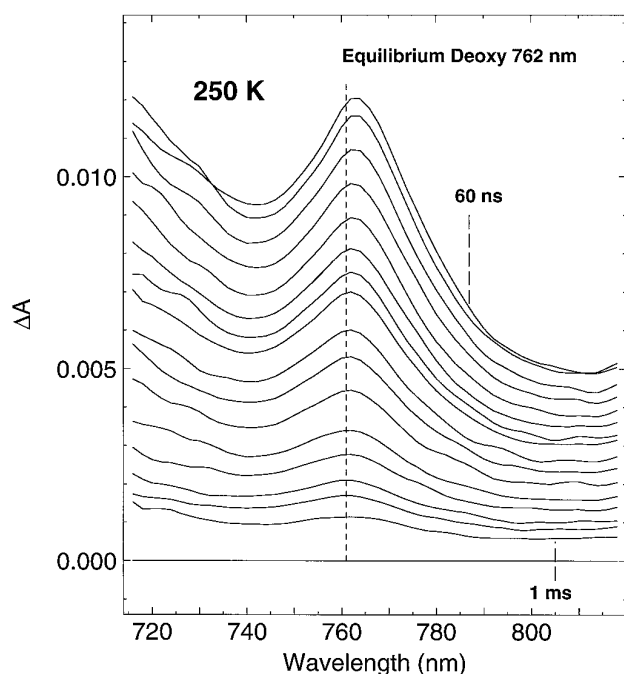[4] S. Franzen, L. J. Moore, and S. G. Boxer, manuscript in preparation.

FIG. 5. **The time-resolved difference absorption spectra of photolyzed myoglobin in the near-infrared region of the spectrum.** The Band III region ΔA is shown for selected times ranging from 60 ns to 1 ms. The frequency shift in Band III of photolyzed MbCO can be seen relative to the position of equilibrium deoxy Band III in deoxymyoglobin (*dashed line* at 762 nm). Transient spectra were offset vertically to facillitate comparison.

FIG. 6. **Comparison of the time dependence of the Soret band shift and Band III band shift in wild-type horse heart myoglobin.** The samples were prepared identically to the sperm whale myoglobin samples described in the text with the exception of the myoglobin concentration in the Band III experiments. Given the low extinction coefficient of Band III ($\epsilon \approx 250$ M^{-1} cm^{-1}), the concentration of heme used in the Band III experiments was 2 mM to obtain a sufficiently high optical density.

is lowered, the protein relaxation rate appears to slow relative to the ligand rebinding rate. At 250 K the matrix processes of ligand rebinding in H93G(1-Me Im) occur on roughly the same time scale as the slowest phase of the Soret band shift relaxation (compare the kinetics in Figs. 4 and 5), leading to kinetic hole burning seen in the CO-bound Soret band shift. By extending measurements to low temperature the dependence of CO rebinding kinetics on the *unrelaxed* population of myoglobins could be studied.

The origin of nonexponential ligand rebinding kinetics is also addressed by the data we present here. The CO rebinding kinetics shown in Fig. 4 exhibit a nonexponential geminate phase in both wild-type Mb and in H93G(L). This suggests that any distribution of protein conformations coupled through the covalent bond to the heme iron is not the sole or even the dominant factor that governs the nonexponentiality. Since the covalent bond between the heme iron and protein backbone in wild-type Mb is abolished in H93G(L), any direct coupling that could be exerted by F α-helix in myoglobin is not present in the cavity mutant. Control over ligand binding affinity and kinetics in hemoglobin by the heme iron out-of-plane coordinate and tilt angle of the proximal histidine depends on the very large (>1 Å) displacement of the F α-helix (38, 39). By contrast, the F α-helix is displaced by only ~0.1 Å in deoxy-Mb. The subtle structural modifications required to modulate the diatomic ligand rebinding barrier in hemoglobin requires several kilocalories/mol of energy to be released in the formation of intersubunit contacts. These factors are missing in Mb, and consequently the control that the F α-helix exerts in myoglobin is much smaller than in Hb. The cavity mutant recombination data provide evidence that the conformational substates responsible for a distribution of rebinding rates in glycerol buffer solutions are likely due to global structural differences or possibly to more localized distal pocket conformational subpopulations.
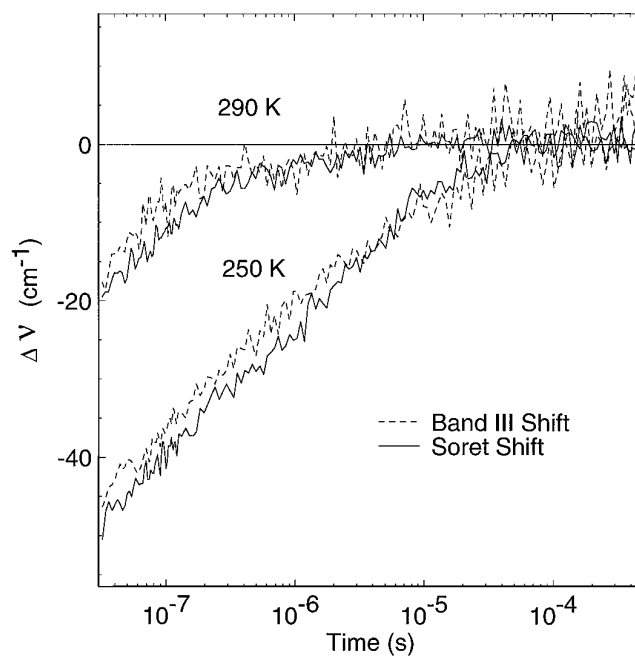
Under physiological conditions protein relaxation is significantly more rapid than CO recombination. However, O_2 recombination is highly nonexponential under these conditions with about half of the dioxygen ligands recombining more rapidly than protein relaxation (as measured by Band III shifts) and half-recombining significantly more slowly. It is still not known whether the different subpopulations of O_2 recombination interconvert rapidly, but it seems likely that they do given that interconversion of CO-bound states in MbCO is rapid. Myoglobin and hemoglobin must physically open to allow CO or O_2 ligand escape following dissociation, but at the same time must protect against autooxidation of the heme iron. Chemical reactions at the heme binding site, including steps leading to autooxidation, are detrimental to the function of oxygen-binding proteins, but are part the normal functioning of redox enzymes such as cytochrome c oxidase, where the bound oxygen is reduced to water (40). The suppression of protonation of oxygen in ligated Hb and Mb may be aided by hydrogen bonding of oxygen to His-64 (41) that is absent in heme enzymes such as cytochrome c oxidase. However, once the ligand is dissociated in globins this protection may be lost, and oxygen recombination in an open pocket conformation could lead to rapid autooxidation. For example, the autooxidation rates of distal mutants that have a more open distal pocket geometry are much more rapid than in wild type (28). This suggests a structure-function relationship in oxygen binding globins where protein relaxation may serve as a gating mechanism to prevent O_2 recombination while the distal pocket is exposed to bulk solvent during opening of the protein to allow O_2 escape. Of course, protein relaxation in the distal pocket could serve other functions as well such as discrimination against CO binding or regulation of ligand dynamics in response to external effectors analogous to the allostery of hemoglobin.

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