Mechanisms of Autoxidation of the Oxygen Sensor FixL and *Aplysia* Myoglobin: Implications for Oxygen-Binding Heme Proteins†

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ABSTRACT: On exposure to oxygen, ferrous heme is thought to autoxidize via three distinct mechanisms: (i) dissociation of protonated superoxide from oxyheme; (ii) reaction between a noncoordinated oxygen molecule and pentacoordinate deoxyheme, and (iii) reaction between a noncoordinated oxygen molecule and an intermediate having water coordinated to the ferrous heme iron. The formation of a hexacoordinate aquomet ($H_2O{\cdot}Fe^{3+}$) species has been proposed to drive mechanism (iii); consequently, heme proteins with a pentacoordinate met (Fe^{3+}) form might be expected to lack this pathway. We have measured the dependence of autoxidation rate on oxygen concentration for *Rhizobium meliloti* FixL and *Aplysia kurodai* myoglobin, which have pentacoordinate met forms. For both proteins, the bell shape of this dependence shows that they autoxidize primarily by mechanism (iii), indicating that a hexacoordinate aquomet species is not required for this mechanism. A novel presentation of the oxygen dependence of autoxidation rates that uses heme saturation, rather than oxygen concentration, more clearly reveals the relative contributions of autoxidation pathways.

Heme proteins that bind oxygen do so only in their ferrous state. Free heme in solution oxidizes to the ferric state very rapidly on exposure to oxygen. In heme proteins that bind oxygen as part of their physiological roles, an important function of the protein matrix is to diminish the tendency of the heme iron to oxidize. The mechanisms of autoxidation, and the roles of various heme pocket side chains in controlling this process, have been examined primarily for vertebrate myoglobins and model compounds (*1*−*3*). The bacterial oxygen sensor FixL is much more susceptible to autoxidation than sperm whale myoglobin (SWMb)[1] (*4*). The full-length FixL from *Rhizobium meliloti* is a homodimeric kinase whose enzymatic activity is regulated by binding of oxygen to a heme domain (*5*). Autoxidation, per se, does not affect the mechanism of kinase regulation, since the heme-controlled inactivation of the FixL kinase occurs in both oxidation states on binding of ligands (*6*). However, to sense oxygen, FixL must be maintained in the ferrous state. FixL differs from typical oxygen-binding heme proteins in several important respects. The ferric heme iron is pentavalent and without coordinated water (*4*, *7*). FixLs binds oxygen with association rates that are over 60-fold slower than those of SWMb, leading to dramatically lower oxygen affinity (*4*). These unusual properties also hold for the isolated heme domain of the protein, RmFixLH, which is monomeric and of approximately the size of myoglobin. There are no exact equivalents in FixL for the structural features of myoglobins, since FixL does not even share with them any recognizable motifs (*8*). Nonetheless, the ways in which a protein matrix modulates the reactivity of a heme prosthetic group are expected to be quite general and to lead to similar mechanisms of autoxidation in oxygen-binding heme proteins. To examine the intrinsic properties of the FixL heme domain apart from effects due to regulation of the kinase, for our studies of autoxidation we have chosen to compare RmFixLH to myoglobin.

Another product of the autoxidation of ferrous heme, besides ferric heme, is superoxide. The initial evidence for this species came from the observation that superoxide dismutase inhibits the co-oxidation of epinephrine in solutions of oxyhemoglobin (*9*). Thus, regardless of mechanism, the net reaction between oxygen and ferrous heme iron is

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^-$$

Reaction Pathways for Heme Protein Autoxidation. The available data on the autoxidation of heme proteins can be accounted for by three mechanistic pathways:

Pathway 1: Dissociation (or Displacement by Solvent) of Protonated Superoxide from Oxyheme. Weiss (*10*) had proposed that oxygen binds to the heme and then dissociates as a superoxide anion:

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[1] Abbreviations: RmFixLH, heme domain of the *Rhizobium meliloti* oxygen-sensing kinase FixL; Mb, myoglobin; SWMb, sperm whale myoglobin; P_{O_2}, partial pressure of oxygen; P_{50}, partial pressure of a gas at which the hemes are half-saturated. The notation for myoglobin mutants is a one-letter code for the original amino acid followed by the position of the amino acid in the protein sequence and then a one-letter code for the replacing amino acid; e.g., H64V SWMb refers to a mutation of histidine 64 to valine in sperm whale myoglobin.

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$$Fe^{2+} + O_2 \rightleftarrows Fe^{2+}O_2 \rightarrow Fe^{3+} + O_2^-$$

As written, this would require an unfavorable dissociation of the negatively charged superoxide from the positively charged ferric heme iron in a hydrophobic environment. Shikama, Caughey, and their colleagues postulated a protonated oxyheme intermediate that would dissociate more readily (*11*, *12*).

Pathway 1

$$Fe^{2+} + O_2 \rightleftarrows Fe^{2+}O_2$$

$$Fe^{2+}O_2 + H^+ \rightarrow Fe^{2+}O_2H^+ \rightarrow Fe^{3+} + HO_2$$

If essentially all autoxidation under oxygen-saturating conditions occurred by dissociation of superoxide from protonated oxyheme, this rate would increase with decreasing pH as the fraction of oxyheme in the protonated state increases. Olson and colleagues have measured the autoxidation rates in air for SWMb mutants ranging widely in their oxygen affinities (*1*). They found that, for each unit pH decrease between 5 and 7, the autoxidation rates accelerate roughly 10-fold. The differences in the autoxidation rates of the different SWMb mutants were plausibly explained in terms of interactions stabilizing or destabilizing the protonated form of oxyheme. The data are consistent with a p*K* for protonated oxyheme in the range of 4−5. On the other hand, the autoxidation of myoglobin from the shark *Heterodontus japonicus* is independent of pH below 6.3, where the strongest pH dependence would be expected if the intermediate were protonated oxyheme (*13*). There is as yet no direct evidence of the dissociation of superoxide from any natural or synthetic oxygen complex of iron.

The superoxide dissociation mechanism in Pathway 1 would result in autoxidation rates directly proportional to the concentration of oxyheme, i.e.

$$k_{ox(1)} = k_{diss}\left(\frac{[O_2]}{K_d + [O_2]}\right) = k_{diss}(Y) \qquad (1a)$$

where k_{diss} is the rate of dissociation of superoxide, $Y = [O_2]/(K_d + [O_2])$ is the fraction of the oxyheme at a given concentration of oxygen, and K_d is the oxygen dissociation equilibrium constant (Figure 1A, first term).

Pathway 2: Outer-Sphere Electron Transfer to a Pentacoordinate Deoxyheme Intermediate. Interestingly, the same hyperbolic dependence of the autoxidation rate on oxygen concentration predicted for the superoxide displacement mechanism is also predicted by a purely outer-sphere mechanism not requiring the dissociation of superoxide from oxyheme (*2*):

Pathway 2

$$Fe^{2+}O_2 \underset{K_d}{\rightleftharpoons} Fe^{2+} + O_2 \xrightarrow[k_1]{} Fe^{3+} + O_2^-$$

This leads to a contribution to k_{ox} proportional to oxygen and to the fraction of protein in deoxy form:

$$k_{ox(2)} = k_1[O_2](1 - Y) = k_1[O_2]\left(\frac{K_d}{K_d + [O_2]}\right) = k_1K_d\left(\frac{[O_2]}{K_d + [O_2]}\right) = k_1K_d(Y) \quad (1b)$$

The reaction between oxygen and pentacoordinate ferrous heme has the same form as eq 1a, the superoxide dissociation reaction, even though the latter depends only on the concentration of oxyheme. This arises because the product of the concentrations of oxygen and deoxyheme is proportional to the concentration of oxyheme. The interpretation of the constant k_1K_d, however, differs from the meaning assigned to the constant k_{diss}. For instance, the outer-sphere electron transfer, unlike the dissociation of superoxide, does not depend on pH.

The Unimolecular Term in the Autoxidation Rate Expression: Combining Pathways 1 and 2. The rate expression of heme proteins is known empirically to contain a term proportional to the concentration of oxyheme. As we have seen, such a term would arise from either Pathway 1 alone, Pathway 2 alone, or a combination of both. As there is only indirect evidence for either pathway in heme proteins, let us combine the unimolecular contributions to k_{ox} for the most general expression:

$$k_{ox(uni)} = (k_1K_d + k_{diss})\frac{[O_2]}{K_d + [O_2]} = (k_1K_d + k_{diss})Y \quad (1)$$

Pathway 3: Outer-Sphere Electron Transfer to a Hexacoordinate Deoxyheme Intermediate. Instead of the autoxidation rate proportional to oxygen concentration that we would observe if only Pathways 1 and 2 contributed to autoxidation, Stratmann and colleagues (*14*) observed a bell-shaped dependence for myoglobin like the one in Figure 1A. At lower saturations, autoxidation does accelerate with increasing oxygen, but it is fastest when the myoglobin is half-saturated with oxygen. Any further increases in the concentration of oxygen slows the autoxidation, which asymptotically approaches a nonzero limiting value.

To explain the region of the Stratmann curve in which the rate of autoxidation slows with increasing oxygen tension, Caughey and colleagues (*12*) have proposed a two-step ligand-mediated electron-transfer reaction. First, a facilitating nucleophile N, e.g., water, binds to the ferrous myoglobin. Since there is no spectroscopic evidence for this binding, it is postulated to be slow and weak. Second, in a bimolecular oxidative step, the hexacoordinate ferrous heme transfers an electron to oxygen via an outer-sphere mechanism:

Pathway 3

$$N + Fe^{2+} \rightleftarrows Fe^{2+}N$$

$$Fe^{2+}N + O_2 \rightarrow Fe^{3+}N + O_2^-$$

In support of this model, a number of workers have shown that ligands of ferric heme enhance the rate of autoxidation (*12*, *15*). Pathway 3 would account for the bell-shaped dependence of the autoxidation rates on oxygen, since this reaction depends on the fraction of deoxyheme as well as

the fraction of oxyheme. This pathway adds a second term to the expression for k_{ox} of the form

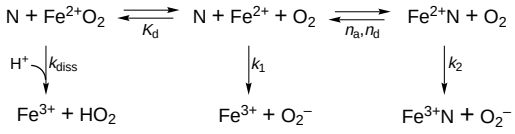
$$k_{ox_{(3)}} = \left\{ n_a[N] \frac{K_d}{(K_d + [O_2])} \frac{[O_2]}{((n_d/k_2) + [O_2])} \right\} \quad (2)$$

where n_a and n_d are the association and dissociation rate constants for the coordination of water (N) to the heme iron and k_2 is the rate of autoxidation of the hexacoordinate deoxyheme intermediate. This contribution to the total autoxidation rate decreases at elevated oxygen and would account for the "oxygen protection" effect.

The observed autoxidation rate dependence on oxygen concentration can be accounted for without any autoxidation by oxyheme (*2*). This scheme, a combination of Pathways 2 and 3, assumes that oxyheme is entirely unreactive, and all autoxidation occurs by reaction between O_2 and deoxyheme (with or without a coordinating nucleophile). Oxygen protection would therefore result from the competition between oxygen (which forms an unreactive species) and solvent nucleophiles (which form a complex that oxidizes faster than pentacoordinate heme) for coordination of deoxyprotein.

Comprehensive Expression for the Dependence of the Autoxidation Rate on Oxygen Concentration. (For a derivation of the rate equations, see Appendix.) If we allow all of these pathways to contribute to the autoxidation rate, we obtain the following scheme and oxygen dependence:

Scheme 1



This leads to the rate equation:

$$k_{ox} = \left\{ (k_{diss} + k_1 K_d) \frac{[O_2]}{(K_d + [O_2])} \right\} + \left\{ n_a[N] \frac{K_d}{(K_d + [O_2])} \frac{[O_2]}{((n_d/k_2) + [O_2])} \right\} \quad (3a)$$

where N is a solvent nucleophile, n_a is the rate of association of N, and n_d is the rate of dissociation of N. This equation can be simplified by grouping some of the constants and substituting for *Y*, the saturation of the protein with oxygen. Let $Y = [O_2]/([O_2] + K_d)$; $k_u = (k_{diss} + k_1 K_d)$; $k_b = n_a[N]$; and $q = n_d/k_2$. Then

$$k_{ox} = \{k_u Y\} + \left\{ k_b (1 - Y) \frac{[O_2]}{(q + [O_2])} \right\} \quad (3)$$

Importance of Reactive Oxygen Species. Since the redox potential for the O_2/O_2^- couple (−0.33 V) is lower than that of the Hb^{III}/Hb^{II} system (−0.046 V), the one-electron transfer from Fe^{2+} to free O_2 required for Pathways 2 and 3 cannot occur spontaneously (*16*). Those reactions are driven by the spontaneous dissociation (K_d) of the superoxide to hydrogen peroxide: $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ (*17*). Thus in all three pathways above, the reaction steps leading to formation of superoxide can be treated as irreversible. Hydrogen peroxide can oxidize both deoxy- and oxyMb, although it oxidizes deoxyMb 100-fold faster (*18*). Several authors have suggested that it is the greater susceptibility of the deoxyheme to peroxide oxidation that causes the oxygen protection effect (*19*). Along the same lines, they have suggested that it is the effect of pH on the disproportionation of superoxide to peroxide that is reflected in the acceleration of the autoxidation rates at acidic pH. However, the pH dependence of the autoxidation rate is unchanged when large concentrations of these enzymes are added; the rates themselves change by less than 30%, and they decrease rather than increase (*1*).

Hexacoordinate Deoxyheme Intermediate. The resonance Raman spectra of metSWMb show that water is coordinated to the ferric heme iron (*20*). In most vertebrate Mbs, this weakly binding ligand is stabilized by hydrogen bonding to a distal histidine. In proteins lacking this stabilization, there is no detectable coordination of water to ferric heme; this is true for FixL, *Aplysia* Mbs, and some mutants of SWMb (*1*, *4*, *7*, *20*−*22*). Even in proteins that coordinate water in their ferric forms, coordination of water to the ferrous heme is so slight as to be undetectable. However, the spectrum of water-coordinated ferrous heme has been measured by reducing ferric Mb at cryogenic temperatures (*23*). The same factors that destabilize water in the ferric forms of some heme proteins would also destabilize the water-coordinated ferrous heme intermediate proposed for Pathway 3 (*12*). Pathway 3 might be lacking in such proteins, if they have a greatly reduced tendency to form the hexacoordinate deoxyheme intermediate. If this were so, the autoxidation of such proteins would not have the bell-shaped dependence on oxygen that is predicted for pathways requiring this intermediate. We examine for the first time the dependence of autoxidation on oxygen concentration for *Aplysia kurodai* Mb and RmFixLH, two heme proteins that are pentacoordinate in their met form. A novel treatment of the oxygen dependence of autoxidation rates reveals more clearly the relative contributions of autoxidation mechanisms.

EXPERIMENTAL PROCEDURES

Protein Purification. RmFixLH was purified as described previously following its overproduction in *Escherichia coli* strain TG1(pRH61) (*4*). Purified *Aplysia kurodai* Mb was provided by Dr. Keiji Shikama.

Autoxidation Rates. Unless otherwise specified, the rates of oxidation were measured for 3−6 μM protein in 100 mM sodium phosphate, pH 7.0. Because of its rapid autoxidation, the measurements were at 25 °C for RmFixLH. The measurements of *Aplysia* myoglobin autoxidation were at 37 °C. Ferric heme proteins were reduced with crystalline sodium dithionite. Deoxyheme protein, free of dithionite, was recovered from a small Sephadex G-25 (Pharmacia) column equilibrated with degassed 100 mM sodium phosphate, pH 7.0, in an anaerobic chamber. The deoxyheme protein (3−6 μM) was transferred to a sealed cuvette equipped with a bubbler and valve. The bubbler cuvette was placed in the thermostated cell holder of a Hewlett-Packard 8452A diode-array spectrophotometer and connected to the precision gas mixing apparatus described below. After the system was flushed with 20% O_2/80% N_2, a programmable

Table 1: Rate and Equilibrium Parameters for the Autoxidation of RmFixLH and Myoglobins

protein	O_2 k_{on} (M^{-1} s^{-1})	O_2 k_{off} (s^{-1})	O_2 K_d (μM)	q	k_u (h^{-1})	k_b (h^{-1})	max rate (h^{-1})
			Pentacoordinate Met				
RmFixLH	0.22	6.8	29	31	0.66	26	6.4
Aplysia kurodai Mb			15	15	0.5	4.9	1.5
H64V SWMb[a]	110	9900	200	200	45		45
			Hexacoordinate Met				
V68T SWMb	2.8	39	14	14	3.0	4.7	3.7
K45E SWMb			8.6	8.6	1.2	0.92	1.2
H62, SWMb	24	130	15	15	0.071	2.5	0.66
V68A SWMb	22	18	4.0	3.8	0.17	0.91	0.33
V68F SWMb	1.2	2.5	5.8	5.4	0.043	1.0	0.28
wild-type SWMb	0.16	14	4.0	3.6	0.054	0.92	0.26

[a] The raw data for SWMb and its mutants were provided by Olson and colleagues (*1*).

valve bubbled this gas periodically through the protein solution. Absorption spectra from 350 to 700 nm were collected automatically at programmed intervals. Autoxidation was followed by the appearance of the met absorption band at 396 nm. All time courses showed clear isosbestic points and were followed to near completion. The rates were calculated from the absorbance change at 396 nm.

Influence of Oxygen on the Rates of Autoxidation. Controlled gas mixtures for bubbling were achieved with an apparatus consisting of a series of mass-flow controllers connected to tanks containing 100% O_2, 100% N_2, 50% O_2/50% N_2, and 10% O_2/90% N_2. The gases from any pair of tanks could be mixed in any proportion to achieve $\pm$ 0.1% of a desired P_{O_2}. The rates of autoxidation were measured as described in the previous section in 0.5%, 1.0%, 2.0%, 3.0%, 5.0%, 10.0%, 20.0%, 50.0%, and 100.0% O_2 (remainder N_2).

RESULTS AND DISCUSSION

Autoxidation Rates of Heme Proteins Can Be Described Solely in Terms of Oxygen Saturation, without Reference to Oxygen Concentration. When the autoxidation rates of heme proteins ranging widely in their oxygen affinities are fitted to eq 3, the fitted parameter $q = n_d/k_2$ is always very close to K_d, which is the experimentally observable P_{50} for oxygen saturation. Olson and colleagues (*1*) have pointed out that from the derivative of the rate expression with respect to oxygen concentration, this would have to be true to account for the familiar empirical observation that the maximum rate of autoxidation via Pathway 3 occurs very near the P_{50}. From measurements of the autoxidation of SWMb at less than half-saturation, where any differences would result in the largest error, we estimate that the difference between q and K_d, if any, is less than 5%. This relationship between n_d, k_2, and K_d permits a dramatic simplification of eq 3: if $q = n_d/k_2 = K_d$, then

$$k_{ox} = \{k_u Y\} + \{k_b Y(1 - Y)\} \quad (4)$$

Although it makes no explicit reference to oxygen concentration, the empirical eq 4 gives a good fit to all heme proteins examined (Figures 1B,D and 2B). This relationship holds despite the fact that equivalent saturations result from vastly different oxygen concentrations for proteins ranging by 2 orders of magnitude in their oxygen affinities. It also holds for proteins differing greatly in their autoxidation rates and the relative contributions of unimolecular and bimolecular mechanisms to this reaction.

Equation 4 accurately describes the empirically observed behavior of a wide variety of heme proteins, regardless of the underlying mechanism responsible for this rate law. Since there is as yet no direct evidence for intermediates such as protonated oxyheme, or solvent-coordinated ferrous heme, playing a role in autoxidation under physiological conditions, the pathways described here may be considered speculative. A number of other mechanistic schemes can result in the same algebraic form. The form of the second term, which is linear in both oxy- and deoxyheme, suggests a reaction between those two protein species. The derivation given here shows that this mathematical form can arise without such a reaction. However, eq 4 fits the empirical data only if the quantity n_d/k_2 always equals K_d, and there is no explanation for this equivalence. The possibility of electron transfer from oxyheme to deoxyheme should be seriously considered, since it leads very naturally and simply to an equation of the correct form without any such assumptions:

$$Fe^{2+}O_2 + Fe^{2+} \rightarrow Fe^{3+} + Fe^{2+}O_2^- \rightarrow Fe^{3+} + Fe^{2+} + O_2^-$$

This reaction involves dissociation of superoxide from ferrous heme, which would be more favorable than a dissociation from ferric heme. As noted in the introduction, the one-electron transfer from Fe^{2+} to free O_2 required for Pathways 2 and 3 cannot occur spontaneously (*16*). Electron transfer to protein from heme-coordinated oxygen is more common in biology than transfer from free oxygen.

Relative Contributions of the First and Second Terms in the Rate Equation 4 for Different Heme Proteins. Table 1 lists the fitted rate constants obtained from the oxygen dependence of the autoxidation rates of RmFixLH, *Aplysia kurodai* Mb, and the wild-type and mutant SWMbs (*1*). To facilitate comparison of the relative contributions of the first and second terms for the various proteins, Figure 2 shows their fitted oxygen dependence curves, normalized to their maximal rates. The representation of oxygen dependence in terms of saturation in Figure 2B is especially convenient for comparing heme proteins with radically different oxygen affinities, since these proteins have very different proportions of oxy- and deoxyheme at a given oxygen tension, each oxidizing by a different mechanism. This novel presentation facilitates an evaluation of the relative contribution of each term by visual inspection. An autoxidation reaction with an oxygen dependence dominated by the first term of eq 4

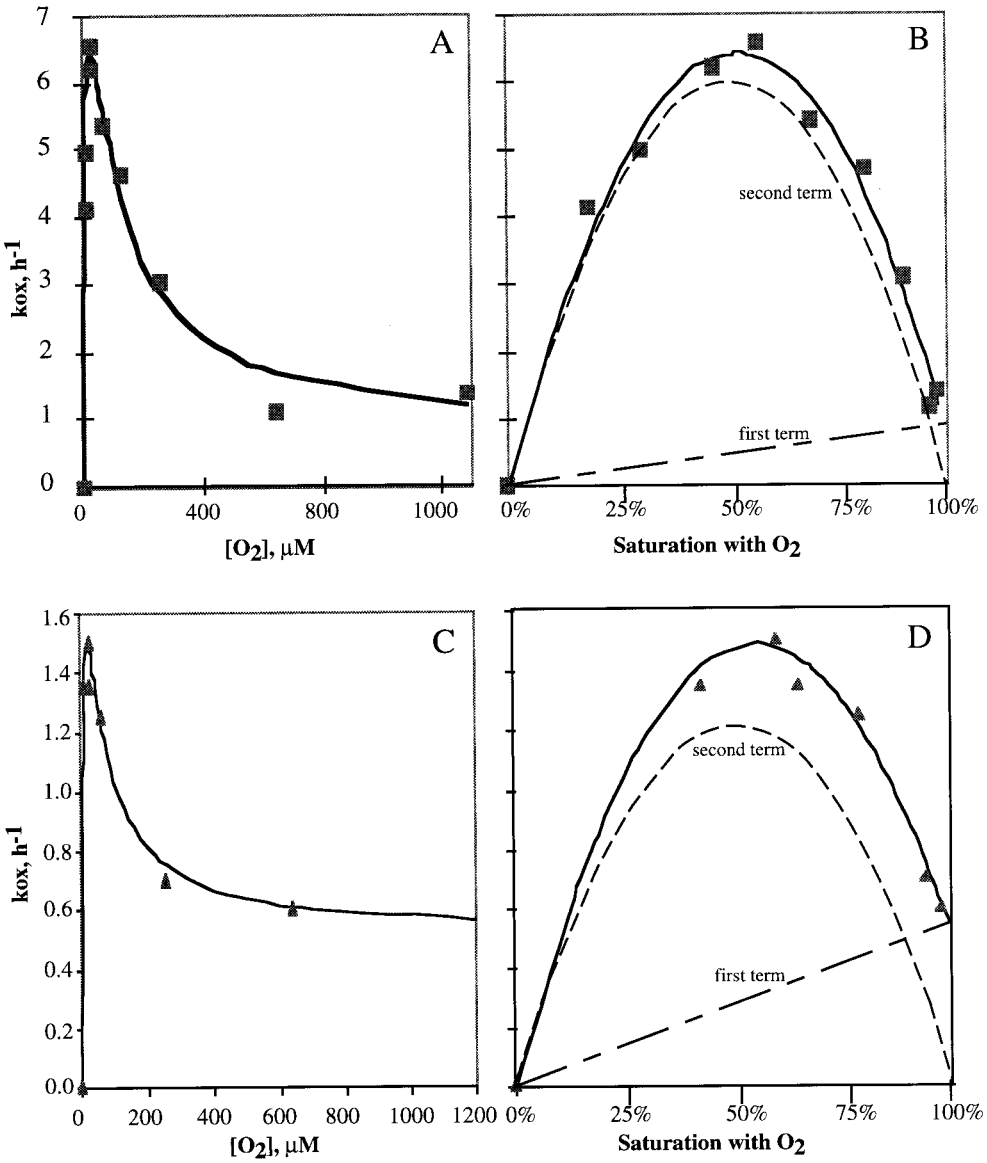


FIGURE 1: Influence of oxygen on the autoxidation of RmFixLH. Panels A and C show the autoxidation rates together with the curves fitted to eq 3. Panels B and D show the autoxidation rates fitted to eq 4. RmFixLH measurements are marked by squares (A, B), while those for *Aplysia* Mb are indicated by triangles (C, D). Lines with alternating long and short dashes show the contribution of the first term in the rate equation to the autoxidation rate, while lines with constant length dashes show the contribution of the second term. Details of the measurements are in Experimental Procedures.

will appear linear, while one dominated by the second term will appear parabolic. The intercept of the curve at 100% saturation with oxygen gives k_u.

Effect of Oxygen Concentration on the Autoxidation of RmFixLH and Aplysia Mb: Implications for the Role of Solvent Coordination. An oxygen protection effect is clearly illustrated for RmFixLH (Figure 1A,B). The rate of autoxidation peaks near the P_{50} for oxygen and slows to 20% of its maximum as the hemes become fully saturated with oxygen. Figure 1A shows the fit to eq 3, with $k_u = 0.66$ h^{-1} and $k_b = 26$ h^{-1}, as well as the individual contribution from each term in that expression. Since $n_d/k_2 \sim K_d$, the data are also a good fit to the empirical eq 4 (Figure 1B). As can be seen from the very hyperbolic shape of the curve for RmFixLH, the relative importance of Pathway 3 is greater for RmFixLH than for any other protein examined, except H64Q SWMb (Figure 2B). *Aplysia kurodai* myoglobin also shows a strong oxygen protection effect; its autoxidation rate when saturated with oxygen is only about a third of its rate when 50% saturated (Figure 1C,D).

The mechanisms that have been proposed to explain the oxygen protection effect involve reaction via a hypothetical hexacoordinate deoxyheme. Ferric heme proteins can be either of the hexacoordinate met type, with a molecule of water coordinated to the heme iron, or of the pentacoordinate met type. It is probably a very fast rate of dissociation of solvent from proteins such as FixL that leads to the very low (undetectable) affinity for solvent. The association rates should be at least as large as that of water-coordinating proteins. Indeed, if the interpretation of k_b is correct, the association rates of ferrous RmFixLH with solvent should be 30 times larger for RmFixLH than for SWMb (Table 1). This is reasonable in light of the extremely accessible heme pocket in FixL (*24*).

Although coordination of water to the ferrous form of either type of protein is spectroscopically undetectable at

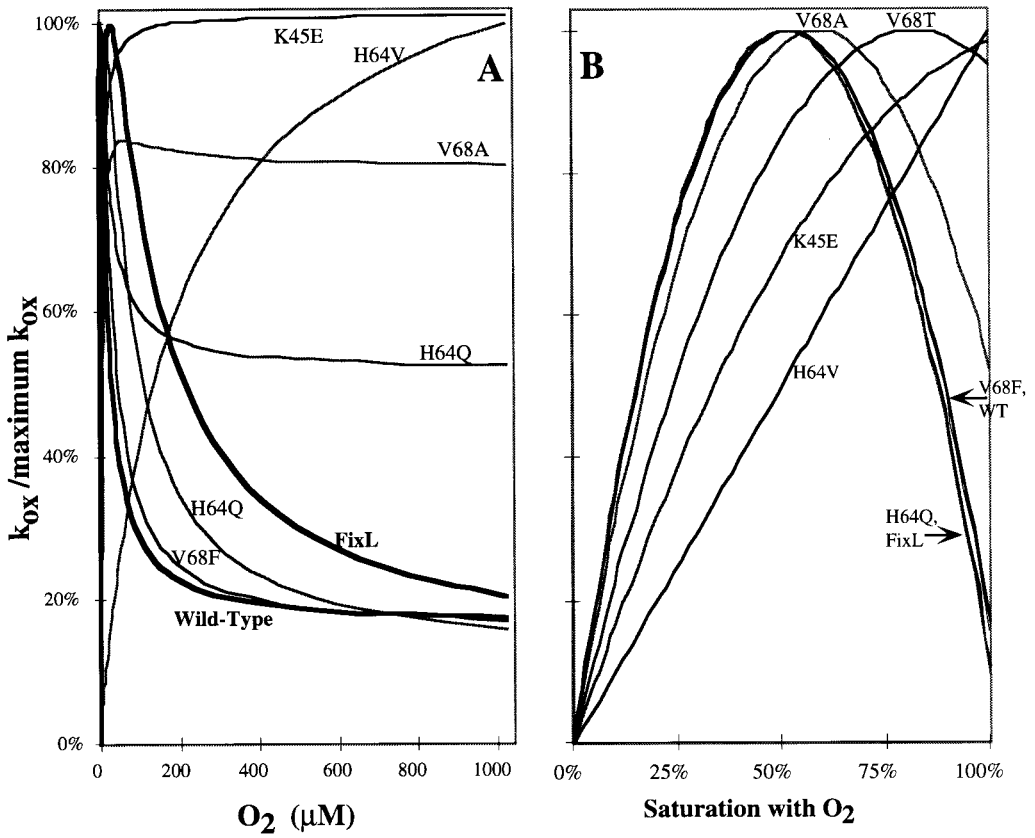


FIGURE 2: Comparison of the autoxidation of RmFixLH to that of the wild-type sperm whale myoglobin and its mutants. The curves show the best fit of the data to eq 3 (A) and eq 4 (B). (See the legend to Figure 1.) Details of the measurements of autoxidation of RmFixLH are in Experimental Procedures. The data for the sperm whale myoglobins are from Brantley et al. (*1*).

room temperature, this intermediate can be observed by reduction of Mb at cryogenic temperatures (*23*). From the available structural data on myoglobin-like proteins, we would expect that any interactions between the residues lining the heme pocket and any coordinated ligand will be essentially the same for the ferrous and ferric forms of a protein. For example, X-ray diffraction analysis shows that the water molecule that in metSWMb is coordinated to the heme iron is still associated with the distal histidine even in deoxySWMb, in which water is not detectably coordinated (*25*). The same factors that cause coordinated water to be less stable in metFixL than in metSWMb would also apply to the ferrous forms of these proteins. It is reasonable to assume that the dissociation rate of solvent from ferrous FixL is also much larger than n_d for myoglobin, leading to a much lower concentration of the solvent-coordinated ferrous intermediate.

The second term of eqs 3 and 4, which is responsible for the oxygen protection effect, has been attributed to Pathway 3. Since this pathway requires the hexacoordinate deoxyheme intermediate, its contribution might be expected to be directly related to the tendency of the ferric form of the protein to coordinate water. Yet no such relationship is consistently seen. Three heme proteins with pentacoordinate met forms were examined: RmFixLH, *Aplysia* Mb, and H64V SWMb. Of these, only H64V SWMb appears to lack an oxygen protection effect. Conversely, K45E SWMb, which has a hexacoordinate met form, shows a far smaller oxygen protection effect than FixL or *Aplysia* Mb, although K45E SWMb would have been expected to have a much higher tendency to form the hexacoordinate deoxyheme intermediate.

How do we account for the absence of any connection between coordination of water and the oxygen protection effect? One possibility is that a mechanism other than Pathway 3 is responsible for this effect. For example, direct transfer of electrons from oxyheme to deoxyheme would lead to the same oxygen dependence. The results are not necessarily inconsistent with the accepted reaction pathways. If the reactivity of the hexacoordinate deoxyheme intermediate is such that it is much more likely to react than to dissociate, then only the association rate for its formation will affect the reaction rates. A higher intrinsic affinity of the deoxySWMb for solvent due to hydrogen bonding would have no effect on its relative concentration, since the intermediate would be as short-lived as its counterpart in FixL or *Aplysia* Mb. As for the apparent absence of Pathway 3 from the H64V SWMb mutant, it may well be that Pathway 3 in this mutant is just as fast as it is for native myoglobins. The very large first term for this mutant would mask the effect of Pathway 3, since the oxygen protection effect is difficult to detect unless k_b is much larger than k_u.

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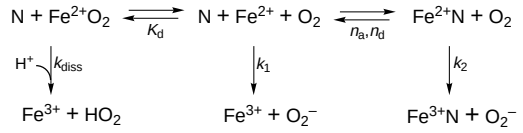
APPENDIX

Derivation of Rate Equations 3a and 4

For the overall reaction scheme below:



$$\frac{\partial Fe^{3+}}{\partial t} = k_{diss}[Fe^{2+}O_2] + k_1[O_2][Fe^{2+}] + k_2[O_2][Fe^{2+}N]$$

Let us assume [$Fe^{II}N$] is small and constant:

$$\frac{\partial[Fe^{2+}N]}{\partial t} = 0 = n_a[N][Fe^{2+}] - n_d[Fe^{2+}N] - k_2[Fe^{2+}N][O_2]$$

$$[Fe^{2+}N] = \frac{n_a[N][Fe^{2+}]}{(n_d + k_2[O_2])}$$

Substituting this expression for [$Fe^{II}N$] gives us

$$\frac{\partial Fe^{3+}}{\partial t} = k_{diss}[Fe^{2+}O_2] + k_1[O_2][Fe^{2+}] + k_2[O_2]\frac{n_a[N][Fe^{2+}]}{(n_d + k_2[O_2])}$$

$$= k_{diss}[Fe^{2+}O_2] + k_1[O_2][Fe^{2+}] + n_a[N][Fe^{2+}]\frac{[O_2]}{((n_d/k_2) + [O_2])}$$

At any time the fraction of ferrous heme that is bound to oxygen and the fraction that is free are given by

$$Fe^{2+}O_2 = YFe_T^{2+} = \frac{[O_2]}{[O_2] + K_d} Fe_T^{2+}$$

$$Fe^{2+} = (1 - Y)Fe_T^{2+} = \frac{K_d}{[O_2] + K_d} Fe_T^{2+}$$

where Fe_T^{II} is total ferrous heme protein. Substituting these expressions into the equation and factoring out Fe_T^{II} results in

$$\frac{\partial Fe^{3+}}{\partial t} = \frac{-\partial Fe^{2+}}{\partial t} = [Fe_T^{2+}]\left\{k_{diss}\left(\frac{[O_2]}{[O_2] + K_d}\right) + k_1[O_2]\left(\frac{K_d}{[O_2] + K_d}\right) + n_a[N]\left(\frac{K_d}{[O_2] + K_d}\right)\frac{[O_2]}{((n_d/k_2) + [O_2])}\right\}$$

Therefore

$$k_{ox} = \left\{(k_{diss} + k_1K_d)\frac{[O_2]}{[O_2] + K_d}\right\} + \left\{n_a[N]\frac{K_d}{[O_2] + K_d}\frac{[O_2]}{((n_d/k_2) + [O_2])}\right\} \quad (3a)$$

We see that n_d and k_2 in the second term cannot be fitted individually but only as a ratio. If $n_d/k_2 = K_d$, which has been the case for all heme proteins examined, then the oxygen concentration can be removed from the equation to obtain an expression entirely in terms of oxygen saturation. Substituting the expressions for Y and $(1 - Y)$ and defining $k_u = (k_{diss} + k_1K_d)$ and $k_b = n_a(N)$ results in the simple expression

$$k_{ox} = \{k_uY\} + \{k_bY(1 - Y)\} \quad (4)$$

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