Communications to the Editor

Uptake and Release of O_2 by Myohemerythrin. Evidence for Different Rate-Determining Steps and a Caveat

Christopher R. Lloyd, Edward M. Eyring,* and Walther R. Ellis, Jr.*

Department of Chemistry, University of Utah Salt Lake City, Utah 84112

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The biological transport[1] of molecular oxygen is carried out by iron- and copper-containing proteins. Refined X-ray structures[2] of members of the iron O_2 carriers, hemoglobins and hemerythrins, reveal no channels for exogenous ligands to directly access the iron sites from the solvent, demonstrating the physiological importance of protein fluctuations. The oxy adducts of these proteins are photosensitive, enabling the study of O_2 recombination with deoxy forms produced by laser flash photolysis. Results to date have been interpreted[3] in terms of consecutive O_2 recombination equilibria for members of both protein families, indicating that O_2 passage through these protein matrices involves multiple barriers. In relating rate and equilibrium data at physiological temperatures for these proteins, it has been tacitly assumed by many workers that $K_{eq} = k_{on}/k_{off}$. In this communication, we report results[4] for *Themiste zostericola* myohemerythrin (Mhr) and demonstrate that this assumption is not valid.

Mhr binds dioxygen by reducing it to hydroperoxide; concomitantly, diferrous deoxyMhr is oxidized to the diferric oxy form. The (hydro)peroxo → Fe^{III} charge-transfer transition, centered at 500 nm, of oxyMhr was used to monitor the kinetics of O_2 uptake and release. Rapid mixing[5] of oxyMhr with sodium dithionite was used to scavenge O_2 in solution, initiating O_2 release from the protein. Rate constants[6] for this reaction as a function of temperature and pressure are plotted in Figures 1 and 2, respectively, and summarized in Table 1. Laser flash photolysis[7] (530 nm, 6 ns pulse) of oxyMhr was used to generate deoxyMhr for kinetic analysis of the O_2 uptake (recombination) reaction; temperature- and pressure-dependent results are plotted in Figures 1 and 2, respectively, and listed in Table 1. The kinetic activation parameters for O_2 release are consistent[8] with a dissociative rate-determining step involving Fe–O bond cleavage. However, the positive volume of activation for O_2 uptake is not consistent with the compressed transition state expected for an associative reaction. Furthermore, Fe–O bond formation should not exhibit a near-zero enthalpy of activation. These results point to a rate-determining step that occurs *prior* to Fe–O bond formation.

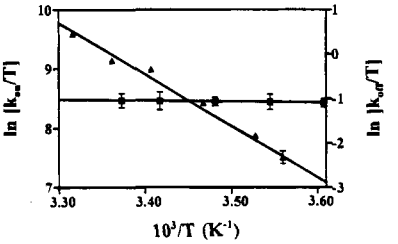

Figure 1. Eyring plots[6] of the temperature dependences of the observed rates (1 atm, pH 8.0) of O_2 uptake (k_{on}, ■) and release (k_{off}, ▲).

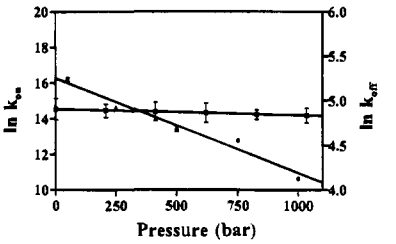

Figure 2. Plots[6] of k_{obs} vs pressure (21.5 °C, pH 8.0) for O_2 uptake (k_{on}, ■) and release (k_{off}, ▲). No pressure-induced protein denaturation occurs at pressures up to 2000 bar.

Table 1. Summary of Rate[a] Data for the Uptake and Release of O_2 by Myohemerythrin

parameter	O_2 uptake	O_2 release
k (21.5 °C; 1 atm)	$(1.40 \pm 0.02) \times 10^6$ M^{-1} s^{-1}	209 ± 2 s^{-1}
k_H/k_D (21.5 °C; 1 atm)	1.01 ± 0.09	1.6 ± 0.1
$\Delta H^{\ddagger}$ (kcal/mol; 21.5 °C; 1 atm)	0.3 ± 0.1	$+22 \pm 1$
$\Delta S^{\ddagger}$ (eu; 1 atm)	-29 ± 1	$+28 \pm 4$
$\Delta V^{\ddagger}$ (cm^3/mol; 21.5 °C)	$+8.4 \pm 0.3$	$+28 \pm 3$

[a] Both reactions are independent of pH in the range 8.0–9.5.

Deuterium isotope effects (Table 1) support our interpretation of the kinetic behavior of the system. $k_H/k_D = 1.6$ for O_2 release, suggesting that rupture of the O–H bond depicted in Figure 3 occurs during the same elementary step as Fe–O bond rupture. The absence of a reciprocal isotope effect for the O_2 uptake reaction indicates, on the basis of microscopic reversibility considerations, that the rate-determining steps for these two reactions do not coincide. Referring to Figure 3, which depicts the minimum number of steps in these reactions, we suggest that k_{-3} is rate-determining during O_2 release, while k_2 limits

(1) For a recent review, see: Jameson, G. B.; Ibers, J. I. In *Bioinorganic Chemistry*; Bertini, I., Gray, H. B., Lippard, S. J., Valentine, J. S., Eds.; University Science Press: Mill Valley, CA, 1994; pp 167–252.

(2) (a) Perutz, M. F. *Trends Biochem. Sci.* **1989**, *14*, 42–44. (b) Stenkamp, R. E. *Chem. Rev.* **1994**, *94*, 715–726.

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(4) Obtained using recombinant wild-type Mhr purified from *Escherichia coli* cell extracts. This protein is structurally and kinetically identical to Mhr obtained from *T. zostericola* retractor muscles (Raner, G. M., Ph.D. Dissertation, University of Utah, 1994). Protein samples were prepared in 300 mM Tris-sulfate buffers (our reported data are for pH 8.0). Deuterated samples were prepared by repeated cycles of diluting metMhr into deuterated Tris-sulfate buffer (prepared using 99.9% D_2O; Cambridge Isotope Laboratories), reduction to the deoxy form with a slight excess of sodium dithionite, and concentration by centrifugal ultrafiltration.

(5) Temperature-dependent data were obtained using a Durrum stopped-flow spectrophotometer. The design of our pressurizable stopped-flow instrument follows that described in the following: van Eldik, R.; Gaede, W.; Wieland, S.; Kraft, J.; Spitzer, M.; Palmer, D. A. *Rev. Sci. Instrum.* **1993**, *64*, 1355–1357. Rates of O_2 release are independent of both $[Na_2S_2O_4]$ and [Mhr].

(6) Experimental uncertainties associated with individual points not containing error bars in Figures 1 and 2 are contained within those points.

(7) The laser flash photolysis system and high-pressure optical cell have been described previously (Ji, Q.; Eyring, E. M.; van Eldik, R.; Reddy, K. B.; Goates, S. R.; Lee, M. L. *Rev. Sci. Instrum.* **1995**, *66*, 222–226). O_2 concentrations were measured with a Corning M90 O_2 analyzer.

(8) van Eldik, R., Ed. *Inorganic High Pressure Chemistry: Kinetics and Mechanisms*; Elsevier: Amsterdam, 1986. The partial molar volume of O_2 is 28 cm^3/mol.

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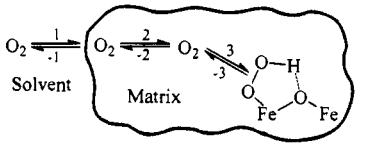

Figure 3. Schematic view of the presence of multiple intermediates during the uptake or release of O_2 by myohemerythrin.

the rate of O_2 uptake.[9] K_{eq} (21.5 °C) for O_2 binding by deoxyMhr, $(2.5 \pm 0.5) \times 10^5$ M^{-1}, measured by spectrophotometric equilibration of mixtures of deoxyMhr and O_2, is not in reasonable agreement with the $(6.7 \pm 0.1) \times 10^3$ M^{-1} value for the rate ratio.

Our work establishes that the kinetic determination of K_{eq}, as k_{on}/k_{off}, for O_2 binding by Mhr is not valid and results in an unacceptable error. Results for other iron O_2 carriers suggest a similar circumstance. Deuterium isotope effects, similar to those noted in Table 1, have been reported[10] for *Phaseolopsis gouldii* hemerythrin. Positive values of $\Delta V^{\ddagger}$ for O_2 uptake by sperm whale myoglobin and bovine hemoglobin have been observed.[3g,11] A flash photolysis study[3c] of O_2 binding to sperm whale myoglobin concluded that the rate-limiting step occurs prior to Fe—O bond formation, as is the case here. Motion of the distal histidine is thought[2a,12] to play a key role in the uptake of O_2 by myoglobin and the α-subunits of hemoglobin. At present, it is unclear which amino acid residues are important determinants of the kinetic behavior of Mhr.

The use of temperature-jump relaxation in perturbing oxy ⇄ deoxy metalloprotein equilibria (e.g., refs 10, 11c, 13) has usually resulted in uniphasic[14] relaxation times, indicating that this method cannot be used to determine the number of steps in ligand binding by these proteins. Since additional relaxation amplitudes have not been detected, we infer that ΔH° values for the steps after the initial Fe—O bond breakage must be very small. Other routine kinetic studies of O_2 uptake and release are also handicapped by a number of observables insufficient to properly treat multistep equilibria such as these. Reported O_2 affinities, obtained under the assumption of a single equilibrium step, therefore only represent *apparent* equilibrium constants.

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(9) Our flash photolysis results are independent of added glycerol (0—50% vol/vol), indicating that k_1 does not limit the O_2 uptake rate.

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(14) A biphasic relaxation spectrum has been reported for a hemerythrin (Bates, G.; Brunori, M.; Amiconi, G.; Antonini, E.; Wyman, J. *Biochemistry* **1968**, *7*, 3016—3020).