LIGAND MIGRATION AMONG PROTEIN CAVITIES STUDIED BY FOURIER TRANSFORM INFRARED/TEMPERATURE DERIVATIVE SPECTROSCOPY*  

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Fourier transform infrared (FTIR) spectroscopy in the CO stretch bands combined with temperature derivative spectroscopy (TDS) was used to characterize intermediate states obtained by photolysis of two sperm whale mutant myoglobin, YQR (L29(B10)Y, H64(E7)Q, T67(E10)R) and YQRF (with an additional I107(G8)F replacement). Both mutants assume two different bound-state conformations, A₀ and A₁, which can be distinguished by their different CO bands near 1965 and 1933 cm⁻¹. They most likely originate from different conformations of the Gln-64 side chain. Within each A substate, a number of photoproduct states have been characterized on the basis of the temperature dependence of recombination in TDS experiments. Different locations and orientations of the ligand within the protein can be distinguished by the infrared spectra of the photolyzed CO. Recombination from the primary docking site, B, near the heme dominates below 50 K. Above 60 K, ligand rebinding occurs predominantly from a secondary docking site, C, in which the CO is trapped in the Xe4 cavity on the distal side, as shown by crystallography of photolyzed YQR and L29W myoglobin CO. Another kinetic state (C⁺) has been identified from which rebinding occurs around 130 K. Moreover, a population appearing above the solvent glass transition at ~180 K (D state) is assigned to rebinding from the Xe1 cavity, as suggested by the photoproduct structure of the L29W sperm whale myoglobin mutant. For both the YQR and YQRF mutants, rebinding from the B sites near the heme differs for the two A substates, supporting the view that the return of the ligand from the C, C⁺, and D states is not governed by the recombination barrier at the heme iron but rather by migration to the active site. Comparison of YQR and YQRF shows that access to the Xe4 site (C⁺) is severely restricted by introduction of the bulky Phe side chain at position 107.

Myoglobin (Mb),¹ the first protein for which structure was solved to atomic resolution (1), is an α-helical polypeptide chain of about 150 amino acids encapsulating a heme group to which small gaseous ligands like O₂, CO, or NO can bind. Early on it was recognized that the ligand binding site in the interior of the protein is not accessible by ligands in the average structure, and it became apparent that ligand binding has to rely on structural fluctuations that provide transient channels for the ligands to enter and exit the protein. Hence, for more than 40 years Mb has played the role of a model system for the investigation of the interrelationships among structure, dynamics, and function in heme proteins and in proteins at large (2–9).

All ligands that bind to the ferrous heme iron can be photodissociated (10), and the ensuing ligand binding reaction can be conveniently studied using time-resolved spectroscopy. After photodissociation at room temperature, the ligand remains momentarily trapped within the protein before diffusing out into the solvent. Intramolecular rebinding to the heme iron, called geminate recombination, is controlled by ligand migration within the protein matrix and thus has been exploited to investigate the internal dynamics in a time range from ps to μs (8, 11, 12). The complexity of the events controlling geminate recombination was particularly apparent in flash photolysis experiments carried out at cryogenic temperatures, which give rise to a sequence of well resolved kinetic features (4, 13, 14).

Propositions as to the nature of the kinetic intermediates included structural transitions of the protein that affect the ligand binding energetics and/or migration of the ligand to different docking sites (13–16). These models were, in the absence of direct structural information, merely speculative. Nevertheless, the kinetic and spectroscopic experiments, particularly those using temperature-derivative spectroscopy (TDS) after extended illumination (17–19), were invaluable to the establishment of illumination protocols that populated photoproduct states to a level amenable to structural studies using x-ray crystallography. The first photoproduct structures of MbCO were obtained at 40 K (20–22) revealing that, after photodissociation, the CO was located in the vicinity of the heme, in the so-called primary docking site, as proposed by IR spectroscopic studies (23). Subsequently, new photoproduct structures of sperm whale Mb mutants became available, showing that CO can migrate away from the primary docking site into internal cavities within the protein (24–27). These cavities have long been known because of their ability to bind an atom of xenon (28, 29). Using nanosecond time-resolved x-ray crystallography at room temperature, Moffat and collaborators (30) recently confirmed that these cavities play a role as transient docking sites in the physiological ligand binding process, as suggested earlier by Elber and Karplus (31). For the
sake of clarity, in Fig. 1 we depict a schematic view of the heme and a few of the key amino acid side chains on the distal and proximal side, as well as the approximate CO locations in the different photoproduction states (A–D) that will be referred to throughout the paper.

Photoproduction structure determination is a difficult pursuit, and it is successful only if an intermediate can be populated to a significant fraction. By contrast, IR spectroscopy is a sensitive technique that allows one to distinguish different species with fractional populations in the percent range. Moreover, measurements carried out as a function of temperature yield information on the energy barriers governing internal migration and rebinding of the photodissociated ligand; particularly revealing are IR-spectroscopic experiments monitoring the stretch bands of heme-bound or photodissociated CO near 5 μm (32, 33). Measurements of the ligand-bound species yield detailed information about structural properties, in particular heterogeneity, in the vicinity of the active site. Multiple lines observed in the wave number range around 1950 cm\(^{-1}\) have been associated with multiple conformations of the protein, called “A” substates (see Fig. 1). For example, native sperm whale Mb exhibits three A substates (A\(_1\) at ~1966 cm\(^{-1}\), A\(_2\) at ~1945 cm\(^{-1}\), and A\(_3\) at ~1933 cm\(^{-1}\) (17, 34). Comparative studies of many distal pocket mutants have shown that multiple A substates originate from electrostatic interactions between the bound CO and different heme pocket environments (23, 35), which alter the electron distribution and bond order of the iron-carbonyl complex and, accordingly, shift the IR stretch frequency (36). The IR bands of CO in aqueous solutions are too broad to be detected. Photodissociated CO trapped inside the protein matrix, however, displays multiple IR lines near 2130 cm\(^{-1}\) (32) that arise from different locations and/or orientations of the CO within the protein. Although their small absorbances and wave number shifts demand careful experiments, these IR bands are useful tools to follow CO trapped inside the protein.

In this work, we address the problem of ligand migration, employing two mutants of sperm whale Mb and exploiting the power of low temperature infrared spectroscopy in combination with a temperature ramp protocol. The general purpose of our work is 2-fold, i.e. to understand the role of the internal cavities and packing defects in controlling ligand migration to the heme iron and to test the power of protein engineering in dictating internal pathways and occupation of the available docking sites (see Fig. 1). The experimental approach of TDS-FTIR spectroscopy enables us to separate different dynamic processes by their different temperature dependences and to determine the enthalpy barriers governing the observed rate processes. Using this technique we investigated two multiple mutants of sperm whale Mb that seemed particularly suitable as model systems in view of their interesting and almost unique properties. The results obtained by FTIR-TDS have been correlated with the crystallographic structure obtained for both mutants in the CO-bound state. The triple Mb mutant referred to as YQR has the substitutions L29(B10)Y, H64(E7)Q, and T67(E10)R; the quadruple mutant YQRF has the bulkier Phe chain protruding into the cavity leading to the Xe4 cavity, which should gate access to this docking site. In this study we present a detailed picture of the structural dynamics of both mutants at low temperatures with particular focus on the effect of the mutations in the ligand migration pathways and discuss the level of consistency with room temperature kinetic experiments and low temperature crystallographic data.

MATERIALS AND METHODS

Sample Preparation—Mutant sperm whale myoglobins YQR and YQRF were expressed in Escherichia coli and purified as described previously (39). Samples were prepared by dissolving the lyophilized protein at a concentration of ~15 mg in cryosolvent (75% glycerol, 25% potassium phosphate buffer (v/v), pH 7) and subsequent reduction with excess dithionite under a CO atmosphere. For the spectroscopic experiments, a few microliters of the protein solution were held between two CaF\(_2\) windows (diameter 25.4 mm) separated by a 75-μm thick Mylar washer.

Cryospectroscopy and Photolysis Setup—The windows were sandwiched inside a block of oxygen-free high conductivity copper mounted on a cold-finger of a closed cycle helium refrigerator (model SRDK-200AW, Sumitomo, Tokyo, Japan), which allowed adjustment of the sample temperature in the range between 3 and 320 K. The temperature was measured with a silicon temperature sensor diode and regulated by a digital temperature controller (model 330, Lake Shore Cryotronics, Westerville, OH). Samples were photolyzed with light from a continuous wave, frequency-doubled Nd:YAG laser (model Forte 530–300, Laser Quantum, Manchester, UK), emitting 300 milliwatts of output power at 532 nm. The beam was split and focused with lenses on the sample from both sides. The standard photolysis rate, \(k_{L}\), was determined as ~20 s\(^{-1}\) at low temperatures. A Fourier transform infrared (FTIR) spectrometer (IFS 66v/S, Bruker, Karlsruhe, Germany) was used to collect transmission spectra in the mid-infrared range between 1800 and 2400 cm\(^{-1}\) at a resolution of 2 cm\(^{-1}\).

Temperature Derivative Spectroscopy—To assess the kinetic properties of different photoproducts, we use TDS, an experimental protocol designed to investigate thermally activated rate processes that are characterized by distributed enthalpy barriers. The method has been described in detail in previous papers (17–19, 40), and thus we give only a short summary here.

In a TDS measurement, the sample is initially perturbed from equilibrium. Here we use laser irradiation under different illumination protocols so as to enhance particular photoproduct species (18). The temperature is subsequently increased linearly in time at a warming rate \(a \approx 5\) mK/s, and spectra are collected continuously, yielding one complete spectrum for every kelvin temperature increase. Difference spectra are calculated from a set of spectra at consecutive temperatures and plotted as a function of temperature in a TDS map, which is a (linearly or logarithmically scaled) contour plot of the absorbance changes on a surface spanned by the wave number and temperature axes.

In the experiments presented here, absorbance changes arise from two different rate processes, CO rebinding to the heme iron and CO moving to other locations. At the lowest temperatures, thermal energy is too small to overcome most of the enthalpy barriers governing these processes. As the temperature increases with time, successively higher barriers can be surmounted. Therefore, the TDS protocol ensures that the different rate processes in the sample are sorted according to their activation enthalpy, which is, to a good approximation, linearly related to the temperature at which the process is observed (40).

RESULTS

Infrared Difference Spectra at 3 K—Infrared difference spectra of the YQR and YQRF mutants MbCO (Fig. 2) show that in the region of the stretch absorption of the heme-bound CO, both mutants display two A substate bands, with slightly different frequencies in YQR and YQRF. In the same figure, the IR region of the photodissociated CO displays a number of bands between 2110 and 2160 cm\(^{-1}\). The two main bands are centered at 2142 and 2144 cm\(^{-1}\); the weaker bands at lower wave numbers, however, cannot be resolved unambiguously in the difference spectrum at 3 K. In MbCO, an IR absorption line near 1965 cm\(^{-1}\) is present in mutants with an apolar distal pocket, for example replacement of the distal His-64(E7)
with a small aliphatic residue (23, 35). In wt MbCO, the A0 line at 1966 cm⁻¹ appears upon protonation of the distal histidine with a pK of 4.5 (41), which forces the imidazole side chain into an alternate conformation away from the distal heme pocket into the solvent so as to better solvate the charged imidazolium side chain. The other two A substates, A1 (1945 cm⁻¹) and A3 (1933 cm⁻¹), are characterized by a neutral imidazole side chain residing in the heme pocket. Johnson et al. (42) measured the exchange kinetics between the A1 and A3 states and suggested that they originate from two slightly different conformations of His-64 in the distal pocket. Additional support for this view was provided recently on the basis of a high resolution crystal analysis (43). An enhanced A1-like absorption was noticed in several mutants modified at residue 29, for example L29A/L29V/L29I/L29F (35). Presumably, interactions between Leu-29 and His-64 are responsible for the occurrence of A1 and A3 in native MbCO.

Although the two mutants studied here have residues Leu-29 and His-64 replaced by Tyr and Gln, respectively, we observe an A state spectrum with bands similar to A0, A1, and A3 of the native protein. Based on data obtained on the H64Q mutant of sperm whale MbCO (35), an A1-like line may have been expected, because the conformation of the glutamine side chain forces its N₆-bound hydrogen in a position similar to that of the imidazole N₆ of His-64. The double mutant L29F/H64Q has a broad, further red-shifted IR absorption at 1938 cm⁻¹ (44), whereas the L29F single mutant has a fairly narrow absorption at 1932 cm⁻¹ (35). This red shift is explained by a positive partial charge near the ligand oxygen originating from the edge of the aromatic residue. In our mutants, the replacement of Leu-29 by Tyr leads to an entirely new A3-type IR band with a dominant line at 1933 (YQR) and 1929 cm⁻¹ (YQRF) (35). Presumably, interactions between Tyr-29 and/or Gln-64 and the bound ligand are weakened or cancel each other. Possible explanations are that the Gln-64 bulky Phe side chain at 107 appears to cause a slightly different stereochemistry of the Y and Q side chains in the distal pocket, as indicated by the additional 4 cm⁻¹ shift to the red. The full width at half maximum of 8 and 9 cm⁻¹ of A3 in YQR and YQRF, respectively, is comparable with that of the A0 and A1 substates in wt MbCO (17).

In both mutants the minority species A0 displays a very broad absorption in the range between 1950 and 1980 cm⁻¹. The high frequency implies that polar interactions between Tyr-29 and/or Gln-64 and the bound ligand are weakened or cancel each other. Possible explanations are that the Gln-64 side chain assumes an isomeric conformation in which either the nonbonded electrons of O₆ are closer to the ligand or the glutamine side chain moves away from the ligand location. The width of the A0 band suggests that this alternate conformation is structurally not well defined.

**TDS after Short Illumination at 3 K**—In these experiments, the sample was illuminated for 1 s at 3 K after which the TDS experiment was started in the dark. The resulting TDS maps in the spectral regions of the bound and photodissociated CO in Fig. 3 show the absorbance change between two successive spectra, taken 200 s and 1 K apart, as a function of temperature in a contour plot. The features in the A state map (Fig. 3, A and C, top) show rebinding in the minority species, A0, taking place predominantly in the region below 40 K for both mutants. In contrast, rebinding from A0 is very different in the two mutants. In YQR, rebinding extends over the entire temperature range up to ~110 K. The integrated absorbances of the A0 and A3 states as a function of temperature (Fig. 3B) clearly show a double-humped structure with maximum rebinding around 40 and 60 K. In the figure, we also display calculated TDS data obtained from fitting multi-gaussian barrier distributions to the TDS data, using pre-exponentials from low temperature kinetic experiments. The peak enthalpies, H, variances, σ, and fractional weights, f, have been compiled in Table I. In YQRF recombination from A3 is maximal at 10 K and gradually decays from there, extending up to ~100 K. There is a substantial population rebounding below ~50 K that can only be fitted with two barrier distributions. Moreover, the rebounding processes above 60 K, which were so apparent in YQR, appear only as shoulders in both A states in Fig. 3D, which presents both measured and fitted integrated absorbances of the two A states as a function of temperature. Fit parameters for YQRF are also included in Table I. The observed heterogeneity in the ligand rebinding properties of A0 is also apparent in Fig. 3C, in which its TDS peak shifts to higher wave numbers with increasing temperature.

The TDS maps of the photodissociated CO in Fig. 3, A and C (bottom), show several positive and negative peaks. These feature the IR properties of the CO in the internal cavities as shown schematically in Fig. 1. If only rebinding processes were present, the map should display negative contours (dashed lines) only, as rebinding leads to an absorbance increase in bound CO bands and a corresponding loss in the photoproduct bands. The FTIR-IR map should display negative contours (dashed lines) only, as rebinding leads to an absorbance increase in bound CO bands and a corresponding loss in the photoproduct bands. In the case of YQRF, we already observed from A3 that conformational changes at the distal pocket are much smaller. Rebinding above 60 K occurs entirely from these two peaks.

**TDS after Slow Cooling under Illumination from 140 K**—For the TDS experiments in this subsection, laser illumination was switched on at 140 K, and the temperature was ramped down to 5 mK/s. After reaching 3 K, the laser was switched off and the TDS experiment started. With this illumination protocol,

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**Table I**

<table>
<thead>
<tr>
<th>Sample/substate</th>
<th>Pre-exponential</th>
<th>Peak 1</th>
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<th>Peak 3</th>
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<tr>
<td></td>
<td>log A/s kJ/mol</td>
<td>a₁ kJ/mol</td>
<td>f₁ %</td>
<td>kJ/mol</td>
</tr>
<tr>
<td>YQR A₀</td>
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<td>0.7</td>
<td>4.7</td>
<td>91</td>
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<tr>
<td>YQR A₁</td>
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<tr>
<td>YQRF A₀</td>
<td>8.60</td>
<td>1.0</td>
<td>1.7</td>
<td>24</td>
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</table>

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FTIR-TDS of Ligand Migration in Myoglobin

ligands can migrate to locations that are separated by larger enthalpy barriers and hence can only be accessed at higher temperatures. Lowering the temperature under illumination ensures that some of the ligands remain trapped in these sites until, in the subsequent TDS experiment, thermal energy has again been increased sufficiently for the barrier to be crossed (18, 19).

The TDS maps of the YQR mutant in Fig. 4A, obtained after slow-cool illumination, show drastic differences when compared with the maps after 1 s of photolysis at 3 K, Fig. 3A. In A3 at 1933 cm⁻¹, the peak at 40 K has essentially vanished, the one at 60 K is prominent, and a new feature appears at 130 K, containing 31% of the population. Although the contours for the weak A0 state are much noisier, it is nevertheless apparent that corresponding states near 60 and 130 K also exist for the minority species. The TDS maps of the YQRF mutant are presented in Fig. 4B. Again, the A state map (top) is very different from the one taken after short illumination but similar to that of YQR; the yield of populating the 130 K peak in A3 is only 12%, however, and thus markedly smaller than in YQR. The peak in A0 corresponding to the 60 K peak in A3 is shifted 10 K higher up, and a minor peak also occurs in the 130 K region.

For the YQR mutant, the absorption band at 2118 cm⁻¹ in the region of photodissociated CO is entirely missing (Fig. 4A, bottom), and the band at 2142 cm⁻¹ has almost disappeared except for a tiny population near 50 K, which comprises those CO molecules that reside in the high energy (temperature) wing of the barrier distribution peaking near 40 K (Fig. 3, A and B). Exchange features are also visible below 30 K arising from a population exchange within the inner doublet at 2127 and 2134 cm⁻¹. It is interesting to note that the exchange changes its direction at ~8 K. Careful inspection shows that, above 8 K, molecules on the red side of the 2127 cm⁻¹ band preferentially exchange with those on the blue side of the 2134 cm⁻¹ band. The high temperature maximum in the A state map at 130 K is associated with two absorption lines in the photoproduct region at 2128 and 2133 cm⁻¹.

In YQRF (Fig. 4B, bottom) a rich variety of bands and dynamic processes can be seen. A strong feature at 2144 cm⁻¹ extends up to 50 K. Below 10 K, a population transfer from a band at 2135 cm⁻¹ to a band at 2119 cm⁻¹ occurs. Above 10 K, the peak at 2135 cm⁻¹ gains population. Whereas it is not clear a priori whether this band is fed by the peak at 2119 cm⁻¹ or by the peak at 2144 cm⁻¹, the analogous behavior of the bands of YQR in this temperature and wave number range in Fig. 4A (bottom), where there is no ambiguity due to the absence of a third band, lends support to the assumption that here we also observe a reversal of the exchange observed below 10 K, as in YQR. It is most remarkable, however, that the bands associated with rebinding near 60 K have wave numbers distinctly different from those observed after 1 s illumination at 3 K (see Table II). This is very different from the behavior of YQR, where the different illumination protocol leads to different amounts of rebinding near 60 K without changes in the peak frequencies. In YQRF, by contrast, not only is the population rebinding near 60 K much larger upon extended illumination, but the bands are at 2119 and 2135 cm⁻¹, as compared with 2123 and 2130 cm⁻¹ after short illumination at 3 K. The bands are also substantially broader after extended illumination, implying a heterogeneous environment of the CO ligands. The feature at 130 K in the A states has two corresponding peaks at 2122 and 2128 cm⁻¹ in the photoproduct region, distinctly different from those found for the 60 K peak.

We note that experiments analogous to those described here were done with slow cooling under illumination to 3 K starting from 80 instead of 140 K (data not shown). The essential features are identical up to 100 K; the peak near 130 K, however, is missing.

**TDS after Slow Cooling under Illumination from 185 K**—The experiments in this subsection are designed to probe long-lived photoproduct states in the presence of large-scale protein motions that are activated above ~180 K (6). To enhance their populations, we have illuminated the samples for 2 h at 185 K and then ramped the temperature down to 160 K at 5 mK/s before starting the TDS experiment.

In Fig. 4C we present the resulting TDS contour plots for the YQR mutant. For the A3 substate (Fig. 4C, top), a broad distribution between 170 and 210 K is seen in the A state map with a maximum at 190 K. The signal in A0 looks complicated, having negative contour lines around 190 K between positive features at both lower and higher temperatures. The negative contours reflect decreasing A0 absorption due to a population transfer to A3. This occurs because the different rebinding barriers in A0 and A3 lead to a non-equilibrium population ratio, A0/A3, which adjusts itself once the temperature is high enough that A state interconversions can occur on the time scale of the measurement as shown earlier for wt MbCO (42, 45). The TDS map of the photodissociated CO in Fig. 4C (bottom) shows a broad line at 2128 cm⁻¹; a weak shoulder at 2135 cm⁻¹ can be seen in the spectra.

The A state TDS map for the YQRF mutant in Fig. 4D has very similar features to YQR, including the A state exchange at 190 K. The contours extend 10 K higher up, implying that rebinding in this mutant is somewhat more difficult than in YQR. The TDS data of the photodissociated CO are spectroscopically identical with those of YQR.

**DISCUSSION**

The TDS experiments allow us to identify for both mutants a number of different photoproduct states from where rebinding occurs within particular temperature ranges. Typically, each of these different states is characterized by two CO bands, although occasionally we need a third band for a fully satisfactory fit. Here we discuss the structural dynamics as deduced from the spectroscopic features and their temperature depen-
differences in the TDS maps, particularly with reference to the region of the photodissociated CO trapped in the protein matrix. At 3 K, the dominant bands of CO ligands in the protein are at 2142 (YQR) and 2144 cm\(^{-1}\) (YQRF) (Fig. 2 and Table II) as compared with the main band at 2131 cm\(^{-1}\) observed in wt MbCO (17). The discussion that follows refers frequently to the schematic structure shown in Fig. 1, displaying the different states of the CO either bound to the heme iron (A states) or trapped at different locations within the protein (B, C, and D states).

In the spectral region monitoring the A states, the TDS experiments on YQR and YQRF reveal a complex sequence of events after 1 s photolysis at 3 K. Separate populations with markedly different rebinding properties exist in both the A\(_3\) and A\(_1\) species, best viewed by comparing the integrated absorbances in Fig. 3, B and D. For rebinding below \(~40\) K, the temperature dependence and thus the energy barriers for the two mutants are quite different (Table I), implying that the additional mutation I107F favors an active site structure that enhances recombination. Upon comparison of the data in Fig. 3, B and D, the most obvious difference between YQR and YQRF is seen in the temperature dependence of rebinding in A\(_3\) (the dominant species) above \(~60\) K. Although both mutants show a rebinding population distributed around 60 K, this is much more pronounced in YQR, accounting for the marked difference in the rebinding behavior of A\(_3\).

The TDS maps in the region of the photodissociated CO (Fig. 3, A and C, bottom) give a detailed picture of ligand dynamics in the protein, which governs rebinding in the temperature range above \(~40\) K. We note that the TDS maps show clearly different spectroscopic bands, which were not resolvable in the 3 K difference spectra in Fig. 2. These bands represent CO in different locations within the protein; their peak wave numbers are compiled in Table II. The spectral changes below 20 K reflect ligand motion within the B states near the heme (Fig. 1) with a net transfer from B\(_2\) to B\(_1\) (shift from 2118 to 2142 cm\(^{-1}\)). This exchange is understood because at 3 K the ligands are photodissociated with a large excess of kinetic energy and trapped in one or the other of two metastable states (B\(_1\) and B\(_2\)). At 10 K, however, thermal energy is sufficient for barrier crossing on our experimental time scale (\(~100\) s), and the population ratio readjusts in accordance with the free energy difference. The same exchange phenomenon was also observed in wt MbCO, where an equilibration occurs below 20 K between B states characterized by IR bands at 2119 and 2131 cm\(^{-1}\) (17, 32). On the basis of their room temperature femtosecond IR experiments, Anfinrud and co-workers (46) have suggested that the photodissociated CO resides in the primary docking site above heme pyrrol C in two orientations (rotated by 180\(^{\circ}\)). This interpretation should also hold at low temperatures for the two mutants. Thus, we tentatively assign the two bands to the CO located in the (B\(_1/B_2\)) site in opposite orientations.

As the temperature is ramped up beyond 20 K, the A\(_3\) contours display an increase in rebinding while simultaneously the TDS map of the photodissociated CO displays a second exchange process. Thus, above 20 K ligands leave the B site either because they rebind or because they escape to other locations characterized by a distinctly different electrostatic environment, as inferred from the spectral changes of the CO. The essentially identical temperature dependence of the two newly arising bands suggests that we are again dealing with the same location but two different orientations of the CO; the smaller band splitting as compared with the previous doublet (Table II) implies a weaker electric field component along the direction of the CO dipole in this location. We assign this photoproduct to the C state (Fig. 1) on the basis of the temperature where this migration process occurs in the TDS map. According to the YQR MbCO photoproduct x-ray structure determined at 30 K (24), the photodissociated CO resides in the Xe4 cavity (see Fig. 1) in the back of the distal pocket in a location very similar to the one found for the L29W MbCO photoprotein (25). Note that the Xe4 cavity, also referred to as C site here, was denoted as B\(_3\) in the latter paper (25). Around 50 K, the B site on top of the heme is entirely depleted by recombination or exchange, and rebinding occurs only from the C site up to a temperature of \(~110\) K.

In the case of the YQRF mutant, the additional replacement of I107 by the bulky Phe in the channel connecting the B site with the C site (see Fig. 1) was expected to introduce a constriction, making CO migration into C' more difficult. Indeed,

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**Table II**

<table>
<thead>
<tr>
<th>Sample</th>
<th>B1/B2</th>
<th>C'</th>
<th>C'(^{\alpha})</th>
<th>C'</th>
<th>D</th>
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<td>110-130 K</td>
<td>180-200 K</td>
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<tr>
<td>Wave number (cm(^{-1}))</td>
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<td>2127</td>
<td>2127</td>
<td>2128</td>
<td>2128</td>
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<tr>
<td>Absorbance ratio</td>
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<td>21 ± 2</td>
<td>21 ± 2</td>
<td>27 ± 2</td>
<td>56 ± 4(^{d})</td>
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<tr>
<td>YQRF MbCO</td>
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<td>2125</td>
<td>2119</td>
<td>2122</td>
<td>2128</td>
</tr>
<tr>
<td>Wave number (cm(^{-1}))</td>
<td>2144</td>
<td>2130</td>
<td>2135</td>
<td>2128</td>
<td>(2135)(^{b})</td>
</tr>
<tr>
<td>Absorbance ratio</td>
<td>(2130)(^{b})</td>
<td>21 ± 2</td>
<td>18 ± 2</td>
<td>26 ± 2</td>
<td>52 ± 4(^{d})</td>
</tr>
</tbody>
</table>

\(^{a}\) After slow cooling under illumination.

\(^{b}\) Minority species.

\(^{c}\) A/B, (2142/2144 cm\(^{-1}\)) only.

\(^{d}\) Strongly temperature-dependent. The ratio increases from 45 (48) at 180 K to 68 (70) at 200 K for YQR (YQRF).
the A state TDS map in Fig. 3D does not reveal a well defined maximum at higher temperatures as in the case of YQR (Fig. 3B). The TDS map in the region of the photodissociated CO, however, shows exchange processes similar to the ones observed in YQR, although with a significantly reduced amplitude. B₁ at 2144 cm⁻¹ feeds population into two states at 2123 and 2130 cm⁻¹, implying that the long tail in the A₃ rebinding (Fig. 3D) arises from a small fraction of photodissociated CO that has escaped into the C' site. Thus the effect of Phe at 107 is to introduce a severe but not complete roadblock in the channel from B to C'. As we discuss below, it is possible however to enhance the different photoproduction populations by extended illumination. Phe-107 not only hinders ligand escape to the C' site but also alters the structure of the Tyr-29 and Gln-64 side chains at the active site. This is supported by the observed shifts of the A substate lines by 4 cm⁻¹ shown in Fig. 2. Thus, the structural changes coupled to Phe at 107 appear to give the ligand better access to the heme iron from the B₁/B₂ states; instead of a peak at 40 K as in YQR, a maximum already occurs at 10 K, and we now need two populations with rather small barriers (Table I) to fit the low temperature integrated absorbance (Fig. 3D).

Upon slow cooling of the sample from 140 to 3 K under illumination, the TDS maps look much different (Fig. 4, A and B), showing that repeated photoexcitation at higher temperatures enables many ligands to escape from the vicinity of the active site. Some rebinding remains even at the lowest temperatures, but the 40 K peak is no longer discernible in A₃. More interesting is the observation that extended illumination has created a population at 130 K in both A substates, assigned to an additional

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**Fig. 3.** A and C, TDS maps of MbCO for YQR (A) and YQRF (C) following photolysis for 1 s at 3 K. Contours are spaced logarithmically. **Solid lines,** absorbance increase; **dashed lines,** absorbance decrease. **Top,** rebinding monitored in the A₀ and A₃ IR stretch bands of heme-bound CO; **bottom,** rebinding and ligand movements among different sites seen in the IR stretch bands of photodissociated CO. **B** and **D,** TDS signals of A₀ and A₃ in MbCO for YQR (B) and YQRF (D) as obtained by integrating the experimental data in the intervals 1960–1980 cm⁻¹ for A₀ and 1920–1940 cm⁻¹ for A₃. **Dashed lines,** TDS signals fitted with gaussian enthalpy barrier distributions with parameters given in Table I; **solid lines,** sum of the individual distributions.
photoproduc state called C’’ from which the CO has to overcome higher barriers for rebinding than from C’. A thermal transition from B1/B2 to C/H11032 is not observed in the TDS map in Fig. 4, as most ligands had already escaped to C/H11032 while we illuminated the sample at the higher temperatures. A significant difference between the two mutants lies in the fact that the positions of the two IR bands associated with CO in the C/H11032 cavity change only weakly with the illumination protocol in YQR (see Table II). In YQRF, however, the C’’ peak positions are distinctly different. It appears that for YQRF, the small fraction of protein molecules in which CO can escape by thermal activation after short illumination at 3 K has particular spectral properties, yielding C/H11032 bands at 2123 and 2130 cm⁻¹. Therefore, under extended illumination conditions, ligand migration to the C’’ site can also occur in YQR despite the hindrance to escape; these molecules are characterized by different C’’ peak positions (with bands shifted to 2119 and 2135 cm⁻¹). From the difference in the C’’ bands of YQR and YQRF (Table II) it is obvious that Phe at position 107, which is in contact with the C’’ cavity (Fig. 1), has a strong influence on the IR bands.

In our TDS experiments, the C’’ photoproduc state, unlike C’, does not become populated to any detectable level by thermal transitions, but its population can be greatly enhanced using extended illumination, thereby providing the ligands with many opportunities to escape to this state instead of rebinding. C’’ is characterized by two IR bands at 2128 and 2133 cm⁻¹ in YQR, and at 2122 and 2128 cm⁻¹ in YQRF (Table II). At present, no direct structural information is available for this intermediate. The essentially identical temperatures of rebinding from the C’ and C’’ photoproduc intermediates for both A0 and A3 substates suggest that rebinding from C’ and C’’ is governed by enthalpy barriers not located in the close vicinity of the ligand binding site. The clear separation of C’’ from C/H11032 in the TDS map and its different doublet of IR bands suggest that the CO migrates to a location in the protein interior different from the Xe4 cavity. An obvious candidate for an alternate location of the CO would be the Xe2 cavity (29), located on the pathway between the C/H11032 and D sites (Fig. 1). We cannot, however, entirely exclude the possibility that the C’’ state may be the result of structural changes within the Xe4 pocket affecting both the energetics of ligand binding and the CO spectra. Again, the IR spectra of this photoproduc for YQRF differ markedly from YQR (Table II), which implies that the CO in the C’’ state is located in the vicinity of Phe-107. Because this side chain separates the Xe4 and Xe2 cavities (Fig. 1), we cannot decide on the basis of this observation alone whether state C’’ corresponds to a CO that has moved to the Xe2 cavity or one that remains in the C’ site but for which the surrounding structure has changed.

In the third TDS experiment, we illuminated the sample above the dynamical transition temperature of ~180 K, where global structural changes of the protein can occur, as demonstrated by
the A state spectrum is red-shifted by 4 cm⁻¹/H11002.

The bands of photodissociated CO near 190 K can be resolved into two bands (at 2128 and 2135 cm⁻¹), which are identical for YQR and YQRF. This is expected for CO sitting in the proximal cavity (Xe1) because the CO absorption within this location should not be sensitive to differences in the distal pocket. The ratio of the integrated absorbances of the IR bands of the bound and photodissociated CO in the D site of YQR (YQRF) is 56 (52), averaged over the temperature range of 180 to 200 K and thus quite large. It increases continuously with temperature across the D state peak, suggesting that recombination above 180 K includes increasingly more CO molecules that have escaped into the solvent and thus do not contribute to the absorption spectrum, because solvated CO cannot be detected.

CONCLUSIONS

Using infrared spectroscopy in the IR stretch bands of the heme-bound and photodissociated CO in combination with three different temperature ramp protocols, we have characterized ligand binding from a number of photoproduct states in the A state spectrum, with defined polarity and stereochemistry.

A particular focus of this work was to study the effect of the replacement I107F in the channel connecting the primary docking site (B₁/B₂ states) near the heme iron to the Xe4 cavity or C' pocket. In YQR, the ligand can easily access this secondary docking site by thermal activation, as observed in the TDS map in Fig. 3, implying a very small barrier from B₁/B₂ to C'. This is fully consistent with laser photolysis experiments and molecular dynamics simulations carried out on the same mutant (38). In wt Mb, a significant population of this site was achievable only by repeated photoexcitation (18, 19), and this may be understood based on the steric effects exerted by Leu-29 in the wt protein (24). In the YQRF mutant, however, the channel is partially blocked so that an increase in the population of the C' state can only be achieved upon extended photoexcitation at higher temperatures. This is consistent with expectations based on the x-ray structure of YQRF compared with YQR (Fig. 1). In general, the photoproduct CO bands consist of doublets (Table II), which shows that the CO can assume, within each cavity, two opposite orientations related by a C₂ symmetry axis. This supports the view that the packing defects hosting the photodissociated CO may indeed be considered docking sites, with defined polarity and stereochemistry.

A number of photoproduct states with CO trapped in the protein have been identified on the basis of both their recollection temperature in the TDS data sets and their IR absorption spectra. These features are characteristics of CO residing in different docking sites within the protein matrix, as indicated schematically in Fig. 1. A primary docking site (B) with the CO very close to the heme iron is taken to correspond to the location immediately above heme pyrrole C observed at very low temperatures in the crystallographic experiments of photoproduct intermediates carried out on wt and mutant MbCOs (20, 22). In this paper we have reported three more photoproduct sites, called C', C'', and D. The barrier from the B to the C' state is very small in YQR and can be overcome above ~20 K thermally by photodissociated CO ligands on our experimental time scale (~100 s). On the other hand, in YQRF the thermally activated transition is possible only for a small fraction of protein molecules. Photoproduct state C' can, however, be populated to a greater extent by extended illumination in the temperature range 100–150 K. This indicates that at low temperature the bulky Phe-107 acts as an efficient roadblock in the channel leading to the C' state cavity (which corresponds to the Xe4 binding site, see below), whereas at somewhat higher temperatures it may allow access of the photodissociated CO to the Xe4 cavity. An additional photoproduct state, C'', has been unequivocally identified in both mutants. Whereas C' and C'' are accessible in the frozen protein, another photoproduct state, called D, can only be populated upon photolysis above 180 K, implying that larger protein fluctuations are needed to open gates for ligands to migrate to the new site.

The x-ray structures of myoglobin photoproducts as obtained at cryogenic temperatures allow us to interpret the photoproduct spectroscopic states in structural terms (Fig. 1). In the B₁/B₂ states, the CO resides near the heme in the primary docking site, whereas it moves to the Xe4 cavity in C' and to the Xe1 cavity in D at higher temperatures. The CO location corresponding to the C' state is still unknown. The C' state IR bands in YQR and YQRF are quite different (Table II) implying that the CO in this location feels the influence of Phe at 107 quite strongly, thus suggesting some connectivity. The working hypothesis originating from these experiments is that the C'' state identified from the TDS maps experiments may be the Xe2 cavity of Tilton et al. (29), which is on the pathway between Xe4 and Xe1 (Fig. 1). In general, the photoproduct CO bands consist of doublets (Table II), which shows that the CO can assume, within each cavity, two opposite orientations related by a C₂ symmetry axis. This supports the view that the packing defects hosting the photodissociated CO may indeed be considered docking sites, with defined polarity and stereochemistry.

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by Scott et al. (12) on the basis of laser photolysis experiments on many single mutants of sperm whale Mb. Our new results lend support to the hypothesis that the Xe-binding cavities define the pathway of internal migration to and from the heme and play a role in the structural dynamics of the protein (48). Because the effect of Xe on the time course of geminate rebinding of O2 at room temperature was clearly proven by Scott and Gibson (49), the conclusions drawn from low temperature crystallography and spectroscopy can be safely extrapolated to physiological temperatures. Even more support for this conclusion is provided by the latest time-resolved x-ray crystallography data taken at room temperature (30). This beautiful work gives very clear evidence of the importance of photoproduct states B and D as transient docking sites of the ligands. The additional photoproduct intermediates C' and C'' reported here could not be detected from the x-ray data, however. From this observation we cannot conclude that they do not exist at room temperature or that they are not relevant in physiological ligand binding. Multiple intermediate states can only be distinguished in any kinetic experiment if their respective lifetimes are well separated in time. By performing the experiment at cryogenic temperatures as we have done here, not only do the lifetimes increase, but the different processes also appear more widely dispersed in time. With this strategy, Nienhaus et al. (19) have shown earlier that additional photoproduct intermediates also exist in wt MbCO crystals.

In summary, using infrared spectroscopy we have been able to draw a detailed picture of CO migration to and recombination from various sites within the protein matrix, over a wide temperature range. These spectroscopic observations have been correlated with the structural data obtained by cryocrystallography of photoproducts. They are generally consistent with room temperature kinetic and crystallographic data obtained after laser photolysis, supporting the significance of our results for the control of ligand binding dynamics and therefore for the biochemistry of O2 transport.

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