The Electron-Transfer Mechanism of Autoxidation for Hemoglobin, Myoglobin, and Their Iron(II) Cyclidene Models

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Abstract: The rates of autoxidation of iron(II) cyclidenes have been studied as functions of dioxygen pressure and with variations in the size of the cavity within which the O_2 molecules must reside in order to bind to the iron atom. As the cavity is opened up, first O_2 and then solvent species can enter and bind to iron, and this is accompanied by changes in the algebraic form of the rate law for autoxidation. Analysis strongly suggests that the molecule of O_2 that oxidizes the iron(II) is not bound, thereby implicating an electron-transfer mechanism for the autoxidation process. The peculiar nature of the O_2 dependences of myoglobin and hemoglobin autoxidations indicates that the same mechanism applies.

Introduction

All known dioxygen carriers undergo autoxidation (oxidation by O_2) in solution, including the natural dioxygen carriers. Typically about 1.5–3% of the hemoglobin in mammalian blood is autoxidized to iron(III)-containing methemoglobin each day,[1,2] but a reduction process maintains the level of methemoglobin below about 1%.[3] The mechanism of the autoxidation process for hemoglobin and myoglobin is important both to health science and as fundamental science. The key to understanding this mechanism has been found by recognizing the importance of early work on the dependence of the Hb and Mb autoxidation rates on dioxygen pressure and the compelling parallel between that behavior and autoxidation patterns found for the iron(II) cyclidene family of synthetic dioxygen carriers. The large changes in structure that are easily made with the cyclidenes have been particularly useful in solving this venerable puzzle.

In 1925 Neil and Hastings reported that high pressures of dioxygen hinder the autoxidation of hemoglobin,[4] and in early studies of the autoxidation kinetics for the natural dioxygen carriers, hemoglobin[5,6] and myoglobin,[7–9] the same highly distinctive dioxygen dependencies were observed, wherein high O_2 pressures actually appeared to protect the substrate from oxidation by O_2 (Figure 1). The rates were found to increase with dioxygen pressure, pass through maxima, seemingly in the vicinity of $P_{O2} = P_{50}$, and then decrease with further increases in P_{O2}. At the same time, the rates were found to be first order with respect to the heme protein.[10] In a searching analysis, Stratmann and George[8] concluded that the behavior could only be explained by assuming the presence of some third iron-containing species in addition to the principal deoxy and oxy forms of the heme proteins; this was prophetic of the model that we are supporting here. Most subsequent investigations of the autoxidation mechanisms of these preeminent dioxygen carriers have failed to consider adequately the results described above. In support of an electron-transfer mechanism, Caughey et al.[11–13] pointed out the difficulty of rationalizing the Hb and Mb autoxidation behavior in terms of either superoxide dissociation[14,15] or bimolecular displacement[16,17] mechanisms that had been proposed for these systems. The simple

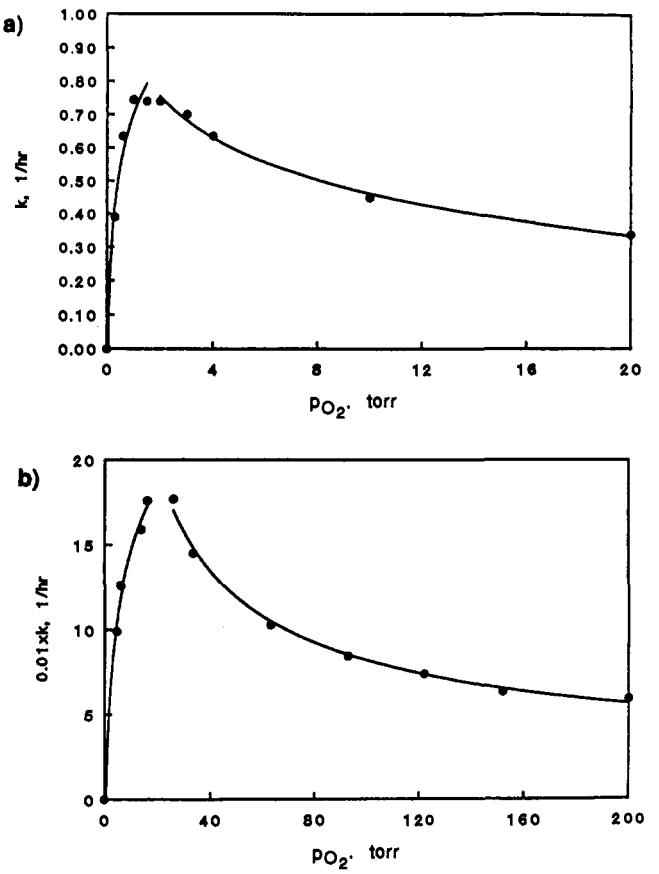

Figure 1. Plot of the dioxygen pressure dependencies of the observed rate constants of autoxidation of (a) myoglobin[8] and (b) hemoglobin.[6]

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(10) It is necessary to not be confused by the well-known occurrence of a similar dioxygen protection that has been observed for free porphyrins and which cannot apply to the heme proteins. That instance of O_2 protection is associated with the μ-peroxo bridge mechanism in which two irons become simultaneously bound to a single dioxygen molecule. Obviously that mechanism cannot occur for Hb and Mb where each iron-containing prosthetic group is sequestered within an individual globular protein. Further, those processes show second-order dependence on the iron concentration.
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first-order dependence of the rate of autoxidation on the concentrations of various potential ligands (N_3^-, SCN^-, F^-) had led to the proposal of a bimolecular displacement of superoxide from the dioxygen adduct by the nucleophile;[18] the strong nucleophile N_3^- displays both pH-dependent and -independent pathways. The bimolecular mechanism has been supported in later studies, as well.[16,17]

The main contending mechanism is an electron-transfer mechanism also suggested by Caughey.[11,12] This is given in eq 1–3. In this mechanism, outer-sphere electron transfer and dioxygen binding are competing reactions. With regard to the

$$Hb + O_2 \rightleftharpoons HbO_2 \qquad K_{O2} \tag{1}$$

$$Hb + L^- \rightleftharpoons Hb(L^-) \qquad k_1, k_{-1} \tag{2}$$

$$Hb(L^-) + O_2 \rightarrow \text{met-Hb}(L^-) + O_2^- \qquad k_2 \tag{3}$$

discussion to follow, it is also significant that, a few years later, Castro and co-workers suggested an outer-sphere electron-transfer mechanism for the autoxidation of coordinately saturated iron porphyrin complexes.[19]

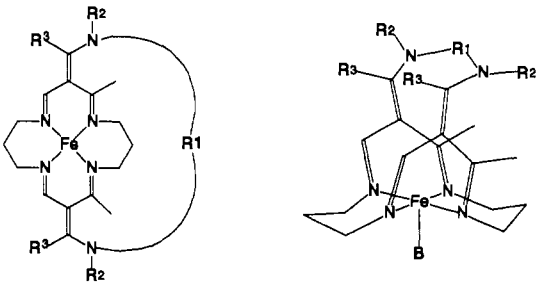
The simplicity of the O_2 binding equilibrium for Mb is attractive for further discussion of the character of the dioxygen dependence of the autoxidation of the natural products. Empirically, the maximum in the rate may be reproduced by eq 4,[8] where K is the

$$k_{obs} = kK[O_2]/\{K + [O_2]\}^2 \tag{4}$$

inverse of the equilibrium constant for formation of the dioxygen adduct and k is a rate constant. The quadratic form of the denominator leads to the prediction that beyond the rate maximum, the autoxidation rate will continuously diminish as the dioxygen pressure increases, but the data indicate an asymptotic high-pressure rate. Consequently, a second reaction pathway must also be involved. Studies on the synthetic iron(II) cyclidenes (structure I) have clarified these relationships.

Some time ago,[20–23] we reported the design and synthesis of the iron(II) cyclidenes, and established this family of complexes as the only fully functional heme protein dioxygen carrier mimics that are totally synthetic, even to the point of containing no porphyrin group. The substituent groups R^1, R^2, and R^3 (structure I) have been varied a great deal and lengthy X-ray crystallographic and dioxygen binding equilibrium studies, especially with the cobalt(II) cyclidenes, have shown that the magnitude of dioxygen affinity can be controlled by those structural variations.[23–31] Structural variants in which the bridge R^1 is a polymethylene group whose length ranges from $(CH_2)_3$ to $(CH_2)_8$ have been particularly useful; as the chain length increases, the dioxygen affinity increases. Further, there is no evidence that a trimethylene bridged species binds dioxygen at all. In fact, the iron(II) cyclidene complex having $R^1 = (CH_2)_3$ shows no affinity for either O_2 or CO. It is the kinetic autoxidation behavior of these polymethylene bridged iron(II) cyclidene complexes that points to the mechanism by which Hb, Mb, and (cyclidene)iron(II) are oxidized by the dioxygen in the air. Early observations revealed that the same dioxygen protection observed for Mb and Hb also occurs for a certain iron(II) cyclidene.[32] Now we report that structural variations provide convincing arguments to distinguish between previously proposed mechanisms for the autoxidation of dioxygen carriers of this class, including hemoglobin and myoglobin.

As the size of the dioxygen binding cavity (structure I) is changed, the dioxygen dependencies of the autoxidation rates of the iron cyclidene complexes change, obeying a variety of rate



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laws, including that observed for Hb and Mb (Figure 2). The facile control over the structures of the cyclidene complexes makes the observed extreme variation in behavior accessible. By altering the bridging group R^1 in the cyclidene ligand the dioxygen binding site can be increased smoothly from the minimum size, which occurs with R^1 = trimethylene, through a maximum lacunar size at octamethylene.[33] Completely open cyclidenes and vaulted cyclidenes provide still larger dioxygen binding sites.[30,34] It would be relatively difficult to vary the size of the dioxygen binding sites in the natural products; however, the distinctive nature of the dioxygen dependence of the autoxidation rates for Hb and Mb has two major consequences that are especially pertinent to the arguments that follow: (1) it implicates those dioxygen carriers in the same autoxidation mechanism as that observed for the iron cyclidenes, and (2) it places them rather precisely in the sequence observed (as O_2 affinity increases) for the iron cyclidenes.

Results and Discussion

Figure 2 depicts the dioxygen pressure dependence of the autoxidation rates of the iron cyclidene complexes as the bridging polymethylene group increases in length, and the rate constants are given in Table I. At C3 (trimethylene bridge), simple first-order dependence on P_{O_2} is observed; opening the cavity to C4 produces saturation behavior; and increasing the cavity size still more to C5 produces the "dioxygen protection" behavior observed for Hb and Mb. The impact in establishing the mechanism is best shown by first considering the C4 case, since saturation behavior is commonplace.

The obvious model to explain the saturation behavior of the tetramethylene bridged iron(II) cyclidene is the long assumed

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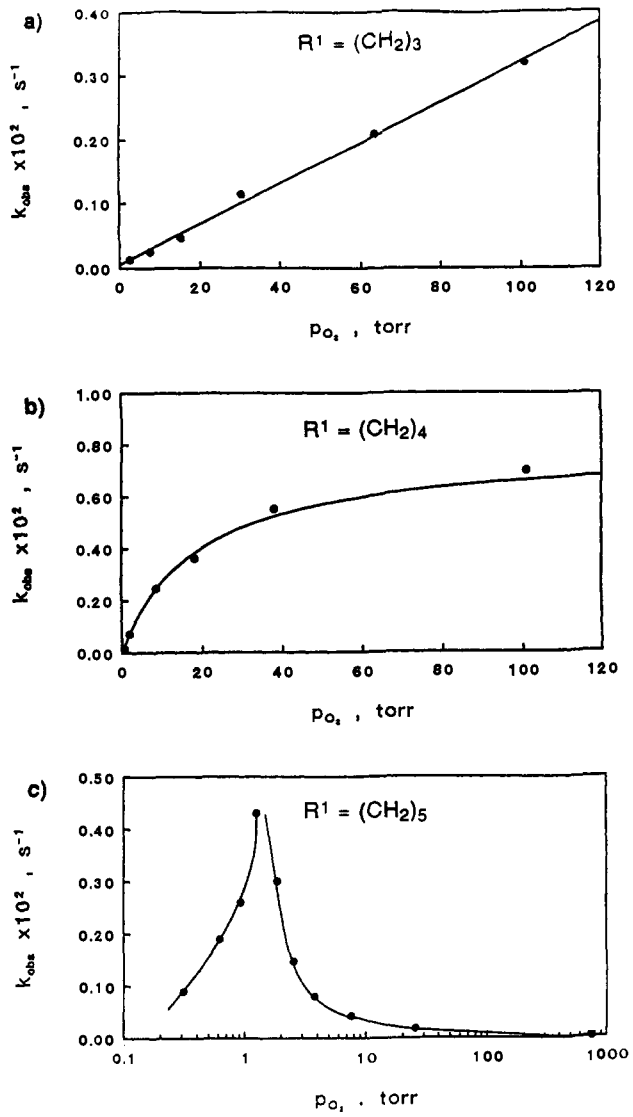

Figure 2. Plot of the dioxygen pressure dependencies of the observed rate constants of autoxidation of iron cyclidene complexes as the bridging polymethylene chain increases in length. The solvent system is MeOH/1 M 1-methylimidazole/0.05 M TBAT (tetrabutylammonium tetrafluoroborate): (a) [Fe^{II}(Me,Me,$(CH_2)_3$)] at −25 °C; (b) [Fe^{II}(Ph,Me,$(CH_2)_4$)] at 0 °C; and (c) [Fe^{II}(Ph,Me,$(CH_2)_5$)] at 0 °C.

superoxide dissociation mechanism (eq 5 and 6). This simply involves preequilibrium formation of the dioxygen adduct which subsequently liberates superoxide[35] and the rate law is given in eq 7. It is, however, necessary to consider a second mechanism

$$Fe^{II} + O_2 \rightleftharpoons Fe(O_2)^{2+} \qquad K_{O_2} \tag{5}$$

$$Fe(O_2)^{2+} \rightarrow Fe^{III} + O_2^- \qquad k \tag{6}$$

$$\text{rate} = kK_{O_2}[Fe^{II}][O_2]/\{1 + K_{O_2}[O_2]\} \tag{7}$$

that predicts precisely the same algebraic form for the rate law. This is an electron-transfer process in competition with the dioxygen binding equilibrium. Equations 8 and 9 present this mechanism and its rate law is given in eq 10. Both mechanisms predict (1) first-order dependence on iron concentration, (2) saturation kinetics with zero-order dependence of the rate on $[O_2]$ at high $[O_2]$, and (3) first-order dependence on $[O_2]$ at low concentrations.

(35) This rate law applies equally well to a bimolecular process in which the superoxide is displaced from the pre-formed dioxygen adduct by a solvent nucleophile.

Table I. Autoxidation Rate Constants for $[Fe^{II}(R^3R^2(CH_2)_n)Cl]^+$ (n = 3–6) in MeOH/1 M *N*-Methylimidazole/0.05 M TBAT

cyclidene	T (°C)	P_{O_2} (Torr)	k_{obs} (s^{-1})
$MeMe(CH_2)_3$	−25	2.53	1.3×10^{-4}
		7.6	2.53×10^{-4}
		15.2	4.68×10^{-4}
		30.4	1.15×10^{-3}
		63.5	2.10×10^{-3}
		101.0	3.20×10^{-3}
$PhMe(CH_2)_4$	0	0.65	1.45×10^{-4}
		1.9	7.13×10^{-4}
		8.7	2.46×10^{-3}
		18.1	3.61×10^{-3}
		38.0	5.53×10^{-3}
		101.0	7.00×10^{-3}
$PhMe(CH_2)_5$	0	0.31	8.94×10^{-4}
		0.62	1.9×10^{-3}
		0.93	2.6×10^{-3}
		1.26	4.3×10^{-3}
		1.86	3.0×10^{-3}
		2.53	1.47×10^{-3}
		3.80	7.91×10^{-4}
		7.60	4.28×10^{-4}
		25.3	1.83×10^{-4}
		751	1.3×10^{-5}
$PhMe(CH_2)_6$[a]	−10	0.30	7.37×10^{-4}
		0.45	1.21×10^{-3}
		0.65	1.24×10^{-3}
		1.4	1.25×10^{-3}
		1.9	1.34×10^{-3}
		5.9	1.20×10^{-3}
		18	9.08×10^{-4}
		27	8.84×10^{-4}
		38	6.67×10^{-4}
		239	5.06×10^{-4}
		745	4.23×10^{-4}

[a] Data in MeOH/0.05 M LiCl.

$$Fe^{II} + O_2 \rightleftharpoons Fe(O_2)^{2+} \qquad K_{O_2} \tag{8}$$

$$Fe^{II} + O_2 \rightarrow Fe^{III} + O_2^- \qquad k' \tag{9}$$

$$\text{rate} = k'[Fe^{II}][O_2]/\{1 + K_{O_2}[O_2]\} \tag{10}$$

The choice between mechanisms (preequilibrium and superoxide dissociation (or displacement) versus competitive equilibrium and electron transfer) rests on the parametric dependence of the two rate laws on dioxygen affinity K_{O_2} and the facile changes that can be made in the structure of the dioxygen binding site of the cyclidene complex. Equation 7 requires the rate of autoxidation to go to zero when the dioxygen affinity (K_{O_2}) vanishes. Chemically, the complex cannot dissociate a superoxide unless it first binds O_2. In contrast, eq 10 only depends on dioxygen affinity in the denominator, and when K_{O_2} goes to 0, the rate law merely changes to simple first order (in $[O_2]$).

Despite repeated efforts with both O_2 and CO, it has always been found that no ligand can enter the cavity of the trimethylene-bridged species, therefore the C3 case qualifies as having zero dioxygen affinity.[36] It follows that the behavior of the C3 bridged complex shown in Figure 2, simple first-order dependence on dioxygen pressure, provides a critical distinction in favor of the competitive equilibrium/electron-transfer mechanism for the autoxidation of the iron cyclidene complexes. The alternative superoxide dissociation (or displacement)[35] mechanism predicts that the C3-bridged complex would not autoxidize, whereas the data in Table I show that the C3-bridged complex autoxidizes the most rapidly of all.

The conclusion that autoxidation occurs by an electron-transfer mechanism is strongly supported by the consequences of opening the cavity further. At C3 nothing enters the cavity; at C4, O_2 enters the cavity and that process competes with electron transfer

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between the iron center and O_2, giving rise to O_2 saturation behavior in the autoxidation process. Opening the cavity still more by adding the fifth methylene group to the bridge produces the C5 complex with the dioxygen protection behavior that is also shown in Figure 2, and which replicates the autoxidation behavior of Hb and Mb. As was deduced by Stratmann and George,[8] a new species must be invoked to explain this more complicated behavior, and that new species simply involves the entering of a solvent molecule into the cavity in competition with both dioxygen binding and electron transfer.[37] Thus eqs 11 and 12 are added to eqs 8 and 9 to produce the overall mechanism, and the steady state (on $Fe^{II}S$) rate law for the newly added path is given in eq 13. At high O_2 pressures, the $[O_2]^2$ term in the denominator

$$Fe^{II} + S \rightleftharpoons Fe^{II}S \qquad k_1, k_{-1} \tag{11}$$

$$Fe^{II}S + O_2 \rightarrow Fe^{III}S + O_2^- \qquad k_2 \tag{12}$$

$$\text{rate} = \frac{k_1(k_2/k_{-1})[Fe^{II}][S][O_2]}{(k_2/k_{-1})K_{O_2}[O_2]^2 + \{(k_2/k_{-1}) + K_{O_2}\}[O_2] + 1} \tag{13}$$

dominates, causing the rate to decrease with further increases in dioxygen pressure. The observation that the autoxidation rate eventually reaches a constant value at very high pressures is explained on the basis of the competing saturation pathway (eqs 8 and 9). At high dioxygen partial pressures, that term in the overall rate law is expected to dominate. Again a close parallel was found by George and Stratmann in the autoxidation of myoglobin; i.e., a two-term rate law was required to explain the limiting high-pressure value of the pseudo-first-order rate constant.[8]

Remarkably, the rich chemical consequences of expanding the cavity size do not cease at the C5 case. Opening the cavity still more yields the C6-bridged compound and its tendency to bind a sixth ligand is so great that the 6-coordinate derivative is a major species at low temperatures, and this produces a most revealing thermochromism.[22] Under strictly anaerobic conditions, an intense absorption band appears at about 500 nm and such spectral features are characteristic of low-spin 6-coordinate iron(II) complexes whose ligands have azomethine donor atoms.[38] The presence of the low-spin complex was confirmed by magnetic susceptibility studies of the solution at various temperatures; for example, at −36 °C about 24% of the iron(II) complex is present in the low-spin 6-coordinate form.[22] The important conclusion is that a low-spin 6-coordinate iron(II) complex is indeed formed in substantial concentrations when the cavity is sufficiently large to accommodate at least the smaller of available ligands.

Now one may ask about the kinetic consequences of this enormous increase in the concentration of what was proposed as a kinetically competent intermediate for the more constrained C5 case. Most simply, the dioxygen dependence of the autoxidation rate shows a relatively broad maximum; however, it is the temperature dependence of the autoxidation rate that is most fascinating. As Figure 3 shows, the rate of autoxidation displays a parabolic dependence on temperature. With cooling from room temperature, the rate at first decreases in a normal manner, but it then increases rather dramatically so that, for example, the rates are about the same at room temperature and at −40 °C. Since the several enthalpies of activation or equilibration associated with a single reaction pathway bear an additive relationship, the parabolic temperature dependence suggests the inception of a second path for autoxidation and is consistent with the overall model presented here. The two pathways involve electron transfer between O_2 and respectively the 5-coordinate deoxy complex and the 6-coordinate solvated complex.

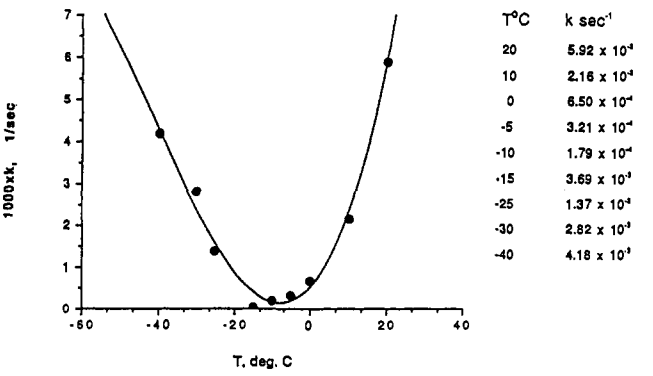

Figure 3. Plot of the temperature dependence of the observed rate constants of autoxidation of $[Fe^{II}(Ph,Me,(CH_2)_6)]$ in acetone/pyridine/water 3:1:1 at 150 Torr of O_2.

Conclusions

A common, unexpected mechanism of autoxidation is established for all well-characterized iron(II) dioxygen carriers of the Pauling type, including hemoglobin, myoglobin, and their cyclidene biomimics. The mechanism involves electron transfer between the iron(II) species and the unbound dioxygen molecule and not the dissociation of superoxide from the dioxygen adduct. The facile variations in structure of the iron(II) cyclidenes made possible the elimination of superoxide dissociation and superoxide displacement mechanisms on the basis of correlations between broadly varying rate laws and dioxygen affinities exhibited by the structural variants. It follows from the electron-transfer mechanism that the dioxygen adducts themselves are inherently stable and that their destruction by O_2 results from competing processes, not from the spontaneous decomposition of the adducts. On the basis of the results reported here, it would be best to look for an electron-transfer mechanism when the autoxidation of iron(II) species is under consideration, rather than the commonly suspected dissociation of superoxide from the dioxygen adduct.

Experimental Section

Iron cyclidene complexes $[Fe^{II}(Me,Me,(CH_2)_3)]$, $[Fe^{II}(Ph,Me,(CH_2)_4)]$, $[Fe^{II}(Ph,Me,(CH_2)_5)]$ and $[Fe^{II}(Ph,Me,(CH_2)_6)]$ were prepared as described earlier.[21,22] All inert atmosphere manipulations were performed under N_2 in a glovebox maintained below 1 ppm of O_2. Spectra were recorded on either a Varian 2300 spectrophotometer or a Hewlett Packard 8452 diode array spectrophotometer, with a 9000 (300) Hewlett Packard Chem Station, and both incorporated flow-through temperature-regulated cell holders, giving a temperature precision of ±0.3 °C. Oxygen/nitrogen gas mixtures were generated with use of Tylan FC-260 mass flow controllers. Typical kinetic experiments involved filling a 1-cm gas-tight cell, fitted with a gas inlet and a bubbling tube, inside the glovebox with a solution of ca. 2×10^{-4} M cyclidene complex. The solvent system was MeOH/1 M 1-methylimidazole/0.05 M TBAT (tetrabutylammonium tetrafluoroborate). Stock solutions of the iron cyclidene in MeOH/TBAT were used, but 1-methylimidazole was added just before the experiment. Solutions were saturated by initial bubbling for 120–180 s with the desired gas mixtures. Pseudo-first-order conditions of constant oxygen concentrations were achieved by occasionally bubbling more O_2 into the solution during the course of the experiment. Kinetic studies were performed with either of the spectrophotometers described above, and the kinetic parameters were evaluated by using either the Hewlett Packard proprietary software or programs written in Basic for the Varian spectrophotometer by Dr. Naidong Ye of this group.

Acknowledgment. Support of this work by the National Science Foundation is gratefully acknowledged.

(37) The intricate variation of rate law with solvent that is observed in the systems reported here is dependent on equilibrium constants appropriate to produce the revealing competitions. The dioxygen affinity depends on axial ligand and solvent, in addition to ligand structure. Consequently, other solvents do not all produce the same behavior; cf.: Sauer-Masarwa, A.; Dickerson, L. D.; Herron, N.; Busch, D. H. *Coord. Chem. Rev.*, Bailar Memorial Volume, in press.

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