Ligand Binding to the Dimeric Hemoglobin from *Scapharca inaequivalvis*, a Hemoglobin with a Novel Mechanism for Cooperativity*

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The homodimeric hemoglobin from *Scapharca inaequivalvis* has an unusual spatial arrangement of the subunits (Royer, W. E., Jr., Love, W. E., and Fender-son, F. F. (1985) *Nature* 316, 277–280). The time course of oxygen and nitric oxide rebinding to this protein following flash photolysis has been measured on a nanosecond time scale. A large amplitude is observed with a half-time of 20 ns (NO). With oxygen the half-time decreases from 70 ns at low fractional photolysis to 30 ns at large breakdown. The second order rate of NO binding is $1.9 \times 10^7$/ms, and is the same as that for oxygen.

Analysis of the geminate data suggests that oxygen and nitric oxide react more rapidly with the heme than in myoglobin, but also escape much more rapidly from its vicinity.

The hemoglobin from *Scapharca inaequivalvis* shows marked cooperativity in ligand binding (1–3). This is manifest in equilibrium experiments, in the accelerating time course of carbon monoxide binding, and in the rates of oxygen dissociation. Cooperativity is unusual in any dimeric hemoglobin, and especially so in a homodimer. It is known that in *Scapharca* hemoglobin the heme groups have a different spatial relation from that of mammalian hemoglobins and are closer together as the E and F helices form the intersubunit contact (4). Now that the high resolution structure has been solved (5), it is apparent that each heme is linked to the other through a salt bridge and two hydrogen bonds. The general arrangement of the heme pocket and of the helices within the subunits is, however, broadly similar to that of myoglobin, although there is an additional helix at the NH$_2$ terminus.

The new structural information lends a special interest to the geminate phenomenon. Such studies (6, 7) have led to a major sharpening of focus in the understanding of ligand binding to hemoproteins. The term describes the situation in which a ligand molecule, after the heme ligand bond has been broken, recombines at the same site rather than escaping to the exterior. The reaction is typically rapid, may show more than one phase, and is independent of the ligand concentra-

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rates are assigned to the initial steps, one must postulate at least one more intermediate species within the protein which can contribute to the observed geminate reaction. In symbols, this can be expressed by writing:

$$A \frac{k_1}{k_2} = B \frac{k_3}{k_4} = C \frac{k_5}{k_6} = D$$

where A represents protein with ligand bound to the heme, B and C are two different species in which a ligand molecule remains associated with the protein subunit in which photodissociation occurred, and D is the final state in which the ligand molecule is no longer associated with a particular heme site.

In this scheme, high rates are assigned to $k_3$ and $k_5$, and the geminate reaction shown in Fig. 1, A, B, and C, is described by $k_4$ and $k_6$. No absolute values for $k_3$ and $k_5$ can be given, though their ratio is well determined. An estimate of the minimum rates required may be made by noting that in Fig. 1D a rate of $1.6 \times 10^{10} \text{s}^{-1}$ has been given for $k_2$ by Gibson et al. (11) for NO myoglobin. To avoid the sharp increase in the amount of unliganded heme shown in Fig. 1D as the flash begins, a rate about five times greater is needed, or about $1 \times 10^{11} \text{s}^{-1}$, corresponding to a half-time of 7 ps or less for both O$_2$ and NO reacting with Scapharca hemoglobin. Although very fast, these rates are not out of line with the fastest rates observed by picosecond spectroscopy (9, 10).

The values for $k_1$ and $k_6$, in contrast, are not greatly different from those reported for several monomeric hemoproteins (9, 12). Approximate numerical values have been assigned by nonlinear least squares minimization, and are given in Table I. The errors in these values cannot readily be determined, but they are thought to be within 20% or so, based on replicate experiments with several preparations and slightly different experimental conditions. The similarity of these numbers expresses the experimental observation that the rate of the slower phase of the geminate reaction is not very different in the proteins studied. In other words, once the ligand has escaped from the immediate vicinity of the heme its further behavior is much the same in every case. This is perhaps an expression of the similarity of the helical structures.

## Table I

<table>
<thead>
<tr>
<th>Protein and ligand</th>
<th>$k_3/k_4$</th>
<th>$k_5$</th>
<th>$k_6$</th>
<th>Fractional photolysis</th>
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<tr>
<td>Scapharca O$_2$</td>
<td>5.6</td>
<td>0.014</td>
<td>0.010</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>5.1</td>
<td>0.013</td>
<td>0.009</td>
<td>0.72</td>
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<td></td>
<td>5.4</td>
<td>0.010</td>
<td>0.008</td>
<td>0.52</td>
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<td></td>
<td>3.5</td>
<td>0.009</td>
<td>0.008</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>2.4</td>
<td>0.010</td>
<td>0.008</td>
<td>0.30</td>
</tr>
<tr>
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<td>2.4</td>
<td>0.009</td>
<td>0.008</td>
<td>0.16</td>
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<tr>
<td>Myoglobin O$_2$</td>
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<td>0.014</td>
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<td></td>
<td>11.0</td>
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<td></td>
<td>7.6</td>
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<td>0.008</td>
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</tr>
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<td>0.014</td>
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<td>590.0</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>2.5</td>
<td>0.0005</td>
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ducible, and well outside the errors defined by the error matrix (13). One experiment using oxygen with 8 different light levels is shown in Fig. 2. The first explanation considered was experimental artifact; so analogous experiments were performed with five different systems all of which show moderate geminate reactions in the same time range. To compare the results with Scapharca the parameters for lines fitted to the data as in Fig. 2 were used. The slope was divided by the intercept in each case, and the results plotted as the histogram inset in Fig. 2. The slopes have both positive and negative values, and the largest positive ratio is only one quarter of that for Scapharca, leaving little doubt that the difference between Scapharca and the other systems is real.

If the effect is real, other ligands may be expected to give analogous results. Unfortunately, the range of ligands available is small. Carbon monoxide cannot be used because the amplitude of the geminate reaction is too small, and with NO the photosensitivity is so low that too small a proportion of ligand can be removed by a flash of the maximum intensity available. Data for NO over the accessible range did not show any effect. Experiments were performed with ethyl and methyl isonitriles, the generous gift of Dr. J. S. Olson, Department of Biochemistry, Rice University, Houston, TX. Both ligands bound, but neither escaped from the protein, more than 90% rebinding within 10 ns of the end of the flash at a rate independent of light intensity. The results with ethyl isonitrile are illustrated in Fig. 3. Analysis shows that the observed rate depends on \( k_t \) in the ABCD scheme and that there is no requirement that the rate parameters be varied with fractional photolysis, although a high proportion of ethyl isonitrile could be removed from species A by the flash. At present, therefore, oxygen is the only ligand to show the effect illustrated in Fig. 2.

The data of Fig. 2 were obtained by fitting a complex reaction to a single exponential. Simulations were therefore carried out in which the time course of ligand rebinding was modeled using the ABCD scheme and the observed profile of the photolysis flash. The resulting simulated course of rebinding was then fitted to a single exponential and a constant term. The rate did not show a significant dependence on photolysis intensity, so the data of Fig. 2 are unlikely to be an artifact of the simple fitting procedure.

Other explanations considered include heterogeneity of the preparation, regarded as unlikely because no effects were seen with other ligands or in measurements of the overall (bimolecular) reaction which showed good linearity over at least 90% of the reaction for several levels of photolysis for both oxygen and NO. The rate of binding, interestingly, was identical for the two ligands, at \( 1.6 \times 10^7 \text{M}^{-1}\text{s}^{-1} \), in agreement with the published result for oxygen (3). The implication, since the rate of reaction of NO at the heme is greater than that for \( O_2 \), is that the same diffusive step limits the bimolecular reaction for both \( O_2 \) and NO.

The oxygen reaction of Scapharca has been followed at 436 nm and at 415 nm, using a range of light intensities. The amplitudes of the excursions at the two wavelengths correlated excellently, so there is no evidence of spectroscopic heterogeneity. Evidence of kinetic heterogeneity has been found, however, in that the distribution of residuals obtained by fitting the time course of oxygen combination to an exponential and a constant was nonrandom for high light intensities, but random for HbCO and low light intensities with Scapharca \( O_2 \).

It is not possible to represent the family of curves in Fig. 1A satisfactorily in terms of the ABCD scheme, primarily because the amplitudes are not reproduced correctly when data for small and large degrees of photolysis are taken together. To deal with the problem, experimental curves were taken singly and represented in terms of the rate parameters. The results have been included in Table I. They show that the ratio \( k_t/k_a \) changes systematically with the proportion of oxyhemoglobin photolyzed, and that \( k_b \) and \( k_a \) also change somewhat. It is necessary to suppose that the effective rates of all three parameters can change in a time no greater than 10 ns or so, since all of the flash experiments in Figs. 1 and 2 start from the same point irrespective of the flash intensity used. This time is short compared to that required for the R to T relaxation of mammalian hemoglobin (14) which, depending on pH, requires 10 to 100 ms. As already mentioned, the hemes are much closer in Scapharca than in mammalian hemoglobin, and the F helices of the subunits are in close contact with the contralateral subunit. Furthermore, it is not necessary to suppose that the R to T transition must take place in order to modify the relative values of \( k_a \) and \( k_b \); the
finding, however, remains unexpected. Further insights may follow completion of the high resolution structure of the deoxy species, defining the structural endpoints of the allosteric transition.

CONCLUSIONS

Although the helical structure of Scapharca is similar to that of myoglobin (4, 5), the geminate behavior with oxygen, and particularly with NO, is different. In myoglobin it seems difficult for NO to escape from the neighborhood of the heme, and a large proportion of photodissociated NO recombines rapidly (half-time 40 ps) with the heme, only a small proportion escaping to give a slower geminate phase and to diffuse to the exterior. With Scapharca, the rate of recombination of NO with the heme is even higher than in myoglobin, but the rate of escape from the vicinity of the heme is likewise fast, limiting the proportion of ligand which rebinds quickly and allowing a large slow (tens of nanoseconds) geminate phase to be seen. In terms of an ABCD scheme, the species B is heavily populated in myoglobin, whereas with the light intensities and length of flash used here, it is never heavily populated in Scapharca. The result is that species C in Scapharca largely takes the place of species B in myoglobin. The larger ethyl isonitrile molecule is unable to escape so readily from the neighborhood of the heme both in myoglobin and in Scapharca, and the pattern of geminate recombination is similar in both.

In examining the reaction of oxygen with Scapharca hemoglobin we have observed, we believe for the first time, a dependence of the rate of the geminate reaction with extent of photolysis which we have failed to associate either with experimental artifact or sample heterogeneity.

The second order rates for oxygen and nitric oxide are the same for Scapharca. This is consistent with limitation by a common step and with previous reports which showed the rate of bimolecular recombination of oxygen to be independent of the extent of photolysis, whereas the rate of CO rebinding is faster with small amplitudes (3). The rate of NO bimolecular recombination is similarly independent of the fraction photolyzed.

REFERENCES