Coupled Oxidation of Heme by Myoglobin Is Mediated by Exogenous Peroxide

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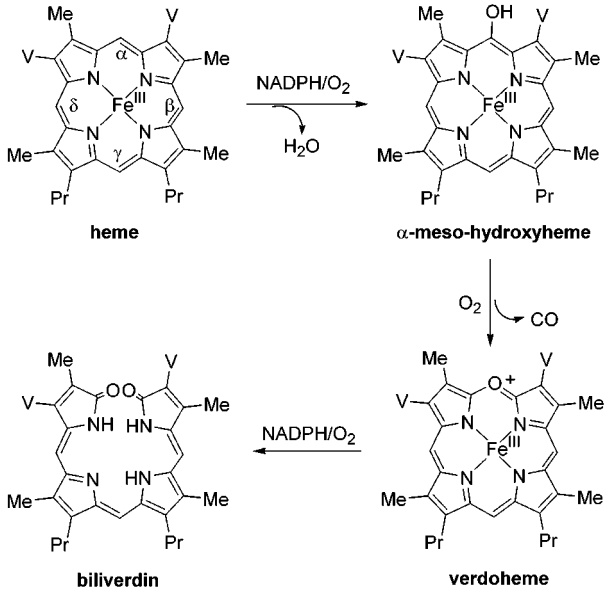
Oxidative degradation of heme is an important catabolic step in biology. The reaction is catalyzed by heme oxygenase (HO) in the presence of O_2 and NADPH-dependent cytochrome P450 reductase (Scheme 1).[1−4] In addition to the elimination of the potentially toxic free heme, this process helps to recycle iron, an essential metal ion of which only a few percent can be obtained from a regular diet.[3] The degradation product, biliverdin, is a precursor of a potent antioxidant bilirubin.[5]

For a long time, heme degradation activity has been observed in several other heme proteins, including myoglobin (Mb)[6−15] and variants of cytochrome b_5[16,17] and cytochrome b_{562},[18] upon their exposure to O_2 and a reductant such as ascorbate. This process is termed coupled oxidation and has been used as a model for HO because it shares several features of HO reactions, including the product formation and, in certain cases, regioselectivity.[3,15] However, important differences have been noted between HO and proteins involved in the coupled oxidation process, including reaction rates, reductant requirements, and heme-binding affinities.[15] A clear understanding of the difference is important in providing insight into the mechanism of not only HO, but also heme catabolism in general.

In the process of studying the reactivity of the myoglobin variant[19] that mimics the heme-copper center in terminal oxidases, we discovered a new important difference between heme degradation catalyzed by HO and by Mb. We found that the coupled oxidation by wild-type sperm whale Mb can be inhibited by catalase. The evidence is consistent with exogenous peroxide as a key component in the observed coupled oxidation.

Scheme 1. Heme Degradation Steps in HO (Me, methyl; V, vinyl; Pr, propionic acid)[3]



The expression and purification of wild-type sperm whale myoglobin (swMb) were carried out as previously described.[19,20] Incubation of 1 mM ascorbate with 3 μM wild-type swMb in 0.1 M (ionic strength) phosphate buffer, pH 7.0 at 37 °C, resulted in a shift of the Soret band from 408 nm to 416 nm and an overall intensity decrease in the Soret region (Figure 1A).[21] During the same period, two new peaks at 545 and 582 nm appeared within the first 90 min and then slowly decayed. The same spectral changes have been observed previously[12−14] and have been shown to be due to the formation of oxyMb, which then decays to Fe(III)-biliverdin. A kinetic trace monitoring the change at 408 nm is shown in Figure 2. This degradation process can be fit to a double exponential, $y = a + b[\exp\{-(k_1t)\}] + c[\exp\{-(k_2t)\}]$, yielding rates of $k_1 = (1.8 \pm 0.3) \times 10^{-2}$ min^{-1} and $k_2 = (1.9 \pm 0.2) \times 10^{-3}$ min^{-1}, which are similar to the rates reported previously.[12−14]

When the above reaction was repeated in the presence of a small amount (0.4 μM) of the peroxide scavenger catalase, only oxyMb (with Soret and α and β bands of 416, 545, and 582 nm, respectively) formation was observed (Figure 1B).[22] No coupled oxidation was detected during the same period. The process monitored at 408 nm now fits to a single exponential (Figure 2), yielding the rate of oxyMb formation, $k = (1.6 \pm 0.1) \times 10^{-2}$ min^{-1}, which is similar to the rate of the first kinetic process (k_1) of the coupled oxidation in Figure 1A.

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(21) The kinetic experiments were performed using a Hewlett-Packard 8453 diode array UV−vis spectrometer. The spectra were recorded starting at 10 s and every 15 min thereafter, over the entire spectral range (300−700 nm), for 10 h. The temperature was maintained at 37 °C using a circulating water bath. All reactions were initiated by the addition of 30 μL of 0.1 mM Mb (final concentration 3 μM) to 1 mL of 0.1 M ionic strength sodium phosphate buffer pH 7 containing 1 mM ascorbate (with or without 0.4 μM catalase). Catalase was added just prior to the start of the reaction. Nonlinear least-squares fitting of the kinetic traces to either a single or double exponential yielded R^2 values >0.99 in each case.

(22) Interestingly, the effect of catalase diminished significantly when its concentration is below 0.2 μM (less than one tenth of an equivalent of the swMb), presumably because a sufficient amount of catalase is required to inhibit the reaction. Furthermore, Cu/Zn superoxide dismutase had no effect on the reaction, suggesting that the reaction does not require exogeneous superoxide.

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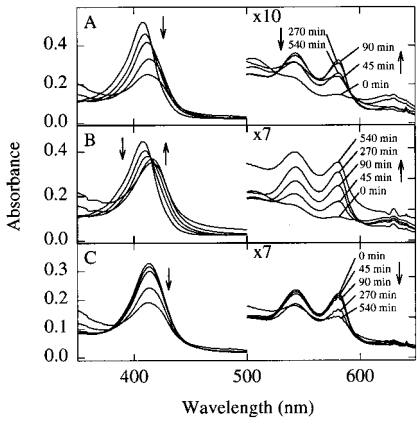

Figure 1. Selected electronic absorption spectra showing the time-dependent changes associated with coupled oxidation of WTswMb in the absence or presence of catalase. (A) Reaction of metMb in the absence of catalase, (B) reaction of metMb in the presence of 0.4 μM catalase, and (C) reaction of oxy-Mb in absence of catalase. Reaction of oxy-Mb in the presence of 0.4 μM catalase resulted in little spectral change other than baseline fluctuation caused by precipitation of a small proportion of the protein during the incubation period (data not shown). The proteins were incubated with 1 mM ascorbate at 37 °C in $\mu = 0.1$ M sodium phosphate at pH 7. See ref 21 for experimental details.

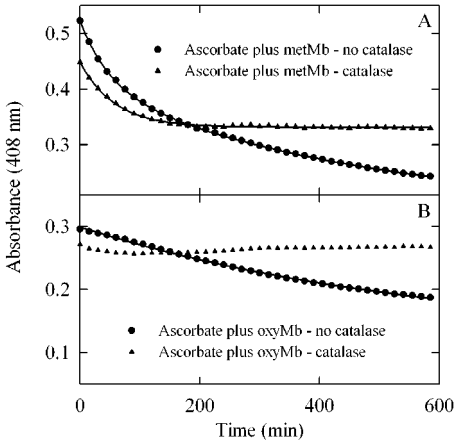

Figure 2. Kinetic traces showing the change in absorption at 408 nm for Mb incubated with 1 mM ascorbate under the conditions described in Figure 1. The lines represent the nonlinear least-squares fit to the data to either a single exponential (for metMb reaction with catalase and oxy-Mb reaction without catalase) or double exponential (for metMb reaction without catalase).

From this finding two points can be concluded. First, k_1 in the reaction without catalase represents the reduction of metMb and formation of oxyMb. The same conclusions have been reached in the previous studies.[12−14] Now addition of catalase allowed us to separate the two kinetic processes and to observe k_1 directly. Second, the coupled oxidation of Mb is due to exogenous peroxide reacting with oxyMb. Generation of H_2O_2 by reaction of ascorbate with oxyMb has been observed before.[23] To support this conclusion, the above coupled oxidation reactions were repeated with oxyMb in the place of the metMb. As expected, the reaction of 1 mM ascorbate with 3 μM oxyMb in air under the same conditions as in Figure 1A resulted in degradation of heme (Figure 1C) with only a single-exponential kinetic process (Figure 2). The observed rate of $(2.4 \pm 0.5) \times 10^{-3}$ min^{-1} is similar to what was obtained by Murakami et al.[14] and is essentially the same as the proposed rate k_2 of coupled oxidation involving metMb (Table 1). However, in the presence of 0.4 μM catalase, no reaction was observed (Figure 2).

Table 1. Reaction Rates of MetMb and OxyMb with 1 mM Ascorbate at pH 7 and 37 °C

	catalase	k_1 (min^{-1}) reduction step	k_2 (min^{-1}) heme decay
metMb	no	$(1.8 \pm 0.3) \times 10^{-2}$	$(1.9 \pm 0.2) \times 10^{-3}$
	yes	$(1.6 \pm 0.1) \times 10^{-2}$	−
oxyMb	no	−	$(2.4 \pm 0.5) \times 10^{-3}$
	yes	−	−

HO reaction is known to proceed through an *internal* peroxy intermediate as a result of reduction of oxyHO by cyt P450 reductase.[1−4] The results presented here show that the process of coupled oxidation in Mb requires *exogenous* peroxide. Furthermore, even though HO can bypass the reduction of oxyHO through a "peroxide shunt" reaction of metHO with exogenous H_2O_2,[3,24,25] the coupled oxidation of Mb using H_2O_2 as a key component is not analogous to the peroxide shunt in HO, because oxyMb is the reactant rather than metMb.[26] Reaction of H_2O_2 with metMb led to high-valent iron-oxo species such as compounds I and II,[15,27] protein cross-linking,[28] and formation of an iron chlorin product,[29] instead of Fe(III)-biliverdin. The high-valent iron-oxo species was clearly ruled out as an intermediate in HO reaction.[1−4] Therefore, these mechanistically important differences must be considered carefully before coupled oxidation is used as a model for HO reactions.

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