Reactions of Sperm Whale Myoglobin with Hydrogen Peroxide

EFFECTS OF DISTAL POCKET MUTATIONS ON THE FORMATION AND STABILITY OF THE FERRYL INTERMEDIATE*

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Distal pocket mutants of sperm whale oxymyoglobin (oxy-Mb) were reacted with a 2.5-fold excess of hydrogen peroxide (HOOH) in phosphate buffer at pH 7.0, 37 °C. We describe a mechanism composed of three distinct steps: 1) initial oxidation of oxy- to ferryl-Mb, 2) autoreduction of the ferryl intermediate to ferric metmyoglobin (metMb), and 3) reaction of metMb with an additional HOOH molecule to regenerate the ferryl intermediate creating a pseudoperoxidase catalytic cycle. Mutation of Leu-29(B10) to Phe slows the initial oxidation reaction 3-fold but has little effect on the rate of ferryl reduction to ferric met-aquo-myoglobin. In contrast, the Val-68(E11) to Phe mutation causes a small, 60% increase in the initial oxidation reaction and a much larger 2.5-fold increase in the rate of autoreduction. Double insertion of Phe at both the B10- and E11-positions (L29F/V68F) produces a mutant with oxidation characteristics of both single mutants, slow initial oxidation, and rapid autoreduction, but an extraordinarily high affinity for O2. Replacing His-64(E7) with Gln produces 3-4-fold increases in both processes. Combining the mutation H64Q with L29F results in a myoglobin with enhanced resistance to metMb formation in the absence of antioxidant enzymes (i.e. catalase and superoxide dismutase) due to its own high pseudoperoxidase activity, which rapidly removes any HOOH produced in the initial stages of autodestruction. This double substitution occurs naturally in the myoglobin of Asian elephants, and similar multiple replacements have been used to reduce selectively the rate of nitric oxide (NO)-induced oxidation of both recombinant MbO2 and HbO2 blood substitute prototypes without altering O2 affinity.

Cell-free hemoglobins, chemically altered or genetically expressed in microbial host systems, have been developed as oxygen-carrying therapeutics. Site-directed modifications are introduced and serve to stabilize the protein molecules in their tetrameric, functional forms (1). Animal studies as well as recent clinical studies have shown that these proteins probably deliver sufficient oxygen to tissues, but concerns still persist regarding the spontaneous oxidation (autoxidation) of hemoglobin and its redox reactions with tissue oxidants that may potentially impede its clinical usefulness (2). Vascular endothelium, a source of a number of oxidants, has emerged as the primary target of hemoglobin-based toxicity due to its proximity to the circulating protein. Hemoglobin, unlike red cells, can diffuse through the endothelial barrier lining the vessel wall, where it can potentially reach nitric oxide (NO), the endothelial derived relaxing factor (1, 3). Reactions of NO with hemoglobin result in the diversion of NO away from the smooth muscle target enzymes, leading to the loss of NO-dependent responses such as vasodilation. Hypertension is a common phenomenon associated with the infusion of hemoglobins in animal models as well as in humans (4–7). The ensuing oxidative reactions of hemoglobin with peroxynitrite (ONOO−), the product of the reaction of NO with superoxide (O2−), hydrogen peroxide (HOOH), or lipid peroxide in the vasculature may exacerbate tissue damage (2).

Under normal conditions, the interplay between NO, O2−, and ONOO− in the vasculature is a delicate balance that must be kept between the pro- and antioxidant processes (8). This balance between NO and O2− can however, be disrupted in favor of ONOO− and HOOH under a variety of nonphysiological conditions (8, 9). Additionally, increased HOOH production is thought to occur under conditions of reperfusion with oxygenated media, in this case hemoglobin (10). Cell culture studies have identified several potential candidates for the agent(s) responsible for the observed cytotoxicity. The oxidative reactions of hemoglobin with HOOH produced largely from endothelial cells are believed to play an important role in hemoglobin-mediated tissue damage (2). Peroxide can induce rapid oxidation of oxyhemoglobin (Fe2+) to methemoglobin (Fe3+). The toxicity of hemoglobin preparations in endothelial cell culture was shown to be dependent on the rate of autoxidation, and it correlated with time in culture and the presence of iron chelators (11).

Hemoglobin induces cellular protective mechanisms and genes that protect against oxidative damage, such as the production of transferrins and heae oxygenase. This phenomenon

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underscores the importance of oxidative stress as a mediator of cell damage (12). The reaction between the oxy or the ferric forms of hemoglobin with HOOH is known to proceed via the formation of the highly reactive oxyferryl complex, Fe(IV)=O detected by optical spectroscopy and a globin-associated free radical detected by EPR (13). These species have been implicated in the mechanism of ischemia/reperfusion injury and most recently were detected in normal human and animal blood (14, 15). A high uncontrollable rate of autoxidation of hemoglobin and vasoconstriction due to the scavenging of nitric oxide were shown to be among the primary constraints in demonstrating the efficacy of some hemoglobin solutions used as blood substitutes (16). The reactions between hemoglobin and tissue oxidants are clearly complex phenomena that must be well understood in order to resolve fully some of the hemoglobin-mediated toxicities.

Strategies to combat oxidation reactions of hemoglobins are evolving. Chemical polymerization of the tetrameric molecule or encapsulation of hemoglobin to inhibit extravasation into the endothelium are among the most commonly used approaches for NO scavenging and unfavorable oxidative side effects. Site-directed mutagenesis of the distal pocket of myoglobin constructs has provided a simple model for the engineering of hemoglobin subunits (17). Both myoglobin and hemoglobin constructs have been produced in which the rates of autoxidation and NO-induced oxidation have been substantially inhibited (18, 19). This result is achieved by filling the distal pocket with larger, apolar residues at position 29(B10) and/or 68(E11). The oxygen affinities of some mutants have also been varied by altering hydrogen bonding to the E7 residue and/or interfering sterically with bound ligand (17, 18). Recently, Doherty et al. (20) have shown that the rate of reaction with nitric oxide determines the hypertensive effect of cell-free hemoglobin. In rats, infusion of recombinant hemoglobins with decreased NO-scavenging activities causes corresponding decreases in the hypertensive responses compared with those of hemoglobins with unchanged NO oxidation rate constants (20).

Here we present data on the interactions of HOOH with oxy and ferric forms of sperm whale myoglobins and the effects of single, double, and triple amino acid mutations at positions His-E7, Leu-B10, and Val-E11 on these reactions. We also identify some of the structural features that regulate the pseudodoperoxidase activity of myoglobin that may be relevant to the design of a stable hemoglobin-based blood substitute. The studies detailed in this report were carried out at concentrations of hydrogen peroxide that are comparable with the levels that are found in vivo. The concentration of peroxide in normal human plasma is between 4 and 5 μM (21). It is not known how much peroxide is actually produced in inflammatory lesions in vivo. However, endothelial cells lining the blood vessels form the initial point of contact with extracellular oxygen carriers and have been shown to be a major source of hydrogen peroxide under both normal conditions and during reperfusion after ischemia (22). Stimulation of various cellular components (platelets, neutrophils and macrophages) attracted to these sites can also contribute to the peroxide pool in the extracellular space. For example, it has been shown that activated polymorphonuclear leukocytes alone can generate up to 200 μM HOOH in vitro (23). Moreover, under conditions of ischemia reperfusion, high levels of nitric oxide have been shown to inhibit glutathione peroxidase, a scavenger of peroxides, leading to accumulation of higher levels of this oxidant (24).

**EXPERIMENTAL PROCEDURES**

**Myoglobin Mutagenesis—**Recombinant sperm whale myoglobins were constructed, expressed, and purified as described previously by Carver et al. (25), Springer et al. (26), and Egeberg et al. (27), using the recombinant gene constructed by Springer and Silgar (28).

**Myoglobin Derivative Preparations—**Myoglobin (Mb) in the ferric (metMb) and ferryl (ferryl-Mb) forms was prepared according to established procedures. Briefly, metMb was prepared by the addition of a 1.2-fold excess of potassium ferricyanide to oxyhemoglobin (oxy-Mb). Unreacted ferricyanide and its reaction products were removed by a two-step gel filtration procedure on Sephadex G-25. The first filtration step (high pH, high salt) was done in 50 mM phosphate (pH 8.3), 1 mM NaCl. The following step (neutral pH, low salt) was done in 50 mM phosphate buffer (pH 7.0) with no added NaCl. Ferryl-Mb was prepared by treating oxy-Mb with a 5-fold molar excess of HOOH in 50 mM phosphate buffer, pH 7.0. Excess peroxide was removed by passing the myoglobin solution through a Sephadex G-25 column (29).

**Autoxidation Experiments—**The in vitro rates of autoxidation of native sperm whale myoglobin and the double mutant (H64Q/L29F) were studied in the absence of antioxidative enzymes (30). Oxy-Mb samples (20 μM in heme) were incubated in air equilibrated with 50 mM phosphate buffer, pH 7.0, at 37 °C in a thermostated spectrophotometric cell, in a Hitachi spectrophotometer (U-2000). Spectral changes in the visible region were followed to near completion for both proteins. The proportions of oxy-Mb, metMb, and ferryl-Mb at various time points were estimated by multicomponent analysis using published extinction coefficients (31). First order autoxidation rate constants were derived from the plot of percentage of oxy-Mb versus time and fitted to a single exponential expression using a nonlinear squares curve fitting routine.

**Kinetic Measurements: Slow Processes—**The slower kinetic processes apparent after mixing oxy-Mb (20 μM heme) with hydrogen peroxide (in a molar ratios of 1:1, 1:2.5, 1:5, and 1:10) were monitored by mixing substrates in the thermostated cell of a rapid scanning diode array spectrophotometer (HP-8453) and collecting spectra as a function of time. All experiments were run at 37 °C, in 50 mM phosphate buffer (pH 7.0) that had been previously treated with Chelex® resin. Initial multicomponent analysis of myoglobin oxidation products was performed according to Whitburn (31) in order to calculate the percentages of oxy-Mb, metMb, and ferryl-Mb at successive stages of the oxidation. Independent estimation of these intermediates along the reaction coordinates and estimation of the rate constants of the interconversions were performed as described below.

**Model for Kinetic Processes—**Estimates for the rates of the slow kinetic processes discussed above were made based on a simple reaction scheme (Scheme 1), which reflects hydrogen peroxide oxidation of both oxyferrous and ferric myoglobins and the autoxidation of the oxyferryl species back to the ferric state.

\[ K_{O_2} \]

\[ K_{HOOH} \]

\[ K_{MbFe^{2+}} \]

\[ K_{MbFe^{2+}O_2}\]

\[ K_{[MbFe^{2+}O_2]} = K_{O_2}[MbFe^{2+}] [O_2] \] (Eq. 1)

\[ K_{HOOH} \]

\[ K_{MbFe^{2+}} + HOOH \]

\[ K_{MbFe^{2+}O_2} \]

\[ K_{MbFe^{2+}O_2} = K_{HOOH}[MbFe^{2+}][HOOH] \] (Eq. 2)

The abbreviations used are: Mb, myoglobin; metMb, metmyoglobin; oxy-Mb, oxyhemoglobin; ferryl-Mb, ferrylmetmyoglobin; HPLC, high pressure liquid chromatography.
The saturation function for hydrogen peroxide binding can be described as follows.

\[ Q = [\text{MbFe}^2+] + [\text{MbFe}^2^+O]_2 + [\text{MbFe}^2^+[\text{HOOH}]] = [\text{MbFe}^2^+] \]
\[ P = Q/([\text{MbFe}^2^+] + K_{\text{HOOH}}[\text{HOOH}]) \] (Eq. 3)

Here \( Q \) is the total concentration of ferrous species in the initial equilibrium, \( K_{\text{HOOH}} \) and \( K_{O} \) are the binding constants for the competing species, and \( P \) (the binding polynomial) is \( Q \) normalized to the concentration of the unliganded ferrous species. At constant oxygen activity, the saturation function for HOOH is as follows.

\[ Y_{\text{HOOH}} = \frac{a \ln P}{a \ln([\text{HOOH}])} = \frac{K_{\text{HOOH}}[\text{HOOH}]}{1 + K_{O}[O] + K_{\text{HOOH}}[\text{HOOH}]} \] (Eq. 5)

The apparent rate of ferrous iron disappearance (\( k_{a} \)) can now be described as the product of the hydrogen peroxide saturation function and the intrinsic rate constant for heterolytic cleavage of bound peroxide with subsequent ferryl formation as follows.

\[ k_{a} = \frac{Y_{\text{HOOH}} \times k_{\text{ferrous}}}{1 + K_{O}[O] + K_{\text{HOOH}}[\text{HOOH}] \times k_{\text{ferrous}}} \] (Eq. 6)

Under conditions of high [HOOH] and low [O], \( Y_{\text{HOOH}} \) in Equation 6 approaches unity, and the following is true.

\[ k_{a} \rightarrow k_{\text{ferrous}} \] (Eq. 7)

Note that this limit is valid even when \([O] = 0\). Likewise, under conditions of high [O] and low [HOOH], \( Y_{\text{HOOH}} \) approaches a limiting value of \( K_{\text{HOOH}}[\text{HOOH}]/K_{O}[O] \), and thus the following is true.

\[ k_{a} \rightarrow \frac{K_{\text{HOOH}}[\text{HOOH}]}{K_{O}[O]} \times k_{\text{ferrous}} \] (Eq. 8)

The experiments detailed in this work were all carried out at relatively low [HOOH]/[O], approximated by Equation 8 above. Thus, mutation-induced changes in \( k_{a} \) can be affected by 1) changes in the relative affinities of the protein for the two competing ligands, 2) a change in the first order rate of ferryl iron formation from the peroxide-ligated species, or 3) a combination of these effects.

The process defined by \( k_{a} \) in the cyclic reaction, Scheme 1, involves the autoreduction of the ferryl iron species back to the ferric state. The mechanism of this process is not well understood, and the identity of the electron donor is unknown. \( k_{b} \) serves as a useful indicator of the lifetime of the ferryl species under the experimental conditions and in the absence of a known exogenous electron donor.

\( k_{b} \) is the second order rate constant for the reaction of the ferric heme protein with HOOH producing a ferryl heme and a protein radical [35, 36]. The lifetime of the protein radical has been reported to be very short with respect to the lifetime of the ferryl heme group [12]. Analogous to the interpretation of the reaction of MbO2 with HOOH, evidence suggests that the oxidation of ferric myoglobin \( (k_{b} \) in Scheme 1) also consists of three discrete steps: 1) dissociation of coordinated water, 2) simple HOOH binding, and 3) heterolytic cleavage of the bound HOOH [33]. At millimolar hydrogen peroxide concentrations (high [HOOH]/[O],) spectral, kinetic, and EPR evidence for the formation of a ferric myoglobin-HOOH complex can be detected in vitro for some mutant myoglobins with apolar replacements of the distal histidine, His-64 [37]. The population of this species in reactions with the native protein is apparently too low for detection by these methods, particularly at low [HOOH] used in our studies.

In combination, the steps characterized by \( k_{a} \) and \( k_{b} \) form a loop, which we refer to as a peroxidase cycle. The rate-limiting step for catalysis is determined by peroxide concentration. Under conditions of relatively high [HOOH], which were employed in our work, \( k_{b}[\text{HOOH}] \) will be large with respect to \( k_{a} \), and autoreduction of ferryl myoglobin will be the rate-limiting step. At low [HOOH], the flux of [HOOH] through the loop will be determined by \( k_{b}[\text{HOOH}] \). Throughout the major portion of the process, the Fe(IV)=O complex will be the predominant spectral species until all HOOH is consumed, at which time the oxidation state of the iron will revert to Fe(III).

When [HOOH] is low, the extent and apparent rate of oxidation of oxymyoglobin by peroxide cannot be properly estimated without taking in account consumtion of HOOH by the peroxidase loop. First, both processes compete for hydrogen peroxide and lower its concentration at different rates. Second, ferric iron formed by the initial oxidation reaction forms the reactive iron species that is active in the peroxidase loop. Estimates of \( k_{b} \) made without taking into account the effects of the peroxidase loop on [HOOH] underestimate the true values. In this work, estimates of \( k_{b} \) were made utilizing a global analysis fit of spectral data to the kinetic model shown above.

Spectral data were analyzed by singular value decomposition (SVD) and nonlinear least squares of the numerical model (30, 38) defined by the following set of rate equations.

\[ \frac{d[HOOH]}{dt} = -k_{f}[\text{ferrous}][\text{HOOH}] \] (Eq. 9)

\[ \frac{d[\text{ferrolyl}]}{dt} = k_{b}[\text{ferrous}][\text{HOOH}] - k_{f}[\text{ferrolyl}][\text{HOOH}] \] (Eq. 10)

\[ \frac{d[\text{ferric}]}{dt} = k_{g}[\text{ferrolyl}] - k_{f}[\text{ferric}][\text{HOOH}] \] (Eq. 11)

\[ \frac{d[HOOH]}{dt} = -k_{b}[\text{ferric}][\text{HOOH}] - k_{f}[\text{ferric}][\text{HOOH}] \] (Eq. 12)

**Stopped-flow Kinetic Measurements: Fast Processes**—Rate constants for the oxidation of ferric myoglobin by HOOH \( (k_{f}) \) were estimated by directly mixing ferric Mb and hydrogen peroxide in an Applied Photophysics stopped-flow spectrophotometer. Reactions were monitored at either 416 or 400 nm at 25 °C in the presence of 25 mM phosphate buffer, pH 7.0. A minimum of 200 data points per experiment were collected and analyzed. Peroxide/myoglobin ratios in these experiments were as low as (1:1) with the maximum value being 10:1. These data were fitted to either a single or double exponential function using the Marquardt-Levenberg fitting routine included in the Applied Photophysics software.

In some experiments, data were analyzed by singular value decomposition and global exponential fitting routines using the Applied Photophysics photodiode array accessory and its relevant software package, Glin.

**RESULTS**

**Reaction of HOOH with the Oxy Forms of Recombinant Myoglobins (His-64)**—Spectral changes accompanying the addition of HOOH to sperm whale oxy-Mb in a molar ratio of 1 heme:2.5 HOOH are shown in Fig. 1 and are similar to those reported by Shikama and co-workers (32, 33). The initial oxidation of the ferrous heme can be seen by the loss of the typical \( \alpha \) and \( \beta \) peaks.
bands of oxy-Mb at 577 and 541 nm and subsequent formation of a transient ferryl intermediate characterized by weaker visible absorption peaks at 545 and 580 nm. This ferryl transient spectrum changes with time to a spectral species with peaks at 510, 550, and 630 nm, characteristic of high spin ferric heme. One factor not accounted for explicitly in our kinetic model is a spectral change caused by the modification of the heme after the initial reaction of HOOH with ferrous myoglobin (36). This modification does not significantly change the rate of the ferric heme/HOOH reaction in subsequent reactions of the cycle (data not included), i.e. the second order rates of the reaction of the native and the modified ferric species with hydrogen peroxide are similar. The "ferric" species in the model corresponds to the sum of all ferric species including those with irreversible modifications to the heme.

Table I shows the results of the analysis of the oxidation profiles of sperm whale myoglobin and a number of distal pocket mutants using low levels of hydrogen peroxide. Spectral time courses were analyzed by singular value decomposition with subsequent nonlinear least squares fitting to the model discussed under "Experimental Procedures." The estimates of $k_1$ (oxidation of the ferrous to the ferryl species) and $k_2$ (autoreduction of the ferryl species back to ferric) given in Table I were determined independently by stopped-flow analysis of the reaction of the ferric proteins with hydrogen peroxide. With the exception of the double mutant L29F/V68F, $k_2$ does not appear to be sensitive to the configurations of the distal pocket for the mutants investigated. Only the rate constant for the L29F/V68F double mutant is significantly different, 3-fold greater than the average estimated for the other proteins. Curiously, the $k_2$ values for the L29F and the V68F mutants are both roughly equal to that for wild-type and native sperm whale myoglobin. Thus, the effects of the single mutations on $k_2$ are not additive in the double mutant, presumably because of the close proximity of the two large benzyl side chains to each other and to the active site.

The Leu-29 to Phe mutation markedly decreases the initial oxidation rate of the oxyferrous species ($k_1$) while having little effect on the rate of autoreduction of the resulting ferryl species ($k_2$). Insertion of valine at this position (L29V) results in a modest increase in $k_1$ and also a 2-fold increase in $k_2$. Although it appears that $k_1$ is inversely correlated with the size of the position 29 residue, decreasing the empty volume further back in the distal pocket by substitution of Val-68 with Phe or Leu has the opposite effect, causing a modest 30% increase in $k_1$. It is therefore reasonable to conclude that both the size and the location of the residue in the heme pocket determine these effects. In addition, the estimate of $k_2$ for V68F is significantly larger than that for the wild type protein. In this case, the double mutant (L29F/V68F) displays a combination of the kinetic properties exhibited by single mutants with a slow $k_1$ (like L29F) and an increased $k_2$ (like V68F) (Table I).

Fig. 2 elaborates on the analysis of wild type sperm whale myoglobin and three of the mutants included in Table I. These three mutants include the two phenylalanine replacements (L29F and V68F) and the double mutant (L29F/V68F). The curves shown in the figure are the model-dependent estimates of the distributions of the ferrous, ferryl, and ferric species as a function of time after mixing with a modest 2.5-fold excess of hydrogen peroxide. Several features illustrate the relationship of the initial rate of oxidation of the ferrous protein ($k_1$) to the rate of the peroxidative loop, which is governed primarily by $k_2$. The high peroxidative capacity for the ferric species caused by the low $k_2$ value is evident in the rapid disappearance of the ferryl transient in Fig. 2.

These properties are further emphasized in Fig. 3, which compares the relative integrated areas under the ferryl curves as a qualitative comparison of the "dose" or time persistence of the ferryl species for the four proteins under the given reaction conditions. Estimates of the amount of ferryl species generated are in the following order: native sperm whale > L29F > V68F > L29F/V68F. Under the conditions in Fig. 3, the reactive ferryl species in the double mutant is cleared from the solution by both the high rate of autoreduction ($k_2$) and the high rate of HOOH consumption ($k_1$) in the peroxidative cycle. The presence of the V68F mutation appears to be responsible for this property. Also shown in Fig. 3 are the relative proportions of ferrous protein remaining after depletion of hydrogen peroxide.
from the reaction mixture when the original HOOH:heme ratio was 2.5:1 (50 μM HOOH, 20 μM heme). It is apparent that, for the four proteins examined, the presence of the L29F mutation in the protein significantly preserves the amount of "functional" protein remaining after the oxidation reaction due to the lower initial rate of reaction of the ferrous mutant with HOOH.

Fig. 3 also emphasizes that these two functional properties, persistence of the ferryl intermediate and stability of the functional ferrous oxidation state, can be altered in an independent fashion depending on the configuration of the residues at positions 29 and 68.

Reaction of Peroxide with Sperm Whale Myoglobin Mutants Containing a Distal Glutamine (Gln-64)—Experiments were carried out by reacting hydrogen peroxide with myoglobin mutants in which the distal histidine was replaced by either the polar amino acid glutamine or the apolar amino acid leucine. With respect to the native protein, the H64Q mutation shows approximately 3-fold increases in both $k_1$ (initial oxidation of ferrous protein) and $k_2$ (autoreduction of the ferryl intermediate) with no corresponding increase in $k_3$ (oxidation of the ferric protein). The increase in $k_1$ and decrease in oxygen affinity ($K_{O2}$), are returned to wild type levels upon formation of the double mutant H64Q/L29F (Table I). A high rate of ferryl autoreduction (i.e. large $k_2$) preserves a more efficient pseudoperoxidase cycle. Like H64Q, the apolar H64L mutation causes a 3-fold increase in the rate of the oxidation of the ferrous protein by HOOH. Values of $k_2$ and $k_3$ are not available from our data. However, Brittain et al. (37) have reported rate constants for the reactions of HOOH with a series of apolar 64 mutants of metMb. The values of $k_3$ for H64V and H64F metMb were 3.0 and 1.5 M$^{-1}$ s$^{-1}$, respectively. These rates are significantly slower than those we have estimated for myoglobins with a polar residue in the distal position (Table I, $k_3$ 5–600 M$^{-1}$ s$^{-1}$) and those measured by Brittain et al. (37) for wild-type and H64Q metMb ($k_3$ = 200–600 M$^{-1}$ s$^{-1}$). Assuming that the H64L mutant also shows a reduced reaction rate with HOOH, the situation is analogous to the slow rate of HCN binding to ferric apolar 64 mutants. In both cases, it is almost certainly the anions, CN$^-$ or HOO$, that bind, and as a result the apolar group is needed to facilitate deprotonation, either directly or through distal pocket water (39).

The distal pocket configuration (H64Q/L29F) corresponds to the in vivo situation seen in the Asian elephant (40), which is one of the few, apparently successful, naturally occurring replacements of the distal histidine in vertebrate myoglobins. It is interesting to note that the addition of the V68F replacement...
to H64Q/L29F myoglobin reverses the effect of the L29F mutation, restoring the value of $k_1$ to the high value seen with the H64Q single mutant. However, the relative effects on $k_1$ and $k_2$ of the addition of the V68F substitution to L29F background are the same whether the distal position (E7) is occupied by histidine or glutamine.

Fig. 4 shows reconstructed spectra of the intermediates in the oxidation of myoglobins that have either histidine (His-64) or glutamine (Gln-64) at the distal position (E7). These representations are the averaged spectra of the individual proteins shown in Table I, grouped to show the general spectral differences between myoglobins with the two E7 configurations.

model-dependent spectrum looks very much like the actual spectra of the oxidation products of the Gln-64 series of mutants. It is likely that significant heme loss and breakdown and globin precipitation take place for these mutants even when oxidized with the moderate ratio of peroxide:heme utilized in these experiments. This is consistent with the normal tendency of the Gln-64 proteins to lose heme at an accelerated rate and precipitate as apoglobin is formed (41).

To estimate the degree of oxidation-stimulated heme loss from these proteins, aliquots of the reaction mixtures of myoglobin and its oxidation products were analyzed by the use of reverse-phase HPLC (42). Protein and heme components were detected by their absorbance at 220 and 400 nm, respectively. Fig. 5a shows typical elution profiles of both the heme and protein fractions of native sperm whale myoglobin untreated with hydrogen peroxide. Fig. 5b shows the averaged amount of heme lost, as calculated from the peak areas at 400 nm, from both His-64 and Gln-64 series before and after treatment with a 2.5-fold molar excess of peroxide. The peak areas at 400 nm were calculated from HPLC profiles such as those in Fig. 1 and were analyzed by HPLC on a Vydac C4 reverse phase chromatography column, as in Ref. 42. b, relative amounts of heme before and after treatment of His-64 and Gln-64 mutants with a 2.5-fold molar excess of peroxide. The peak areas at 400 nm were calculated from HPLC profiles such as those in a. Values are mean ± S.D. (n = 3) for both His-64 and for the Gln-64 series. It appears that ∼20% of the heme was lost when His-64 mutants reacted with HOOH as compared with ∼90% heme loss in the case of Gln-64 series.
Gln-64 mutants compared with a modest decrease (−9%) in case of the His-64 mutants.

Literature values of the autoxidation rate constants (17, 18) are plotted versus the initial HOOH oxidation in Fig. 6 for the mutants examined in this study. It appears that $k_{\text{auto}}$ is roughly correlated with $k_1$ for 9 out of the 10 myoglobins reported in Table 1. However, a fair amount of scatter is observed (ρ = 0.54). We carried out some autoxidation experiments under more relevant conditions, i.e. under air at 37 °C, pH 7.4, with no added antioxidant enzymes for two representative proteins, native and H64Q/L29F sperm whale myoglobins. The initial rate of autoxidation for sperm whale myoglobin and for H64Q/L29F were comparable under our conditions (0.109 and 0.106 h⁻¹ for native myoglobin and the double mutant, respectively).

Long term stability studies in the absence of antioxidant enzymes showed that the double mutant (H64Q/L29F) is a more stable protein. At the end of 46 h of incubation, there was 65% metmyoglobin present in the H64Q/L29F solutions as compared with almost 97% in the native protein. The reason for this increased stability is the −4-fold greater rate of autoreduction of the H64Q/L29F ferryl intermediate ($k_2$), which enhances the removal of HOOC by the peroxidase cycle. Although the H64Q/L29F double mutant has a similar initial rate of autoxidation, the mutant metmyoglobin formed is a 4-fold better catalyst for removing the resultant HOOC, which ordinarily would accelerate the oxidation process as described previously (43). Thus, the H64Q/L29F combination produces a myoglobin that is significantly more resistant to HOOH damage without altering significantly its oxygen binding properties and initial rate of autoreduction.

**DISCUSSION**

Mechanistic analysis of the reaction of hemoglobins/myoglobins with HOOH has revealed the formation of a higher oxidation state, the ferryl heme iron (Fe(IV)), which can be detected by optical spectroscopy (30, 32, 34), and a globin-associated free radical, which can only be detected by EPR spectroscopy (14, 37). Despite its transient nature and ultimate self-destruction, ferryl hemoglobin and ferryl myoglobin can peroxidize lipids, degrade carbohydrates, and cross-link proteins (44). The globin radical of ferryl hemoglobin was recently detected by EPR in normal human and animal whole blood (13, 14). The source of HOOH in blood was reported to be the product of dismutation of O₂⁻ produced via the autoxidation of the intraerythrocytic hemoglobin (14). This reaction, interestingly, occurs despite the presence of normal blood reducing systems (14). A recent study on the reactivity of hemoglobin toward low density lipoproteins has shown that both the oxidation and the cross-linking of the LDL proteins can be initiated by the heme-globin radical (45). One concern, relevant to the use of hemoglobin as an oxygen therapeutic, is that *in vivo* production of both globin-based and heme-based radicals can occur under ischemia and reperfusion in patients with diminished ability to control oxidative reactions of hemoglobin.

Although, no *in vivo* evidence exists to attribute the cytotoxicity seen with cell-free hemoglobins directly to the ferryl species, several protective strategies are under consideration to control or reduce the levels of these highly reactive intermediates. Several chemical strategies are aimed at cycling of ferryl back to ferrous hemes, by attachment of catalase-like activity to hemoproteins using redox-active compounds such as nitroxide (46) or directly cross-linking the red cell antioxidative enzymes superoxide dismutase and catalase to the hemoglobin molecules (47). Other approaches include site-directed mutagenesis that targets distal heme pocket amino acids to sterically limit access of oxidants to the heme iron while maintaining the normal oxygen delivering capabilities of the protein (17, 20).

In recent years, sperm whale myoglobin has been successfully used as a subunit protein prototype to engineer safer, second generation hemoglobin-based blood substitutes (17–20). One approach for constructing a low oxygen affinity blood substitute prototype is to weaken the hydrogen bonding between bound O₂ and the E7 residue in myoglobin (17–20). Replacement of His-64(E7) with Gln causes a 5-fold reduction in oxygen affinity. Apolar substitutions (i.e. H64F) produce dramatic, 100–1000-fold, reductions in affinity (18). However, these substitutions at E7 are not useful, because they dramatically increase the rate of autoxidation, making the protein too unstable for clinical use. On other hand, the Leu-B10 → Phe mutation decreases the rate of autoxidation 10-fold, but increases oxygen affinity by 15-fold (17, 18). The large benzyl side chain inhibits autoxidation reactions by both stabilizing bound O₂ and by filling the space adjacent to the bound oxygen, preventing protonation by solvent water. The Phe-B10 substitution is also effective in selectively inhibiting NO-induced oxidation of oxymyoglobin (19). When Leu is replaced with the even larger Trp residue (L29W) at the B10-position, steric hindrance becomes the dominant factor, and both NO binding and NO-induced oxidation of myoglobin are markedly decreased (19). This approach has been explored further by examining the effects of large B10, E11, and G8 substitutions on the rates of NO-induced oxidation of the α- and β-subunits in recombinant human hemoglobin (20). A series of recombinant hemoglobins have been made with varying oxygen binding kinetics and reactivities with NO with apparent success in some *in vivo* models (20). This approach has provided protein engineering strategies for designing hemoglobin-based oxygen-
Reactions of Mutant Myoglobins with Peroxide

have suggested that the H64Q mutation is fairly conservative with respect to the size and polarity of residue 64 (51). This conclusion explains the presence of Gln-E7 in a few naturally occurring hemoglobins (e.g. opossum (52), Aскаris (53), and Lucina pectinata (54) and myoglobins from elephant (40) and shark (55)). Our data clearly demonstrate that combining H64Q with L29F produces a double mutant that is much more resistant toward oxidation than native sperm whale myoglobin. This H64Q/L29F mutation maintains a normal or slightly lower oxygen affinity, large association and dissociation rate constants for O₂ uptake and release, and normal resistance to autoxidation in the presence of catalase and superoxide dismutase (56). The enhanced resistance to oxidation in the absence of these antioxidant enzymes is due to the larger rate of autoreduction of the ferryl H64Q/L29F intermediate. As a result, the ferric form of the double mutant is a much better pseudoperoxidase, facilitating the rapid removal of HOOH before it can react with the remaining reduced H64Q/L29F oxy-myoglobin. Thus, the double substitution produces a protein with a more effective pseudoperoxidase activity. This protection against further HOOH oxidation may account for the combined H64Q/L29F replacement in the elephant myoglobin.

The enhanced enzymatic-like ability of H64Q/L29F to consume HOOH and to autoreduce the ferryl intermediate back to a less toxic ferric heme is clearly a desirable property in an oxygen-carrying agent. In endothelial cell cultures, chemically modified hemoglobin-blood substitutes were less effective in removing HOOH added to the medium than unmodified hemoglobin. This suppressed pseudoperoxidase activity, due possibly to chemical modifications, correlated with the formation of a long lived ferryl hemoglobin that was able to induce apoptotic cell death (57). We believe that the H64Q/L29F mutation or combinations similar to it can be developed further as useful blood substitute prototypes. One problem, however, remains unresolved, and that is how to overcome the enhanced hemin dissociation and degradation inherent with replacement of His-64 with Gln, either singularly or in combination with aromatic residues of the B10 and E11 positions. It is interesting to note that in the case of opossum hemoglobin (Gln-E7 in α-subunits), the enhanced susceptibility of its hemoglobin toward oxidative damage by peroxide (58) is counterbalanced by the presence of a more effective NADH/NADPH-dependent methemoglobin reductase system than that found within human red cells (59).

In summary, we have shown that replacing Leu-B10 with Phe in myoglobin generally promotes resistance to HOOH-induced oxidation. The large benzyl side chain in L29F mutant also serves to protect the FeO₂ complex from direct reactions with NO and from autoxidation (18, 19). Combining this substitution with a Gln substitution at the E7-position maintains the size and polarity of residue 64 and increases the rate of ferryl reduction but has little affect on O₂ affinity. The net result is a blood substitute prototype with enhanced pseudoperoxidase activity. Consequently, a small amount of oxidation of H64Q/L29F myoglobin produces a ferric protein that rapidly removes HOOH, preventing any further oxidation of remaining ferrous myoglobin.

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Reactions of Mutant Myoglobins with Peroxide

2037

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