Studies on Hemerythrin. I. Thermodynamic and Kinetic Aspects of Oxygen Binding*

G. Bates,† M. Brunori, G. Amiconi, E. Antonini, and J. Wyman

ABSTRACT: Hemerythrin is a respiratory protein, unique in that each oxygen binding site is composed of two nonheme ferrous atoms. A study of the oxygen binding behavior of the hemerythrin of Sipunculid *Sipunculus nudus* has been carried out. The oxygen equilibrium shows only a slight site–site (homotropic) interaction, the value of the Hill constant, n, being between 1.0 and 1.4. There is no Bohr effect. The equilibrium constant is $K = 1.0 \times 10^5$ M^{-1} at 25°. The dissociation of oxyhemerythrin in the presence of sodium dithionite was studied by the stopped-flow method. The process does not adhere to strict first-order kinetics, but tends to slow down as the reaction proceeds. The first-order rate constant during the initial part of the reaction is 120 sec^{-1}. Temperature-jump relaxation studies indicate a complex relaxation spectrum. Two major processes are present, a faster one in the microsecond range, τ_1, and a slower one in the millisecond range, τ_2. A plot of the dependence of $1/\tau_1$ upon the concentration of the reactants shows a linear relationship, indicating that τ_1 reflects a simple bimolecular process with an "on" constant of 2.6×10^7 M^{-1} sec^{-1} and an "off" constant of 650 sec^{-1}. The second relaxation time, τ_2, is concentration independent over the range examined ($1/\tau_2 = 100$–130 sec^{-1}).

The respiratory proteins may be divided into three classes, based on the chemical nature of the oxygen binding site. These are: (1) the heme proteins, which bind one molecule of oxygen per ferrous iron protoporphyrin complex; (2) the hemocyanins, which bind one molecule of oxygen per two nonheme copper atoms; and (3) the hemerythrins, which bind one oxygen molecule per two nonheme iron atoms. It is with the latter class that this paper is concerned.

One of the interesting aspects of the hemerythrins is the occurrence of a distinct species variation among them, both in structural and functional properties. Thus the hemerythrin of the brachiopod *Lingula* exhibits both a site–site (homotropic) interaction and a Bohr effect (heterotropic interaction), while that of the sipunculid *Phascolosoma agassizii* shows neither (Manwell, 1960). Fortunately a number of comprehensive reviews of the hemerythrin literature have appeared in recent years (Manwell, 1960, 1964; Jones, 1962; Ghiretti, 1962; Boeri, 1963).

Of the hemerythrins, only that of the Sipunculid *Phascolosoma gouldii* has received extensive physicochemical characterization. Klotz and his coworkers (Keresztes-Nagy and Klotz, 1963, 1965) have shown that this protein has a molecular weight of 107,000 and report that it is composed of eight probably identical, reversibly dissociable subunits. Each molecule possesses 16 iron atoms, or 2/subunit. The amino acid sequence of the subunit is now partly known (Groskopf *et al.*, 1966).

The hemerythrin with which this article is concerned, that of sipunculid *Sipunculus nudus*, has received less attention. The reported iron content is 0.87% (Holleman and Biserte, 1958) and the protein is known to bind one oxygen molecule per two ferrous atoms (Boeri and Ghiretti-Magaldi, 1957). The molecular weight, based on osmotic pressure measurements, has been reported as 66,000 (Roche and Roche, 1935); however, preliminary experiments of sedimentation equilibrium from this laboratory indicate a value closer to 100,000. The minimum molecular weight calculated from the iron content is about 6400 (or 12,800 per site); consequently each molecule should contain from six to eight binding sites.

In this laboratory we have been concerned with structural–functional relationships exhibited by respiratory proteins (Rossi-Fanelli *et al.*, 1964; Wyman, 1964). Since hemerythrin represents a relatively little-studied class of such proteins, its functional properties seemed to be of considerable interest, particularly in comparison with those of the hemoglobins, as a means of obtaining a better understanding of respiratory proteins in general. For this reason we have investigated the equilibria and kinetics of the reaction of *S. nudus* hemerythrin with oxygen.

Experimental Section

Protein Isolation. S. nudus worms were obtained fresh and alive from commercial fishermen in Taranto (Italy).

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† Present address: Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Md. U. S. Public Health Service Postdoctoral Fellow I-F2-GM-32,946-01 of the National Institute of General Medical Sciences.

The coelomic fluid was drained off and kept in a closed plastic bottle at 0° until arrival at the laboratory. The protein was isolated by a method described by Ghiretti (1962), involving the washing and centrifugation of the coelomic cells, their lysis, and further centrifugation to remove the cell debris. It was necessary to avoid prolonged exposure of the isolated protein to atmospheric oxygen tensions, since this leads to oxidation of the iron to the ferric form. The protein solution was placed in a tonometer, deoxygenated by evacuation, and stored under argon in the cold. Under these conditions the protein remained unchanged in all its properties for at least 1 month.

Analytical ultracentrifugation and starch gel electrophoresis of the hemolysate showed the hemerythrin to be contaminated by a colorless protein component. This was removed by DEAE-cellulose chromatography.[1] After this treatment the hemerythrin showed a single component in the ultracentrifuge ($s_{20,w} \sim 5$ S); in gel electrophoresis, however, two bands were present. Purification by chromatography did not affect any of the properties that we were studying and thus was frequently omitted.

Spectroscopic Data. Spectroscopic work was carried out with either a Cary 14 or Beckman DK-1 recording spectrophotometer. The extinction coefficient of oxyhemerythrin at 500 mμ, on the basis of iron molarity, is 1.1×10^3 M^{-1} cm^{-1}. Throughout the paper we report protein concentrations in terms of oxygen binding equivalents per liter, where one oxygen binding site is composed of two iron atoms. In this case the extinction coefficient at 500 mμ is twice the value above, *i.e.*, 2.2×10^3 N^{-1} cm^{-1}. The figure was obtained by a sodium thiocyanate iron analysis, as outlined by Vogel (1961). The same value for the extinction coefficient has been reported earlier for *S. nudus* (Boeri and Ghiretti-Magaldi, 1957) and also for *P. gouldii* (Kerestez-Nagy and Klotz, 1965).

Oxygen Equilibrium. The oxygen dissociation curves were determined by a spectroscopic method of Rossi-Fanelli and Antonini (1958). This involves the assumption that the spectral change is proportional to the degree of saturation. We found no evidence to the contrary. Two wavelengths were used, 510 and 570 mμ, at both of which oxygenation leads to an increase in absorbancy. Exact experimental conditions are reported in the legends of the figures.

Stopped-Flow Measurements. The kinetics of the dissociation of oxyhemerythrin were measured with a Gibson-Durrum stopped-flow apparatus equipped with a 2-cm observation tube. The dead time of the instrument was 3.5 msec. Generally the kinetics were followed at 500 mμ.

Temperature-Jump Measurements. These were carried out using an Eigen–De Maeyer-type instrument built by the Messanlagen Gesellschaft (Göttingen). A cell having a capacity of 7 ml and a light path of 1 cm was used for all the experiments. The temperature change was between 3 and 5° in the different experiments. The instrumentation and principles of temperature-jump relaxation measurements have recently been reviewed (Eigen and De Maeyer, 1963; Czerlinski, 1966).

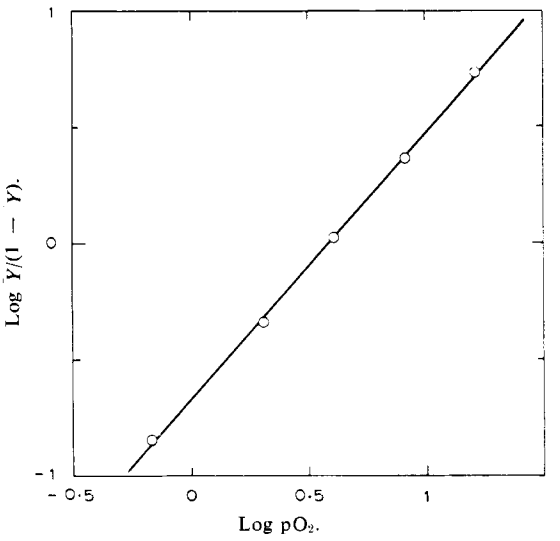

FIGURE 1: Hill plot of the oxygen equilibrium of hemerythrin at pH 7.0, 0.15 M potassium phosphate buffer, 20°. The straight line drawn through the points corresponds to a value of n of 1.15.

Results

Oxygen Equilibrium. The experimental data for the oxygen equilibrium obtained with the usual procedure are shown in Figure 1 in terms of a Hill plot. The value of the Hill parameter, n, was always slightly greater than 1 and in the best experiments close to 1.1, indicating at most only a slight cooperative interaction between the sites. The highest values of n, which in one or two cases ran up to 1.4, are somewhat suspect, due to the progressive development of a slight amount of turbidity in the course of the measurements, probably due to surface denaturation during equilibration of the solution with the gas phase.

In order to avoid development of the turbidity, one experiment was performed by adding O_2 in solution to the deoxyhemerythrin solution. A careful spectroscopic analysis showed that in this case the fractional saturation was independent of wavelength from 600 to 350 mμ. The value of the affinity constant determined by this method was identical with that obtained by the method discussed above; the value of n, however, was unity within experimental error.

A Van't Hoff plot of the effect of temperature on the oxygen equilibrium is presented in Figure 2. The curve is linear within the limits of experimental error, and the slope corresponds to an enthalpy change given by $\Delta H = -13.5$ kcal/mole of oxygen bound. This value includes the heat of solution of oxygen ($\Delta H \sim -3.0$ kcal/mole at 20°).

The value for the association equilibrium constant obtained from the partial pressure of O_2 necessary to

[1] A satisfactory procedure is to employ a linear gradient in ionic strength from 0.01 M Tris (pH 7.3) to 0.01 M Tris plus 0.2 M KCl. Details regarding chromatographic behavior of hemerythrin will be published elsewhere.

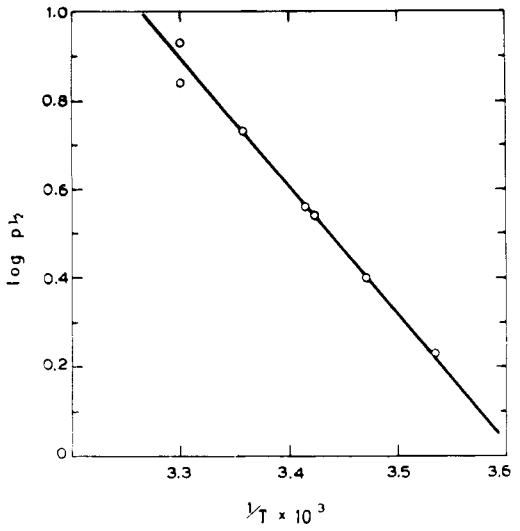

FIGURE 2: Log $p^1/_2$ as a function of $1/T$. The straight line corresponds to a $\Delta H = -13.5$ kcal/mole. At 25° the equilibrium constant $K = 1.0 \times 10^5$ M^{-1}.

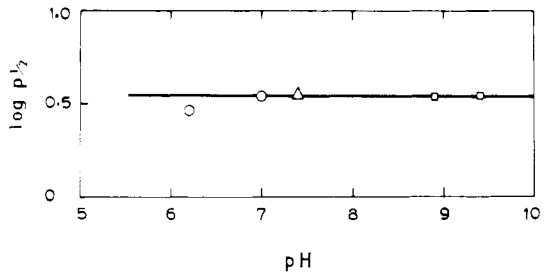

FIGURE 3: Log $p^1/_2$ as a function of pH. Buffers used were: (□) 2% borate, (○) 0.2 M potassium phosphate, and (△) 0.01 M Tris–0.05 M NaCl.

produce 50% saturation (*i.e.*, $p^1/_2$) is $K = 1.0 \times 10^5$ M^{-1} at 25° and pH 7.0. The lack of a Bohr effect over the pH range from approximately 6 to 9.5 is demonstrated by Figure 3. Various buffers were used but all gave essentially the same results.

Kinetic Studies. An attempt to determine the rate of combination of deoxyhemerythrin with oxygen by rapid mixing methods was unsuccessful. Even at very low protein and oxygen concentrations, the reaction was too rapid to be resolved by a flow method.

The dissociation reaction, however, was accessible to measurement. The time course of the reaction following mixing of sodium dithionite and oxyhemerythrin is plotted as a first-order function in Figure 4. The results show that the reaction does not follow first-order kinetics, tending to slow down by 20–40% as it proceeds. The meaning of this is not clear. It might indicate either a heterogeneity of the hemerythrin or a difference of properties between different sites on the same molecule. In Table I are listed first-order velocity constants for the initial part of the reaction with various dithionite concentrations. It will be seen that over a 16-fold range in dithionite there is only a 10% variation in the rate constant. This indicates that the reaction is essentially zero order with respect to dithionite and that the dissociation of the oxyhemerythrin is the rate-limiting step according to

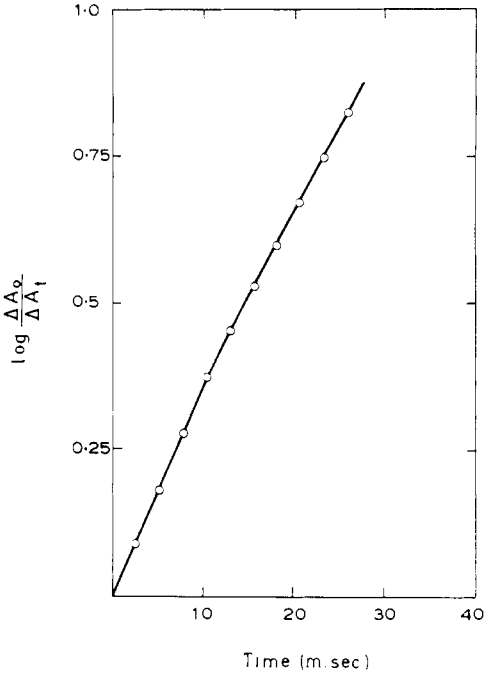

FIGURE 4: Kinetics of deoxygenation of oxyhemerythrin by dithionite at pH 7.0, 0.1 M potassium phosphate buffer, 20°. Observations at λ 500 mμ; protein concentration = 2×10^{-4} binding equiv/l.

TABLE I: The Initial First-Order Rate Constant for the Dissociation of Oxyhemerythrin as a Function of Sodium Dithionite Concentration.[a]

Sodium Dithionite Concn	Obsd First-Order Rate Constant (sec^{-1})
1.0	74
0.25	70
0.0625	67

[a] The highest concentration is approximately 1%. Conditions are the same as reported in Figure 4.

$$HmrO_2{}^2 \xrightarrow{k_1} Hmr + O_2$$

$$O_2 + Na_2S_2O_4 \xrightarrow{k_2} \text{products}$$

where k_1 is the rate-limiting step and thus a true measure of dissociation rate.

The activation energy for the dissociation was studied over a temperature range from 12 to 25°. The value calculated from the Arrhenius plot shown in Figure 5 is

[2] $HmrO_2$ indicates oxyhemerythrin sites. Hmr indicates deoxyhemerythrin.

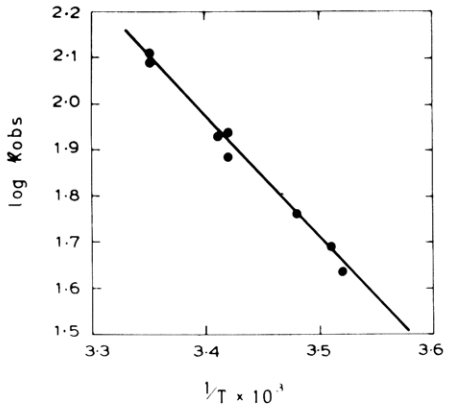

FIGURE 5: Log initial rate constant for deoxygenation of oxyhemerythrin by dithionite as a function of $1/T$. The straight line corresponds to an activation energy of 12.7 kcal/mole.

12.7 kcal/mole. The initial value of the dissociation velocity constant at 25° is 120 sec^{-1}.

Temperature-Jump Relaxation. The temperature-jump measurements reveal the presence of two main processes, a faster one, corresponding to a relaxation time τ_1, and a slower one corresponding to τ_2. These are shown in Figures 6 and 7, respectively. In addition there is a small, very rapid initial decrease in absorbancy which is too fast to be resolved by our instrument. This can be clearly seen in Figure 6. The two main relaxation processes, corresponding to τ_1 and τ_2, persisted under a variety of conditions of pH, buffer, and protein purity and freshness. In particular both the amplitude and the rate of the second process is independent of the presence of ferric hemerythrin. The relative amplitudes of the changes corresponding to τ_1 and τ_2 differ with the wavelength (see below). At very low concentrations of protein, however, the second phase (corresponding to an increase in absorbancy) could not be detected.

The optical density change accompanying the temperature jump accords with the decrease of enthalpy associated with oxygenation. In Figure 8 the total absorbancy change for a given temperature jump from time zero to time infinity is compared with the static difference spectrum of oxyhemerythrin *vs.* deoxyhemerythrin. Since the kinetic data give only *relative* values of difference spectra, they were reduced so as to give the best fit. In view of the large errors involved in the temperature-jump measurements the two sets of data may be regarded as in agreement.

The fast process, illustrated in Figure 6, can always be described by a single relaxation time, τ_1. A series of experiments at various protein concentrations and partial saturations were carried out to study the dependence of $1/\tau_1$ upon the concentration of the reactants. Figure 9 shows a plot of $1/\tau_1$ as a function of the sum of the concentration of free oxygen in solution (O_2) and the concentration of free binding sites (Hmr) for various protein concentrations (from 0.08 to 7.2 $\times$ 10^{-4} binding equiv). The relation is linear over the concentration

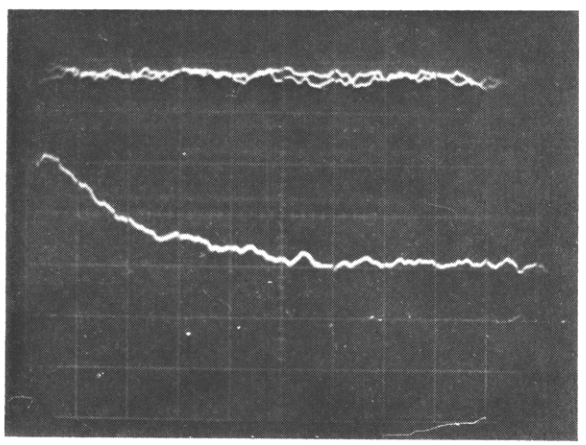

FIGURE 6: Oscilloscope trace of a temperature-jump experiment on oxyhemerythrin at pH 7.0, 0.1 M potassium phosphate buffer. Sweep time, 100 μsec/large scope division; λ 500 mμ; protein concentration, 4 $\times$ 10^{-4} binding equiv/l; fractional saturation, 0.36; discharge, 26 kV giving a temperature charge of approximately 3°; temperature after jump, 25°.

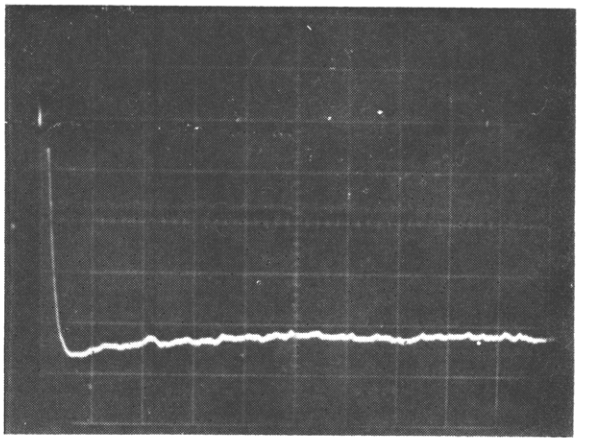

FIGURE 7: Oscilloscope trace of same experiment as reported in Figure 6 with a sweep time of 5 msec/large scope division.

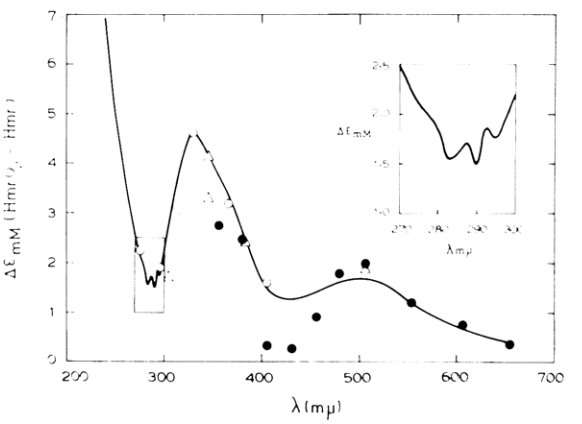

FIGURE 8: Comparison of the static difference spectrum of oxyhemerythrin *vs.* deoxyhemerythrin (solid line) with the kinetic difference spectrum obtained by temperature jump at pH 7, 0.1 M potassium phosphate buffer; constant discharge, 26 kV; (●) protein concentration, 4 $\times$ 10^{-4} binding equiv/l.; $\bar{Y}$ = 0.36; (○) protein concentration, 0.56 $\times$ 10^{-4} binding equiv/l.; $\bar{Y}$ = 0.36; (△) protein concentration, 1.61 $\times$ 10^{-4} binding equiv/l.; $\bar{Y}$ = 0.4.

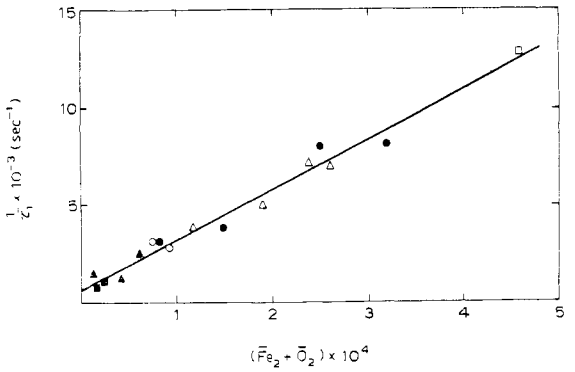

FIGURE 9: Dependence of $1/\tau_1$ upon the concentration of the reactants expressed as the sum of the free binding sites ($\overline{Fe_2}$) and free oxygen ($\overline{O_2}$). The experiments were performed at various protein concentrations: 0.08–0.18, 0.6–0.7, 1.6, 2.5–2.8, 3.4–4, and 7.2×10^{-4} binding equiv per l.

range examined, as would be expected for a bimolecular reaction. The slope corresponds to a combination velocity constant of 2.6×10^7 M^{-1} sec^{-1}; the intercept to a dissociation velocity constant of 650 sec^{-1}.

The second relaxation time, τ_2, is much greater than τ_1 and to a first approximation appears to be independent of the concentration of the two reactants over the range examined ($1.3–3.5 \times 10^{-4}$ M). The value of $1/\tau_2$ is between 100 and 130 sec^{-1}. This relaxation time, τ_2, is not evident at 600 mμ, but shows a monotonic increase in amplitude as the wavelength is varied down to 300 mμ.

Discussion

The equilibrium studies suggest that the oxygen equilibrium of the hemerythrin of *S. nudus* is simple, as if all the sites were alike and independent. It is doubtful whether occasional values of n greater than 1 are significant. There is no Bohr effect and the enthalpy change ($\Delta H = -13.5$ kcal/mole) accompanying oxygenation is similar to that reported for other hemerythrins and for hemoglobins (Manwell, 1964; Rossi-Fanelli *et al.*, 1964).

The kinetic behavior is more complex. The dissociation of the oxyhemerythrin, as measured by the addition of dithionite in a stopped-flow apparatus, is not a simple first-order process, suggesting that there may be two types of binding sites or possibly some other form of heterogeneity.

One of the drawbacks in the treatment and the interpretation of the temperature-jump data is that no detailed picture of the binding site can be given at this time. The stoichiometry shows that one molecule of oxygen is bound for each two iron atoms present in the protein. We do not know, however, whether both iron atoms are actually involved in the binding of the ligand, and if so whether they are equivalent. The abscissa employed in Figure 9 is based on the assumption, made in the absence of any definite structural information, that one oxygen molecule combines with *two* iron atoms. The straight line drawn through the points corresponds to a velocity constant of 2.6×10^7 M^{-1} sec^{-1}. When we plotted the data on the alternative assumption that only one iron atom is involved in the binding of an oxygen molecule, we obtained a graph in which the points, within experimental error, were fitted equally well by a straight line. In this case the slope of the line gave a velocity constant equal to essentially half the previous value. At the present time, due to scatter of the data, it is impossible to decide between these two alternatives by carrying out a least-squares fit.

The existence of a second relaxation time, τ_2, and the difference in the values of the dissociation velocity constant for O_2 as obtained by flow ($k_{off} = 120$ sec^{-1}) and by temperature jump ($k_{off} = 650$ sec^{-1}) indicate that the reaction is more complex than a simple bimolecular process and involves intermediate stages, possibly representing conformational transitions. At present, however, our data do not allow us to assign any precise mechanism to the reaction. It is hoped that future studies will give us further insight into the nature of the hemerythrin binding site and allow a better explanation of the basic oxygenation studies presented here.

Acknowledgments

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