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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 Fenderson, 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 1.6×10^7/M·s, 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 concentration in solution. In a flash photolysis experiment, an intramolecular, or in the case of hemoglobin, an intrasubunit event, requires that the rate of geminate recombination be independent of the extent of photolysis. The hemoglobin from *S. inaequivalvis* is unusual in having rather similar rates of geminate recombination with O_2 and NO as ligands, and in showing a dependence of geminate rate on the extent of photolysis. These features must be related to its unusual structure.

MATERIALS AND METHODS

Hemoglobin from *S. inaequivalvis* was prepared as described in Ref. 3. It was stored in liquid N_2 until use. All experiments were performed in 0.05 M KP_i, pH 7, at 20 °C. All procedures were as described previously, except that experiments on geminate recombination were performed using the cavity-dumped dye laser and Tektronix oscilloscope and camera system described by (8).

RESULTS AND DISCUSSION

The course of ligand rebinding after photolysis by a relatively short photolysis flash is shown in Fig. 1, *A*, *B*, and *C*. The results with O_2 and NO are quite similar except that the proportion of geminate recombination is greater for NO than for O_2. If the recombination phase of the reaction is fitted to an exponential and a constant term, with oxygen as ligand, the exponential accounts for 0.4 of the total absorbance excursion. The proportion depends somewhat on the conditions of the experiment, but varies little with total energy. In one experiment, over an 8-fold range of photolysis intensity the proportion of geminate lay between 0.41 and 0.38, with the larger proportion associated with the greater intensity, so in this respect the protein is homogeneous. With NO as ligand, in the same experiment, the exponential term accounted for 0.68–0.73 of the observed reaction. Much more light is required to bring about dissociation of NO, and, with the apparatus used, less than half of the total ligand can be removed.

The course of ligand removal during the flash is quite similar for both ligands and is represented by the rising limbs of the records in Fig. 1, *B* and *C*. They are remarkable for their near linearity in the range examined, and are in contrast with the results obtained using myoglobin and NO (Fig. 1*D*). With the flash intensities used, and the assumption of a true quantum yield of 1, as established by Jongeward *et al.* (9), and Petrich *et al.* (10) for several hemoproteins and ligands, behavior of *Scapharca* requires that very high rates be assigned both to ligand recombination in the immediate vicinity of the heme, and to the rate of escape of ligand from it. If very high

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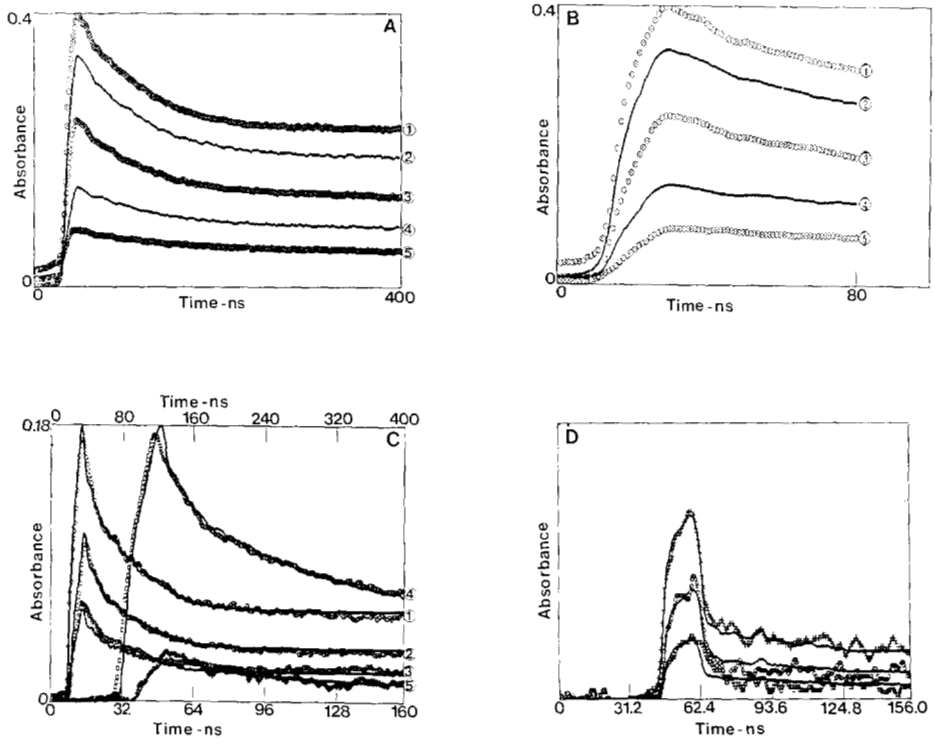

FIG. 1. **Geminate recombination of O_2 (*A* and *B*) and NO (*C*) to *Scapharca* hemoglobin.** Geminate recombination of NO to horse heart myoglobin (*D*). The points are observed, the *light lines* in *C* and *D* are the solutions to the set of differential equations representing the scheme given in the text. The parameters used are in Table I. The light intensities used were: O_2, 0.5, 0.25, 0.125, 0.0313, and 0.0156; myoglobin NO, 1.0, 0.5, and 0.25. All observations were at 436 nm. Concentration 80 μM heme, path 1 mm. Two time scales were used in *C*, the upper relates to traces 1–3, the lower to traces 4 and 5. The relative intensities were 1, 0.5, 0.25, 1, and 0.125, respectively.

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:

$$A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B \underset{k_4}{\overset{k_3}{\rightleftharpoons}} C \underset{k_6}{\overset{k_5}{\rightleftharpoons}} D$$

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_2 and k_3, and the geminate reaction shown in Fig. 1, *A*, *B*, and *C*, is described by k_4 and k_5. No absolute values for k_2 and k_3 can be given, though their ratio is well determined. An estimate of the minimum rates required may be made by noting that in Fig. 1*D* a rate of 1.6×10^{10}/s 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. 1*D* as the flash begins, a rate about five times greater is needed, or about 1×10^{11}/s, 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_4 and k_5, 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

Rates of ligand binding to Scapharca hemoglobin and to some monomeric hemoproteins

Data for myoglobin are taken from Gibson *et al.* (11), except for ethyl isonitrile which is from Jongeward *et al.* (9). Data for other hemoproteins are from Ref. 12. Data for ligand binding to *Scapharca* were obtained by nonlinear least squares optimization in terms of the scheme:

$$A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B \underset{k_4}{\overset{k_3}{\rightleftharpoons}} C \underset{k_6}{\overset{k_5}{\rightleftharpoons}} D$$

All rates are per nanosecond. NA, not applicable; ENC, ethyl isonitrile.

Protein and ligand	k_2/k_3	k_4	k_5	Fractional photolysis
Scapharca O_2	5.6	0.014	0.010	0.87
	5.1	0.013	0.009	0.72
	5.4	0.010	0.008	0.52
	3.5	0.009	0.008	0.42
	2.4	0.010	0.008	0.30
	2.4	0.009	0.008	0.16
Myoglobin O_2	4.2	0.009	0.014	NA
α chains O_2	11.0	0.036	0.081	NA
β chains O_2	7.6	0.015	0.013	NA
Scapharca NO	125.0	0.021	0.008	NA
Myoglobin NO	470.0	0.009	0.014	NA
α chains NO	590.0	0.048	0.060	NA
β chains NO	210.0	0.011	0.020	NA
Scapharca ENC	10.0	0.140	0.005	NA
Myoglobin ENC	0.2	0.097	0.007	NA
β chains ENC	2.6	0.0005	0.0003	NA

In the course of preparing the experimental data with *Scapharca* for Fig. 1, the latter part of the traces, *i.e.* after the end of the flash, was fitted to a single exponential and a constant term. In the case of oxygen, the rate of the observed reaction varied systematically with the proportion of oxygen removed. The effect is not large, about 2-fold, but is repro-

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_4 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

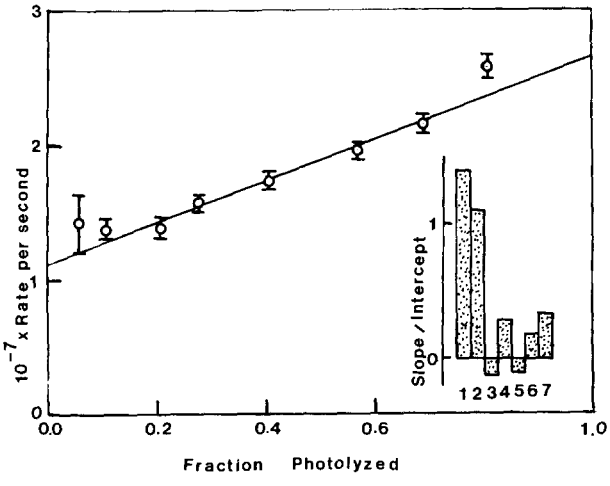

FIG. 2. **Relation between the observed rate of geminate recombination of O_2 with *Scapharca* hemoglobin and fractional photolysis.** The *ordinate* is 10^{-7} × rate of recombination, and the *abscissa* fractional photolysis. The *inset* shows the slope of the line in the main part of the figure divided by the intercept (*Bar 1*). The other *bars* represent analogous experiments performed with: *Scapharca*-O_2 (*Bar 2*), myoglobin-O_2 (*Bar 3*), hemoglobin A-O_2 (*Bar 4*), β-SH hemoglobin chains-O_2 (*Bar 5*), horse Hb-CO in bis-Tris buffer, pH 7 (*Bar 6*), and horse Hb-CO in 0.1 M KP_i (*Bar 7*).

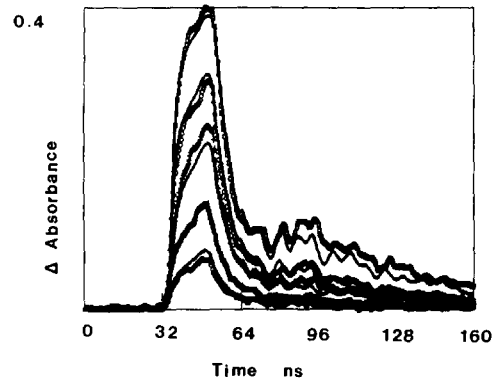

FIG. 3. **Absorbance changes following flash photolysis of *Scapharca*-ethyl isonitrile complex.** The concentration of ligand was 10 mM, of hemoglobin 80 μM, path 1 mm, observation at 445 nm. Temperature 20 °C. The *arrows* show the length of the flash; the relative light intensities were, from above down, 1.0, 0.5, 0.25, 0.125, and 0.063. *Ordinate*, absorbance change, deoxyhemoglobin increases upwards. *Abscissa*, time in nanoseconds. The *continuous lines* are computed solutions using the parameters $k_2 = 1.5$, $k_3 = 0.15$, $k_4 = 0.14$, $k_5 = 0.005$ per ns.

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) rate of 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 1.6×10^7/Ms, 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. 1*A* 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_2/k_3 changes systematically with the proportion of oxyhemoglobin photolyzed, and that k_4 and k_5 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_2 and k_3; 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

1. Chiancone, E., Vecchini, P., Verzili, D., Ascoli, F., and Antonini, E. (1981) *J. Mol. Biol.* **152,** 577–592
2. Ikeda-Saito, M., Yonetani, T., Chiancone, E., Ascoli, F., Verzili, D., and Antonini, E. (1983) *J. Mol. Biol.* **170,** 1009–1018
3. Antonini, E., Ascoli, F., Brunori, M., Chiancone, E., Verzili, D., Morris, R. J., and Gibson, Q. H. (1984) *J. Biol. Chem.* **259,** 6730–6738
4. Royer, W. E., Jr., Love, W. E., and Fenderson, F. F. (1985) *Nature* **316,** 277–280
5. Royer, W. E., Jr., Hendrickson, W. A., and Chiancone, E. (1989) *J. Biol. Chem.* **263,** 21052–21061
6. Austin, R. H., Beeson, K. W., Eisenstein, L., Frauenfelder, H., and Gunsalus, I. C. (1975) *Biochemistry* **14,** 5355–5373
7. Duddell, D. A., Morris, R. J., Muttucumaru, M. J., and Richards, J. T. (1980) *Photochem. Photobiol.* **31,** 4831–4839
8. Gibson, Q. H., Wittenberg, J. B., Wittenberg, B. A., Bogusz, D., and Appleby, C. A. (1989) *J. Biol. Chem.* **264,** 100–107
9. Jongeward, K. A., Magde, D., Taube, D. J., Marsters, J. C., Traylor, T. G., and Sharma, V. S. (1988) *J. Am. Chem. Soc.* **110,** 380–387
10. Petrich, J. W., Poyart, C., and Martin, J. L. (1988) *Biochemistry* **27,** 4049–4060
11. Gibson, Q. H., Olson, J. S., McKinnie, R. E., and Rohlfs, R. J. (1986) *J. Biol. Chem.* **261,** 10228–10239
12. Olson, J. S., Rohlfs, R. J., and Gibson, Q. H. (1987) *J. Biol. Chem.* **262,** 12930–12938
13. Bevington, P. I. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, p. 154, McGraw-Hill Inc., New York
14. Sawicki, C. A., and Gibson, Q. H. (1976) *J. Biol. Chem.* **251,** 1533–1542