Hemerythrin's oxygen-binding reaction studied by laser photolysis
(oxygen-carrying proteins/geminate recombination/diffusion)

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ABSTRACT The dioxygen-iron bond in oxyhemerythrin is shown to be photosensitive. The recombination reaction after photodissociation depends strongly on solvent viscosity. In water (η = 1 centipoise or 1 x 10^{-3} Pas) the recombination is monophasic and second-order in solvent oxygen concentration, with a bimolecular rate coefficient of 2.9 x 10^{5} M^{-1} s^{-1}. In a glycerol/water mixture (η = 180 centipoise) a concentration-dependent geminate recombination process is also seen. This opens a class of proteins to study by flash photolysis.

Hemerythrins are one of the three classes of oxygen-carrying proteins, the others being hemoglobins and hemocyanins. The oxygen-binding site of hemerythrin is a nonheme binuclear iron complex, in contrast to the binuclear copper complex of hemocyanin and the heme group of hemoglobin. Hemerythrin of the marine worm Sipunculus nudus is an octamer with total molecular weight 100,000. Each subunit contains one oxygen-binding site. No cooperativity exists among the subunits in vitro (1). The kinetics of the oxygen-binding reaction have been studied by stopped flow (2) and temperature jump (3), and a significant difference has been noted between the reaction rates of hemerythrin from two different species: stopped-flow experiments on Golfingia gouldii hemerythrin give a second-order recombination rate coefficient of 7.4 x 10^{5} M^{-1} s^{-1}; S. nudus hemerythrin combines at a rate 3.5 times faster so that it escapes the resolution of the stopped-flow technique, but temperature jump determined its rate to be 2.6 x 10^{5} M^{-1} s^{-1}.

Initiating the recombination reaction by flash photolysis has provided a wealth of information on the oxygen-binding reactions of heme proteins (4–10). Flash photolysis is a powerful technique: it is rapid, after photodissociation it leaves the system otherwise relatively undisturbed, and it creates an initial state different from the states produced by either temperature jump or stopped flow. In particular, flash photolysis has been used to probe the diffusion of small ligands within proteins. Frauenfelder and coworkers (9) have shown that geminate recombination in heme proteins can be slowed into the microsecond (and longer) time range by means of low temperatures, high viscosities, or both. We report here flash photolysis experiments on oxyhemerythrin, which has not been previously known to be photosensitive. Our work should open the door to a fruitful extension of low-temperature kinetic studies to nonheme oxygen-carrying proteins.

MATERIALS AND METHODS

We prepared the hemerythrin from the marine worm S. nudus collected on the Brittany coast by the Centre National de la Recherche Scientifique Marine Biological Station at Roscoff, France. Standard procedures (11) were used to prepare the oxyhemerythrin solution, which was diluted to have an absorbance of 1 per cm at 500 nm for the photolysis experiments. Usually the sample was buffered at pH 7 with potassium phosphate; some experiments at pH 8.2 (Tris/acetate) showed no difference. The glycerol/water mixtures (3:1, vol/vol; 79% glycerol by weight) were prepared by using pure dry glycerol (Merck). We were able to verify that the optical spectrum was the same in the glycerol/water solvent as in water. The samples were equilibrated with a controlled atmosphere in the lateral bulb of a 5 x 10 mm optical cell, by allowing a stream of water-saturated gas to flow over the solution under slow stirring. Gas mixtures of known composition were obtained by using pure oxygen, air, or argon and calibrated Brooks flowmeters. The equilibration time was 30 min for water, and glycerol/water mixtures were reequilibrated at 293 K for several hours prior to introduction of concentrated oxyhemerythrin. Temperature adjustments took only a few minutes and we assumed that no reequilibration of the oxygen concentration occurred between 293 K and 278 K in glycerol/water. Gas solubilities for 1 atmosphere (101 kPa) partial pressure of oxygen were taken from published tables (12): O_2 = 1.38 mM and 1.95 mM at 293 K and 278 K, respectively, in water and 0.3 mM at 293 K in glycerol/water.

A Q-switched, frequency-doubled neodymium laser produced a 530-nm actinic light pulse with a half-width less than 20 ns. Recombination of the hemerythrin photodissociated by the light pulse was monitored by the optical absorption increase at 500 nm. The details of the apparatus have been described (13). The slow component of the oxygen recombination was recorded with a 10-μs time constant and the fast component with a time constant of about 10 ns. Due to the different monitoring light systems for the two time ranges, it was not possible in this work to measure data in the time range between 1 and 10 μs.

RESULTS

A laser pulse of 530 nm transiently bleaches the 500-nm absorption band of oxygenated hemerythrin in solution (Fig. 1). Three observations lead us to the conclusion that this bleaching is caused by the photolysis of the iron–oxygen bond: (i) The variation of the initial signal amplitude at various monitoring wavelengths between 380 nm and 600 nm follows closely the difference spectrum between oxyhemerythrin and deoxyhemerythrin. Notably there is a maximum bleaching signal at 500 nm and a minimum at about 440 nm. (ii) Converting all the oxyhemerythrin to methemerythrin by adding an excess of potassium ferricyanide destroys the signal. (iii) The decay rate of

Abbreviations: Hr, deoxyhemerythrin; HrO_2, oxyhemerythrin; cF, centipoise (1 centipoise = 1 x 10^{-3} Pas).
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the transient increases with increasing oxygen concentration in the solution.

The quantum yield for photodissociation of the oxygen ligand was estimated from the size of the signal 20 ns after the laser pulse, using the known difference absorbance coefficients for deoxy–oxhemerythrin. This assumes that the transient absorption spectrum is the same as that of deoxhemerythrin, and that no faster processes are hidden at times less than 20 ns. Our estimate of the quantum yield is approximately 0.03, with a fractional photolysis of 5% in water (input energy 50 mJ).

The recombination rates in water are illustrated in Fig. 2. There the rate of the single exponential is plotted against the total concentration of deoxyhemerythrin and free oxygen immediately after the flash. The bimolecular rate coefficients are $1.8 \times 10^9$ M$^{-1}$ s$^{-1}$ at 278 K and $2.9 \times 10^9$ M$^{-1}$ s$^{-1}$ at 293 K to an accuracy of $\pm 10\%$. We have also measured the rate coefficient in air-saturated solutions at other temperatures between 303 K and 278 K. Their temperature dependence can be fit with an Arrhenius relationship: $k = A \exp(-E_a/RT)$, with activation energy $E_a = -4.4 \pm 0.5$ kcal/mol (1 kcal = 4.18 kJ) and log $A/s^{-1} = 11 \pm 1$. $R$ is the gas constant and $T$ is in kelvin.

Our observed recombination kinetics in a high-viscosity medium differ greatly from those in a low-viscosity one. In water the recombination is a simple exponential, pseudo-first-order in oxygen concentration. In glycerol/water with a viscosity 50–200 times greater, the recombination is biphasic, only the slower process rate varying with concentration (Figs. 1 and 3).

The biexponential recombination reaction in the viscous glycerol/water solvent (viscosity $\eta = 55$ cP at 293 K, 185 cP at 298 K) has a bimolecular phase comparable to the single phase in water, although it is smaller in amplitude and slower. The amplitude difference is taken up by a process more than three orders of magnitude faster. The total signal remains about the same in both media, so apparently the quantum yield is unaffected by the solvent change. In Fig. 3, we have illustrated on a log-log graph the two processes at high viscosity compared with the single exponential in water. The rate of the slower process remains pseudo-first-order in concentration. In contrast, the fast process is not concentration dependent. We can express the observed kinetics by

$$N(t) = N_f \exp(-\lambda_1 t) + N_i \exp(-\lambda_2 t). \quad [1]$$

$N(t)$ is the fraction of deoxhemerythrin not recombined at time $t$ after the photolysis. The prime on $\lambda$ indicates a second-order rate coefficient in M$^{-1}$ s$^{-1}$ for the slow phase. $N_i$ and $N_f$ are the magnitudes of the slow and fast phases and $\lambda_1$ is the rate coefficient of the fast phase. Table 1 lists the measured parameters $N_i, N_f, \lambda_1$, and $\lambda_2$.

**INTERPRETATION**

Because we see biexponential kinetics we propose a two sequential barriers model to facilitate our discussion of the ligand's movement to and from the binding site:

$$\text{Hr} + \text{O}_2 \rightleftharpoons \text{Hr*O}_2 \rightleftharpoons \text{Hr} + \text{O}_2. \quad [2]$$

**Table 1. Parameters of Eq. 1 describing the recombination of O$_2$ with hemerythrin in glycerol/water (3:1, vol/vol):**

<table>
<thead>
<tr>
<th>$T, \text{K}$</th>
<th>$\lambda_1, \text{s}^{-1}$</th>
<th>$N_i$</th>
<th>$\lambda_2, \text{s}^{-1}$</th>
<th>$N_f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>278</td>
<td>$1.2 \times 10^4$</td>
<td>0.92</td>
<td>$5.3 \times 10^2$</td>
<td>0.08</td>
</tr>
<tr>
<td>283</td>
<td>$1.7 \times 10^4$</td>
<td>0.83</td>
<td>$1.8 \times 10^2$</td>
<td>0.16</td>
</tr>
</tbody>
</table>

The rates are determined within 10% at 278 K and to within 20% at 293 K.
Biophysics: Alberding et al.

Table 2. Rates for the two-barrier model (Eq. 3) in glycerol/water

<table>
<thead>
<tr>
<th>T, K</th>
<th>η, cP</th>
<th>k₁, s⁻¹</th>
<th>k₂, M⁻¹</th>
<th>k₃, s⁻¹</th>
<th>ηk₂, s⁻¹</th>
<th>ηk₃, s⁻¹</th>
<th>ηk₄, cP</th>
</tr>
</thead>
<tbody>
<tr>
<td>278</td>
<td>185</td>
<td>1.0 × 10⁷</td>
<td>5.8 × 10⁷</td>
<td>9.0 × 10⁴</td>
<td>1.1 × 10¹0</td>
<td>1.7 × 10⁸</td>
<td></td>
</tr>
<tr>
<td>293</td>
<td>55</td>
<td>1.4 × 10⁷</td>
<td>2.2 × 10⁷</td>
<td>2.8 × 10⁷</td>
<td>1.2 × 10¹0</td>
<td>1.5 × 10⁸</td>
<td></td>
</tr>
</tbody>
</table>

These values are derived from the experimental results of Table 1.

Hr is deoxyhemerythrin, HrO₂ is oxyhemerythrin, and Hr*O₂ represents the photodissociated hemerythrin with the oxygen encased in the protein. The rate k₂ = k₂[O₂]. Though this simple scheme suffices to describe the kinetics we see, kinetic studies over a greater temperature range may reveal additional steps. The exact solution of the system described by Eq. 2 has the form of Eq. 1. To a good approximation it may be written as

\[ N(t) = \left( k_1/k_{12} \right) \exp(-k_1t) \]

\[ + \left( k_2/k_{12} + k_3 \right) \exp(-k_2t/k_1) \]

We assume here that k₂ >> k₁, so that we can ignore the dissociation rate. The known dissociation rate of oxygen, 51 s⁻¹, would seem to support this (2). For our present purposes, the rate of equilibration between Hr*O₂ and Hr + O₂ is k₂ = k₂ + k₃ = k₁ + k₂ + k₃ = k₁ + k₂ + k₃[O₂].

In Table 2 we show the barrier rates derived from this model. It is interesting to note that the rates k₂ and k₃ scale nearly inversely proportionally with the solvent viscosity. Beece et al. (14) and Austin and Chan (10) have shown that the effect of solvent viscosity on the reaction barriers cannot be ignored. Beece et al., in their study of carboxymyoglobin in numerous solvents of various viscosities, have demonstrated that the viscosity’s influence becomes more important in the regions near the protein’s surface. This would seem to agree with our data if we envisage k₂ to be the rate of direct recombination at the binding site and the rates k₁ and k₃ to apply nearer the surface.

It can be seen that the model accounts for both monophasic kinetics in water and biphasic kinetics in glycerol/water if we allow the rates k₂ and k₃ to scale with viscosity and consider the extreme cases of fast and slow equilibration.

(i) In water, at low viscosity, fast relaxation prevails: k₁ >> k₀ and only the slow process dominates with rate k₂ = k₁k₂/k₃ = k₂k₃/k₂. This rate is in fact second order because only k₂ should be proportional to the oxygen concentration in the solvent. In addition, if both k₁ and k₂ are proportional to η⁻¹, k₂ should not vary with small changes in solvent viscosity, as long as the fast relaxation limit is respected. This is indeed the case. When we measure the rate in a sucrose/water mixture with a viscosity of 1.5 cP at 293 K—i.e., the same viscosity as that of pure water at 278 K—the reaction rate remains the same as in pure water at 293 K, where η = 1 cP (see Fig. 2).

(ii) By raising the viscosity from 1 to 55 cP we go to the regime in which the equilibration is slower than the direct recombination rate. This characterizes the slow equilibration limit, k₂ ≣ k₁, which is completely described by Eq. 3.

DISCUSSION

There are two major points to this communication: the dioxygen bond in hemerythrin is photosensitive, and the kinetics at high viscosities show a geminate recombination phase. Thus, the viscosity-dependent multiplet-barrier model of Beece et al. (14) should also be applicable to hemerythrin.

Previous temperature-jump work with S. nudus hemerythrin (3) agrees with our results in water. In contrast, stopped-flow reoxygencation of G. gouldi hemerythrin (2) has been reported to be 1/3.5 times as fast. Fast photolysis experiments similar to this work might determine whether this species dependence is due to the global protein structure or to the local properties of the binding site.

Of more fundamental interest is the comparison of the reaction barriers posed by hemerythrin’s binuclear iron site with the heme site of proteins such as hemoglobin and myoglobin.

Previous work with heme proteins led to a proposed general model of several sequential barriers to ligand recombination within the protein (6, 9). More recent work (14) has emphasized the effects of solvent viscosity on the barriers; in particular, the concept of Kramers (15) that the rate should be proportional to (viscosity)⁻¹ has been shown to be fruitful. Our work here supports these conclusions in a nonheme protein, although some differences are also seen.

The large role that viscosity plays in the barriers is evident from the appearance of a fast phase at high viscosities; further, the dependence of the rates k₂ and k₃ on viscosity is strongly brought out in Table 2, where we see that the actual height of the outer barrier must be considerably smaller than the apparent height if viscosity is not accounted for. The inner barrier, as determined by k₁, shows evidence of a much smaller viscosity dependence. One difference in our work from the heme protein work of Beece et al. (14) is the exponential time dependence of our geminate phase, whereas in refs. 6 and 9 the geminate phase was nonexponential. Apparently in hemerythrin the inner barriers are large enough that the geminate phase is visible at a high enough temperature that the postulated conformational relaxation rate of ref. 9 is larger than k₂. A similar result is evident in the work of Duddel et al. (7) and Alpert et al. (8).

Our results on hemerythrin underline the influence of external solvent viscosity on ligand migration, although a full temperature and viscosity dependence study should be made of this protein to ascertain if any major difference exists between hemerythrin and heme proteins, and if any correlations exist between x-ray structure and ligand mobility.

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