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Ligand Binding to a Hemoprotein Lacking the Distal Histidine

THE MYOGLOBIN FROM APLYSIA LIMACINA (Val(E7))*

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The time course of ligand recombination to the myoglobin from *Aplysia limacina*, which has Val(E7), was measured following photolysis by flashes of 35 ps to 300 ns with a time resolution of 10 ps or 1 ns. CO shows only bimolecular recombination. O_2 has a small geminate reaction with a half-time of tens of picoseconds, but no nanosecond geminate reaction. NO has two picosecond relaxations with half-times of 70 ps (15%) and 1 ns (80%) and one nanosecond relaxation with a half-time of 4.6 ns. The bimolecular rates for O_2 and NO are the same: 2×10^7 M $^{-1}$s^{-1}. Methyl and ethyl isonitriles have a geminate reaction with a half-time of 35 ps. Ethyl isonitrile has, in addition, a nanosecond relaxation (25%) with a half-time of 100 ns. *t*-Butyl isonitrile has four geminate relaxations (10 ps, 35 ps, 1 ns, and 1 μs).

Analysis of the results suggests much easier movement of ligand between the heme pocket and the exterior than in sperm whale myoglobin (His(E7)). The reactivity of the heme is little different, placing the effect of the differences from sperm whale myoglobin on the distal side of the heme.

The continuing development of laser technology in recent years has rekindled interest in the study of the kinetics of ligand rebinding by hemoproteins using optical methods, and data have even been acquired using subpicosecond flashes (1, 2). These papers give references to some of the many workers who have described relaxations in a time range from seconds to a few picoseconds. All times less than a microsecond relate to geminate recombination, in which photodissociated ligands recombine with the heme group without leaving the protein molecule in which they were bound. A full description of the behavior of a protein calls for observations over a wide range of times, so the matrix of possible combinations of ligands and time ranges has been fully defined only for a few proteins.

The myoglobin from the mollusc *Aplysia limacina* (3) has been selected for detailed study because the residue E7, the distal histidine in most hemoproteins, is replaced by valine (4). This change has the significant functional consequence that oxygen dissociation from *A. limacina* myoglobin is faster than that from sperm whale or horse myoglobins (5, 6), where a hydrogen bond to the distal histidine stabilizes this ligand, but is much slower than in the corresponding single residue sperm whale myoglobin mutant (13). The crystal structure of *A. limacina* myoglobin, available at 1.6-Å resolution for the Met derivative (7), suggests that an arginine residue (E10) may interact with the bound ligand.

This paper gives data for the myoglobin from *A. limacina* in the time range from 10 ps and above for six ligands: oxygen, carbon monoxide, nitric oxide, and methyl, ethyl, and tertiary butyl isonitriles. The functional properties of the molecule are widely different from those of other myoglobins so far studied.

MATERIALS AND METHODS

A. limacina specimens were collected in the Bay of Naples (Italy), and myoglobin was extracted as described earlier (3). It was stored as a precipitate in neutralized saturated ammonium sulfate solution.

All the experiments described in this paper were carried out in 0.1 M KP_i buffer (pH 7.0) freed from oxygen before addition of the desired ligand.

The protein was dissolved in buffer just prior to use and diluted to the desired concentration (50-75 μM); some samples were dialyzed overnight to remove residual ammonium sulfate, but no effects attributed to the salt were observed.

For experiments on the carbon monoxide and nitric oxide derivatives, the sample was prepared in a 1-mm quartz cell sealed to a tonometer, reduced under nitrogen with minimal sodium dithionite, and finally equilibrated with the gaseous ligand at the desired partial pressure (usually 1 atm for CO and 0.02-0.1 atm for nitric oxide). Oxygenated myoglobin was usually prepared from carbon monoxide-saturated samples by replacing the gas phase with air or oxygen and allowing the gas to consume the excess dithionite; this procedure gave better samples than reduction under nitrogen with removal of excess dithionite by gel filtration.

Samples of methyl, ethyl, or tertiary butyl isonitrile-saturated myoglobin were prepared in sealed 1- or 2-mm quartz cells, reduced as described above, and allowed to react with 5-10 mM ligand (final concentration).

The spectrum of unliganded and liganded myoglobins was recorded using a Cary 14 spectrophotometer over the wavelength range of 600 to 400 nm. Protein concentration was measured after each experiment by replacing the ligand with NO and using the published extinction coefficient for this derivative (8).

All reagents were of analytical grade.

Data Collection—Three types of laser flash were used with three systems for data collection. Picosecond photolysis flashes were obtained from a frequency-doubled mode-locked YAG laser (Model 571, Quantel, Santa Clara, CA), giving up to 10 mJ in 35 ps at 532 nm.

Two nanosecond flash lengths were obtained from a PhaseR Model 2100 B flash lamp pumped dye laser with rhodamine 575 (Exciton Chemical Co., Dayton, OH). In cavity-dumped mode, up to 60 mJ was delivered in 17 ns, and in normal operation, 300 mJ in 300 ns.

For observation in the picosecond range, a conventional pulse probe arrangement was used, splitting the 532 nm output of the YAG laser, using part of the beam for photolysis. The remaining part was passed

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through a Raman shifter to give an observation beam at 436 nm from the first anti-Stokes line. The pulse output was detected with a fast photodiode (type FND 100, EG&G, Salem, MA), driving an amplifier interfaced to a PC AT microcomputer through an A/D board (Model DAS50, Metrabyte, Taunton, MA).

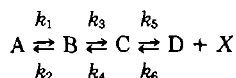
Nanosecond observations were made in real time with a photomultiplier (Model 4818 Hamamatsu, Middlesex, NJ) driving an oscilloscope (Model 7104, Tektronix, Beaverton, WA). The traces were digitized with a camera and frame acquisition board interfaced to the microcomputer. The light source was a 75-watt xenon arc pulsed to 200 × normal brightness.

Millisecond observations were made by using a photomultiplier and amplifier to drive the Metrabyte DAS50 A/D board. The longer time observations with the oscilloscope and with the Metrabyte A/D board could be made with any of the photolysis flash sources, as required. The observing beam came from a 75-watt xenon arc run at constant current. The light was passed through an interference filter or through a dark blue glass filter before reaching the cuvette to reduce the photochemical effect of the observing beam. Beyond the sample, it passed through a second filter to aid in rejecting scattered light from the photolysis flash and through a small grating monochromator (Spex Industries Inc.) to reach the photomultiplier.

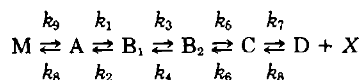
Data Reduction—Data in the millisecond range were collected to measure overall bimolecular rates and were in the form of voltage as a function of time. They included a group of points collected immediately before the photolysis flash as a base line. These were averaged and used to convert the voltages to absorbance differences as a function of time. These, in turn, were converted to concentrations. The logarithm of the ratio of free ligand to free hemoprotein was plotted against time, and the slope was taken as a measure of the second-order rate of recombination.

Data in the nanosecond range were reduced to absorbance in the same way. Experiments with oxygen, carbon monoxide, and methyl isonitrile did not show nanosecond relaxations. Data for ethyl isonitrile (Fig. 3) were represented by the sum of a constant term representing the ligand which escaped from the protein (to recombine subsequently on a millisecond time scale) and a single exponential, reported as a rate in Table I. Data for *t*-butyl isonitrile, which shows several relaxations, were plotted in semilogarithmic form; and the components were extracted by successive subtraction and replotting the remainder, starting with the slowest component.

In treating the data for NO, recent work by Petrich *et al.* (2) has been taken into account explicitly in the algorithm used to obtain the rates. These workers have described a transient species, named Hb*(II) by them, with absorbance at longer wavelengths (450 nm) than those of most myoglobin derivatives. It relaxes to liganded myoglobin at a rate of 230 ns^{-1} and is the principal early photoproduct, accounting for 0.7–0.85 of the quanta absorbed by the protein-ligand complex. Furthermore, Jongeward *et al.* (1) and Petrich *et al.* (2) reported that NO shows two phases of geminate rebinding to sperm whale myoglobin (Mb)[1] in the 1st ns after photolysis. The minimal scheme



is no longer sufficient to accommodate the results and has been expanded to become



where M is the species Hb*(II) from Ref. 2. B_1 and B_2 represent the two picosecond steps in NO rebinding, C is a geminate intermediate on a scale of nanoseconds, A is the stable liganded form of the protein, and D is the stable unliganded form that the ligand rebinds to in the bimolecular reaction. The labeling has been chosen to allow A, C, and D to retain their previous significance.

The parameters in the scheme above were estimated by nonlinear least-squares optimization, comparing numerical solutions of the corresponding set of differential equations with the experimental results.

To obtain apparent quantum yields, the absorbance excursion at the end of the laser pulse was measured using several light levels, and the logarithms were plotted against light intensity. The same procedure was followed with MbCO as a standard. The ratio of the initial slope of the plots, when corrected for the relative extinctions of MbCO and the derivative under study at the wavelength of excitation, was taken as a measure of the apparent quantum yield, assuming a yield of 1 for MbCO.

[1] The abbreviations used are: Mb, myoglobin; MbCO, carbon monoxide myoglobin; MbNO, nitric oxide myoglobin.

RESULTS

Gaseous Ligands—The time course of the absorbance change after laser photolysis of carbon monoxide- or oxygen-saturated *A. limacina* myoglobin was followed at 436 nm over four different time intervals (1 ns, 200 ns, 100 μs, and 1 ms); three different flash lengths were used (35 ps, 17 ns, and 300 ns). Apart from a small and very rapid optical transition observed during the 35-ps laser pulse with oxymyoglobin, but not with carbon monoxymyoglobin, only the bimolecular rebinding process was observed (Fig. 1 and Table I). The quantum yield estimated from the experiments carried out with both the 35-ps and 17-ns laser pulses was close to 1 for CO and 0.1 for oxygen (Table II).

The properties of the nitric oxide derivative of *A. limacina* myoglobin change with time. Although its spectroscopic properties remain unchanged for days or even weeks after preparation, the second-order rate constant for recombination after photolysis, initially the same as that of oxygen, increases as much as 2-fold as the sample ages. The transition is complete in a few hours, so all experiments were carried out as soon as

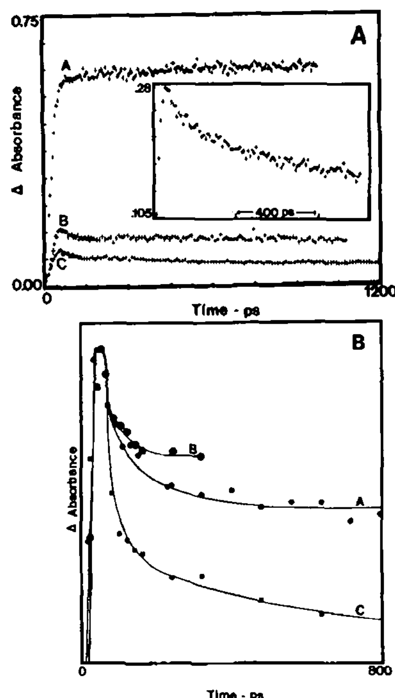


FIG. 1. **Time course of absorbance changes observed following photolysis by 35-ps pulse at 532 nm.** *A*, CO (*trace A*), O_2 (*trace B*), and NO (*trace C*) derivatives. The *ordinate* shows the absorbance change measured at 436 nm and normalized to the same light value for each ligand. The concentration of heme was 75 μM; the path length was 1 mm; and the temperature was 23 °C. The proportions of ligand removed (CO, 0.32; O_2, 0.07; and NO, 0.36 before normalization) were measured at the time of maximum absorbance change and correspond to the apparent quantum yields given in Table II. *Inset*, experiment with NO showing initial portion of trace enlarged. *B*, Methyl (*trace A*), ethyl (*trace B*), and *t*-butyl (*trace C*) isonitrile derivatives. Ligand concentrations were 7 mM for methyl and 5.5 mM for ethyl and *t*-butyl isonitriles. Path length was 2 mm. Other conditions were as described for *A*. The proportion of ligand removed was 0.95.

TABLE I

Rate constants for the relaxations observed after photolysis of A. limacina myoglobin

The relative amplitudes of each phase depend on flash length and are not reported. The picosecond flash at 532 nm had a full-width of 35 ps; the nanosecond flash at 576 nm had a length of 17 ns and was approximately rectangular. The hemoprotein concentration was near 0.1 mM in each case. All observations for the gases were made at 436 nm and for the isonitriles at 445 nm. The temperature was 20 °C. Data for isonitriles are from Ref. 14. The rates for NO were obtained by optimization using the MAB_1B_2CD scheme (see "Materials and Methods").

	Ligand					
	CO	O_2	NO	MeNC[a]	EtNC	tBuNC
Geminate			$10\ ns^{-1}$	$13\ ns^{-1}$	$20\ ns^{-1}$	$70\ ns^{-1}$
			$1.8\ ns^{-1}$			$2\ ns^{-1}$
			$0.2\ ns^{-1}$		$7\ \mu s^{-1}$	$15\ \mu s^{-1}$
						$0.7\ \mu s^{-1}$
Bimolecular	$0.65\ \mu M^{-1}\ s^{-1}$	$24\ \mu M^{-1}\ s^{-1}$	$20\ \mu M^{-1}\ s^{-1}$	$0.6\ \mu M^{-1}\ s^{-1}$	$0.54\ \mu M^{-1}\ s^{-1}$	$0.008\ \mu M^{-1}\ s^{-1}$

[a] MeNC, EtNC, and tBuNC, methyl, ethyl, and *t*-butyl isonitriles, respectively.

TABLE II

Apparent quantum yield at the end of a photolysis flash

The experiments were performed under the conditions given in the legend to Table I. See Footnote *a* for definitions of abbreviations.

Flash length	Ligand					
	CO	O_2	NO	MeNC	EtNC	tBuNC
35 ps	0.9	0.1	0.13	0.5	0.5	
17 ns	0.9	0.1	0.025	0.4	0.4	0.03
300 ns	0.9	0.1	0.015			

possible after preparation of the sample; the geminate relaxations of photolyzed MbNO, however, and the quantum yields measured after each laser pulse (300 ns, 17 ns, and 35 ps) do not appear to depend on the age of the sample.

Photolysis of nitric oxide-saturated *A. limacina* myoglobin is followed by a sequence of three geminate relaxations with half-times of 70 ps, 0.5–1 ns, and 4.5 ns. After completion of the geminate phases, 70% of the ligand photolyzed by a 17-ns laser pulse remains free and recombines in a second-order reaction (Fig. 2). The response to this steep-sided but relatively long flash is illustrated. It is unusual in that both in the experiment and in its representation by the scheme discussed under "Materials and Methods," the population of deoxy-Mb does not increase nearly as fast as the flash turns on (half-time < 1 ns). Formally, this is because the fitted rate for the conversion of B_1 to B_2 is much higher than in other NO derivatives that have been examined. As a result, B_1 is never heavily populated, and its rapid reconversion to A cannot combine with the flash intensity to give a relatively small but rapid increase in the concentration of deoxy-Mb which would correspond to beginning with a step-up in Fig. 2. The absorbance excursion in Fig. 2 is ~10 times as large as would be seen with native sperm whale MbNO, where an immediate step-up in Mb concentration is prominent. This result may also be taken as evidence that species B_1 and B_2 are populated sequentially, rather than in parallel.

As a consequence of the multiexponential time course of NO rebinding, the apparent quantum yield (ϕ) measured immediately after the shortest available flash (35 ps, $\phi = 0.13$) is considerably higher than that observed for nanosecond pulses (17 ns, $\phi = 0.025$; 300 ns, $\phi = 0.015$). When determining the quantum yields for O_2 and NO, it is impossible to approach closely to full photolysis with short flashes because, at high light intensities, irreversible destruction of the heme occurs with bleaching of the solution, as has been observed previously (1, 2). This bleaching depends on the rate of photochemical work, not on the total numbers of quanta delivered. Its relation to light intensity is consistent with a two-photon process, perhaps involving the species Hb*II of Petrich *et al.* (2). In calculating quantum yields from picosecond experiments, data from lower light intensities were used, and the absorbance change for full photolysis was taken from experiments with longer flashes or from static spectra.

Isonitriles—The reactions of several isonitriles with sperm whale myoglobin, which have been studied both on nanosecond and picosecond time scales (1, 5), show widely different patterns of behavior. Three members of the series, the smallest, methyl, and the bulky *t*-butyl, were studied together with ethyl isonitrile. Following photolysis by a 35-ps flash, ~30% of ethyl and methyl isonitriles rebound in a rapid (~20 ns^{-1}) geminate reaction (Fig. 3). This was the only geminate reaction of methyl isonitrile, but a small proportion (25%) of ethyl isonitrile rebound with a half-time in the range of 100 ns. The apparent quantum yield for both ligands following nanosecond flashes is much higher than that for sperm whale Mb (5), at ~0.5, but does not approach 1, even when measured at 20 ps following 35-ps flashes.

Tertiary butyl isonitrile showed a wide range of rebinding rates, and at least five parameters are needed to describe its reaction with *A. limacina* myoglobin; they are listed in Table I. The apparent quantum yield after a 17-ns flash is similar to that of NO. No sound measurement could be made using the 35-ps flash because at least one of the intermediates has a half-life below the range of the apparatus (10 ps).

DISCUSSION

Information about events in the protein following photolysis can be obtained from optical experiments only if geminate recombination occurs. The experiments with carbon monoxide, which does not show geminate behavior on any of the time scales examined and which has an overall apparent quantum yield approaching 1, allow only the obvious conclusion that there is no significant pathway for this ligand other than dissociation with escape to the solution. Experiments with oxygen were similar; but in this case, although no geminate behavior was seen on a nanosecond or microsecond time scale, the apparent quantum yield at 20 ps is only 0.1. The observed picosecond absorbance changes were small, so dissociation of oxygen from the heme with the appearance of the deoxy spectrum is a rare event. The results for both these ligands are similar to those for sperm whale myoglobin, although oxymyoglobin does show a nanosecond geminate reaction accounting for ~30% of the ligand dissociated. The bimolecular rates for both ligands are in good agreement with published values (6) and are also similar to those for sperm whale Mb (5).

The reactions with NO are more complex and widely different from those with sperm whale myoglobin. Two relaxations are seen on the picosecond scale, the faster of which accounts for 15% of the total change and which has a half-

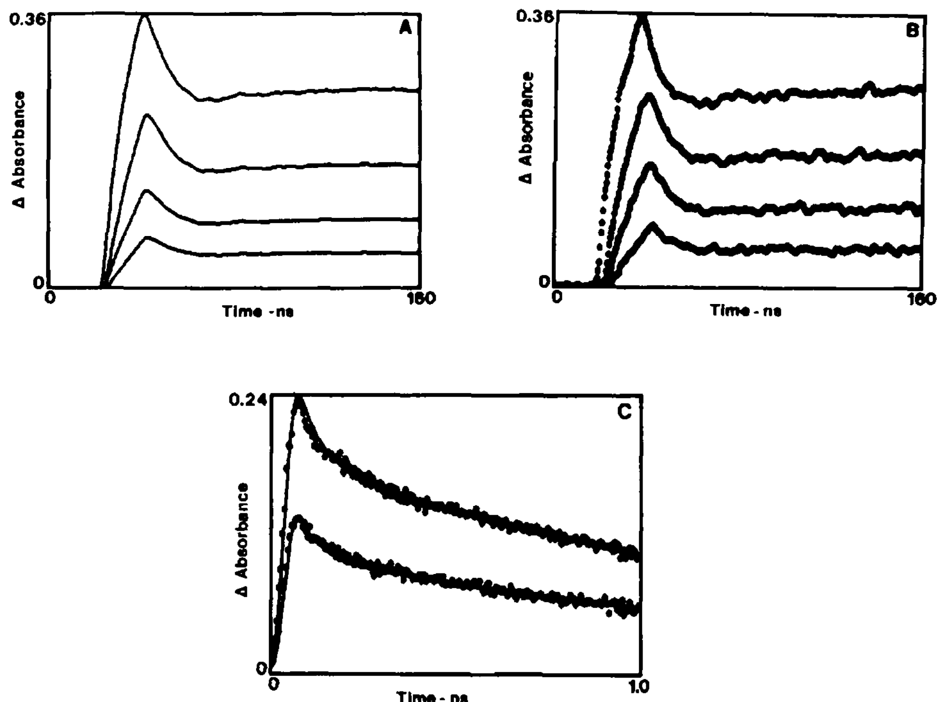


FIG. 2. **Time course of absorbance change observed with *A. limacina* MbNO following photolysis with 35-ps flash (at 532 nm) and with 17-ns flash (at 576 nm).** Observation was at 436 nm; path length was 1 mm; and temperature was 20 °C. Four light levels of the 17-ns flash were used (2.2 (A), 1.1 (B), 0.6 (C), and 0.3 (D) × 10^9 s^{-1}) based on the extent of photolysis of a solution of sperm whale MbCO. The *ordinate* is a measurement of the amount of *A. limacina* Mb present before, during, and after the flash. The maximum breakdown shown corresponds to 0.57 of the sample. *A* shows computed values (*continuous lines*) of the concentration of Mb derived from the MAB_1B_2CD scheme (see "Materials and Methods") using the values: k_2, 8; k_3, 10; k_4, 1.8; k_5, 1.2; k_6, 0.3; and k_7, 0.04 ns^{-1}. The effective proportion of quanta was taken as 0.21. *B* shows the corresponding data. Each reaction is represented by 400 points taken from the oscilloscope. *C* shows the picosecond results for the same sample as calculated using the same rate parameters as for *A* (*continuous lines*) and as observed (*points*). The pulse profile was assumed to be gaussian and was standardized using MbCO.

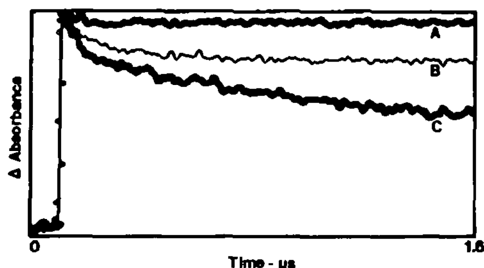


FIG. 3. **Absorbance change after photolysis of methyl (*trace A*), ethyl (*trace B*), and *t*-butyl (*trace C*) isonitrile *A. limacina* myoglobins by 35-ps flash.** The records cover 1.6 μs in each case and have been normalized to simplify comparison. The *ordinate* is the normalized absorbance excursion recorded with a time constant of 1 ns using the Tektronix system (see "Materials and Methods").

time of 70 ps. The major relaxation has a half-time of the order of 1 ns. Sperm whale Mb has also been reported to show two relaxations (1, 9), the faster of which is about two times as fast as the corresponding relaxation of *A. limacina* Mb and accounts for two-thirds or more of the observed picosecond change. Its slower minor relaxation is 10–20 times faster than the corresponding reaction for *A. limacina* Mb.

In the nanosecond range, both proteins show geminate recombination; but whereas the observed rate for sperm whale Mb is at the limit of experimental resolution (>1 ns^{-1}), the rate for *A. limacina* Mb is slower at 0.2 ns^{-1}. Furthermore, after a 17-ns flash, the proportion of geminate recombination is about one-third for *A. limacina* Mb, but more than two-thirds for sperm whale Mb. Data collected during the flash show that the absorbance profile for sperm whale Mb follows the flash profile more closely than that for *A. limacina* Mb. As the slower of the two relaxations observed in the picosecond experiment with *A. limacina* Mb (0.7 ns^{-1}) is clearly faster than the relaxations observed during and after the 17-ns flash, at least three intermediates must be postulated to account for the results.

The reaction with NO was treated by assuming the expanded scheme set out under "Materials and Methods," including species M, taken to have a half-life of 3 ps and the same absorbance at 436 nm as that of MbNO (2). The corresponding set of differential equations was used to describe experiments on both nanosecond and picosecond scales, and all observed relaxations were assumed to arise from sequential reactions. This kinetic assumption is independent of chemistry or structure and does not imply linear progress of ligand from the heme to the exterior, although it does specify that all ligand molecules follow the same pathway. In reality, parallel pathways and dead end paths are at least as probable but are not required by the data and have the disadvantage of introducing large numbers of parameters which cannot be defined. They are therefore less suitable where the aim is to permit compact representation and comparison of data sets rather than to develop a plausible mechanism.

In this framework, picosecond data for *A. limacina* Mb resemble those for native sperm whale myoglobin qualitatively in showing two relaxations (1, 2), but differ quantitatively. The ratio of the two rates is 5:1 for sperm whale Mb but 20:1

for *A. limacina* Mb. Furthermore the faster reaction accounts for 66% or more of the total change in sperm whale Mb, against 15% for *A. limacina* Mb. In experiments with the single residue mutant of sperm whale Mb, H64V (prepared by E. Carver (Department of Biochemistry, Rice University) from a gene described by Springer and Sligar (15) and Springer *et al.* (16)), the reaction with NO was scarcely changed by the substitution.[2] Speculation on the mechanism of this effect is premature, but it may be recalled that Gibson *et al.* (17) found large kinetic effects of substitutions in the C-E region of naturally occurring plant hemoglobins. Effects of residues relatively remote from the heme are not implausible.

Taken together, the effect of these kinetic differences is that escape of an NO molecule from *A. limacina* Mb is 10 times more likely, following photolysis, than escape from sperm whale myoglobin. This difference does not have a single cause, but arises from both nanosecond and picosecond reactions.

The three isonitriles examined differ significantly from one another and behave quite differently in *A. limacina* Mb as compared with sperm whale Mb (1, 5). The data for picosecond and nanosecond experiments cannot be included in a single broad statement. Jongeward *et al.* (1) have reported that, following photolysis of sperm whale methyl and ethyl isonitrile myoglobins, ~25% of the ligand rebinds with rates of 13 and 5.5 ns^{-1}, respectively. *A. limacina* Mb shows similar behavior, but the rates are closer together at 13 and 20 ns^{-1} for these ligands. For *t*-butyl isonitrile, Jongeward *et al.* (1) reported that 75% of the ligand rebinds to sperm whale myoglobin at a rate of 26 ns^{-1}. With *A. limacina* Mb, an absorbance change at or beyond the limit of resolution of the apparatus (70 ns^{-1}) was succeeded by a much slower reaction (30% of the whole) at 2 ns^{-1}, in observations restricted to 436 nm.

There are marked differences between the two myoglobins in the nanosecond range. After photolysis of sperm whale methyl and ethyl isonitrile myoglobins, ~80% of both ligands rebinds at rates of 0.025 and 0.1 ns^{-1}, respectively (1, 5). With *A. limacina* Mb, less than 5% of methyl isonitrile rebinds in this time range even when a 35-ps flash is used for photolysis to minimize pumping of ligand, whereas some 25% of ethyl isonitrile rebinds at a rate of 0.007 ns^{-1}. The bulkier *t*-butyl isonitrile shows more complex behavior, with some 65% rebinding to *A. limacina* myoglobin at the unusually low rate of 0.0007 ns^{-1}, whereas another 30% rebinds at the more ordinary rate of 0.015 ns^{-1}.

As with other proteins that have been studied in detail, the reactions of *t*-butyl isonitrile are different from the other members of the series. The bimolecular binding rate is slow, perhaps because appreciable distortion of the protein may be required to permit its entrance. Recent crystallographic work (12) has shown substantial movement of the distal histidine in sperm whale myoglobin on binding ethyl isonitrile, and even larger movements may be called for with *t*-butyl isonitrile. Once in, however, the molecule does not readily leave either the vicinity of the heme, as shown by the extensive rapid geminate reactions, or the more remote regions, as is required to account for a geminate reaction with a half-time of 1 μs.

The same qualitative explanation of the data offered for NO applies to methyl and ethyl isonitriles also; these relatively bulky ligands are better able to escape from the *A. limacina* protein in competition with rebinding to the heme than they are from sperm whale myoglobin. As a corollary to the proposal that escape from the protein is easier in *A. limacina* Mb than in sperm whale myoglobin, entry to the protein may also be expected to be easier (k_6 in the ABCD scheme). A numerical estimate of the value of k_6 for NO made by combining the values of the parameters in the scheme with the overall rate (see Ref. 5) gives the rate as 0.04 M^{-1} ns^{-1}, as compared with the value of 0.045 M^{-1} ns^{-1} for sperm whale myoglobin given in Ref. 5. This result is consistent with the bimolecular rates of oxygen and NO binding, which are also similar in *A. limacina* and sperm whale myoglobins. Analogous calculations for methyl and ethyl isonitriles give ~12 μM^{-1} s^{-1} (sperm whale Mb, 0.15) and ~2 μM^{-1} s^{-1} (sperm whale Mb, 0.08), respectively. Evidently, the general expectation is borne out for the isonitriles, but not for NO.

Data for the bimolecular reactions of sperm whale myoglobin with the substitution His(E7) → Val have recently been given (13). The mutant differs much more from the native protein than *A. limacina* Mb does; the rate of oxygen binding is 10 times greater than that for *A. limacina* Mb, whereas the oxygen dissociation rate of 0.23 × 10^5 s^{-1} is far greater than that reported for *A. limacina* Mb (6). As oxygen binding is probably limited by diffusion up to the heme rather than by the rate of binding to it, the mutant, with its high rate of reaction, should, like *A. limacina* Mb, show very little geminate rebinding. The relatively low rate of oxygen dissociation from *A. limacina* Mb, which is only 7 times greater than that from native sperm whale Mb, implies stabilization of the oxygen complex by hydrogen bonding. In native sperm whale Mb, the bond is thought to be to the distal histidine (E7). In *A. limacina* Mb, the corresponding bond may be to Arg(E10), which has been observed, in crystallographic studies of unliganded Fe(III) derivatives, to swing toward the heme group (7).

There is little evidence to suggest that the reactivity of the heme iron in *A. limacina* Mb is much different from that in sperm whale myoglobin. The rate of CO binding, expected to be limited by heme reactivity, is, like the rate of CO dissociation, little different in the two proteins. The dissociation velocities for methyl and ethyl isonitriles, when corrected for the very different geminate reactions of the two proteins, are also of the same order, reflecting similar heme-ligand reactivity in both.

Overall, the results appear consistent with the general conclusion that the structural differences between *A. limacina* and sperm whale myoglobins are expressed primarily on the distal side of the heme.

The quantum yields obtained with the various ligands and with the different flash lengths correlate well with the data for the geminate reactions. Where there are no geminate reactions in the nanosecond range, as with CO and O_2, the quantum yield is the same whatever the flash length used in measuring it. With NO, the apparent yield varies with flash length, in good agreement with the amplitude of the nanosecond geminate reaction.

The isonitriles are qualitatively similar to the gaseous ligands, but have a much higher quantum yield, approximating 0.5. In agreement with results with sperm whale myoglobin (1), very fast reactions reducing the apparent quantum yield are less prominent with ethyl and methyl isonitriles than with *t*-butyl isonitrile and are less than with O_2 and NO. Again, with *A. limacina* Mb, very rapid reactions must account for more of the total quanta absorbed than with sperm whale Mb. It is also possible, of course, that only a proportion of the molecules are photosensitive; but our observations give no information on this point.

[2] R. S. Blackmore and Q. H. Gibson, unpublished data.

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