H$_2$O$_2$-mediated Cross-linking between Lactoperoxidase and Myoglobin

ELUCIDATION OF PROTEIN-PROTEIN RADICAL TRANSFER REACTIONS*

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The H$_2$O$_2$-dependent reaction of lactoperoxidase (LPO) with sperm whale myoglobin (SwMb) or horse myoglobin (HoMb) produces LPO-Mb cross-linked species, in addition to LPO and SwMb homodimers. The HoMb products are a LPO(HoMb) dimer and LPO-(HoMb)$_2$ trimer. Dityrosine cross-links are shown by their fluorescence to be present in the oligomeric products. Addition of H$_2$O$_2$ to myoglobin (Mb), followed by catalase to flush excess H$_2$O$_2$ before the addition of LPO, still yields LPO cross-linked products. LPO oligomerization therefore requires radical transfer from Mb to LPO. In contrast to native LPO, recombinant LPO undergoes little self-dimerization in the absence of Mb but occurs normally in its presence. Simultaneous addition of 3,5-dibromo-4-nitrosobenzenesulfonic acid (DBNBS) and LPO to activated Mb produces a spin-trapped radical electron paramagnetic resonance signal located primarily on LPO, confirming the radical transfer. Mutation of Tyr-103 or Tyr-151 in SwMb decreased cross-linking with LPO, but mutation of Tyr-146, Trp-7, or Trp-14 did not. However, because DBNBS-trapped LPO radicals were observed with all the mutants, DBNBS traps LPO radicals other than those involved in protein oligomerization. The results clearly establish that radical transfer occurs from Mb to LPO and suggest that intermolecularly transferred radicals may reside on residues other than those that are generated by intramolecular reactions.

Amino acid centered radicals are now established as catalytic intermediates in a variety of enzymes, including cytochrome c peroxidase (1), prostaglandin H synthase (2), ribonucleotide reductase (3), pyruvate-formate lyase (4), and galactose oxidase (5). Protein radical formation not associated with the normal function of the protein is also observed, as, for example, in the reaction of Mb with H$_2$O$_2$ (6–13). Both the catalytic and incidental protein radicals are most commonly generated by a metal-catalyzed reaction within the protein itself, but the formation of protein radicals by reaction of the protein with an exogenous species such as the hydroxyl radical is also known (14, 15). Protein radicals, particularly those not intrinsic to the catalytic mechanism, can lead to cross-linking of the protein, cleavage of the protein backbone, and the formation of protein peroxyl radicals and protein peroxydes (16). These radical reactions can be physiologically relevant, as in the formation of a tyrosine-cross-linked protective shell by sea urchin ovoperoxidase (17). In most situations, however, protein radicals facilitate pathological or toxicological processes (18). Despite their importance, the factors and mechanisms that control the formation, localization, delocalization, and propagation of protein free radicals remain obscure.

The reactions of sperm whale and horse heart MetMb with H$_2$O$_2$ have served as a useful model for the investigation of protein radicals. The early observation by EPR of a protein radical in the reaction of MetMb with H$_2$O$_2$ has been more precisely defined by recent work (12, 19). Site-specific mutagenesis, in conjunction with protein digestion, peptide sequencing, mass spectrometry, and EPR spectroscopy, has established that MetMb cross-linking involves the formation of Tyr-103 and Tyr-151 dityrosine cross-links (10, 12, 13). Tyr-146, the third tyrosine in the protein, forms a radical, but it may not participate in chemical reactions (15). Of the two tryptophan residues, Trp-7 and Trp-14, only Trp-14 appears to be involved in the formation of a protein-peroxy radical that can co-oxidize styrene (13, 19). Evidence has also been obtained for the formation of a radical located on Cys-110 (20), which is unique to human myoglobin, and on an aliphatic MetMb amino acid residue (21). All of these protein radicals are associated with the formation of a ferryl (Fe$^{IV}$O) species in the reaction of MetMb with H$_2$O$_2$. Recent work indicates that the reaction of MetMb with H$_2$O$_2$ initially yields a transient Compound I-like species, in which the ferryl is coupled to a porphyrin rather than a protein radical (22, 23). It is therefore likely that the protein radical is generated by subsequent rapid electron transfer from the protein to the porphyrin radical. The residue that provides the electron that directly quenches the porphyrin radical is not known, but the resulting protein radical appears to transfer efficiently from one residue to another over considerable distances. Thus, radical reactions involving Tyr-103, Tyr-151, Trp-14, Cys-110 (human Mb only), and Lys-42 have been identified, and mutation of any one of these residues only suppresses the reactions at that specific site. Furthermore, the Tyr-151 radical is still formed, even though the closest Tyr-151 ring carbon is 12 Å from the heme iron, when Tyr-103 and Tyr-146, which are closer to the heme, have been mutated to phenylalanines. A relay mechanism for dispersal of the free...
radical center is therefore not a prerequisite for radical forma-
tion at a distal residue in MetMb.

LPO, a mammalian peroxidase, catalyzes the physiologically rele-
vant oxidation of thiocyanate to antibacterial products, as well
as the oxidation of iodide, bromide, and conventional per-
oxidase substrates such as ABTS and guaiacol (24). Its cata-
lytic mechanism involves reaction with H$_2$O$_2$ to form a Com-
pound I species with a Fe$^{IV}=$O ferryl species and a porphyrin
radical cation. This initial Compound I decays to a second
units/mg protein; vine milk were acquired from Sigma. The commercial LPO (80–150
A relevant oxidation of thiocyanide to antibacterial products, as

in the formation of a dityrosine cross-link involving residue Tyr-289 in each of two enzyme
molecules (27). Spin trapping/EPR evidence was also obtained
for the formation of protein radicals at sites other than the
tyrosine involved in the cross-linking reaction (27). The roles
of these protein radicals in LPO function remain unclear, al-
though a previous study has argued that the protein radicals
may be required for certain catalytic functions (26), and auto-
catalytic covalent binding of the prothetic heme group to the
LPO protein can be formulated as a radical process (28). As
though the evidence is more sparse than that for MetMb, it

The resulting solution was applied to a 2.6 × 20-cm Sepharose Fast
Flow column (Amersham Pharmacia Biotech) equilibrated with 50 mM
potassium phosphate, pH 6.8. The column was washed with 50 mM
potassium phosphate buffer (pH 6.8) and then eluted using the stepwise
procedure described in the text (0–450 mM NaCl). The fractions corre-
sponding to LPO-HoMb heteromers (as assessed by SDS-PAGE analy-
sis) were pooled in two lots. Each lot was desalted and concentrated by
ultrafiltration (Centricon YM 30; Amicon; molecular weight cut off
= 30,000). The concentrated samples contained about 4 mg total protein/ml
and were stored at 4 °C for subsequent analysis.

**Cationic Ion-exchange Chromatography—** A stock solution of 72 μM
LPO and 741 μM HoMb was prepared in 50 mM potassium phosphate
buffer, pH 6.8. The LPO-HoMb stock solution was incubated with H$_2$O$_2$
for 15 min at 25 °C. Excess H$_2$O$_2$ was then consumed by incubation with catalase (1.25 units/ml) for 15 min.

**Reverse Phase HPLC Analysis of the LPO-HoMb Heteromers—** A di-
tyrosine standard or LPO-HoMb hydrolysate samples were injected onto a
Vydac 218TP54, 4.6 × 250-mm, C18 reverse phase HPLC column. The
elution was carried out with a linear gradient rising from 50% solvent A to
10% solvent B in 45 min (solvent A, 0.1% trifluoroacetic acid in water;
solvent B, 0.085% trifluoroacetic acid in acetonitrile). A rapid gradient
to 90% solvent B and then back to 100% solvent A was then run to
regenerate the column. The eluent was monitored with a UV-visible
detector set at 280 nm. Fluorescent compounds were detected with a
PerkinElmer Life Sciences 650-10S fluorescence spectrophotometer
(λ excitation = 380 nm, λ emission = 410 nm) coupled in-line with the HPLC system.

**Preparation of the Dityrosine Standard—** Dityrosine was prepared using the enzymatic method of Amado et al. (32) and purified by reverse
phase HPLC as described previously (27).

**Spin Trapping Experiments—** EPR measurements were performed with an ER/200D EPR spectrometer operating at 9.80 GHz with a TM
resonator. First derivative absorption spectra were obtained with the
following settings: microwave power, 25 mW; center field, 3480
gauss; time constant, 100 ms; sweep time, 50 s; modulation, 0.32
millitesla at a frequency of 100 kHz; and total sweep width, 125 gauss.

Spectra were taken at 18 °C to 22 °C. The magnetic field range and
center were estimated by comparing the EPR spectrum from a LPO/
H$_2$O$_2$ reaction mixture with that of the stable nitrosos compound potas-
sium nitrosodisulfonate. The potassium nitrosodisulfonate splitting
was taken to be 13.091 gauss, and the center peak was taken
to correspond to a g of 2.0056 (33). The reactions were all performed in 50
mM potassium phosphate solution (pH 6.8) containing 200 μM diethyl-
enetriaminepentaacetic acid to inhibit possible catalysis by trace tran-
sition metals.

**RESULTS**

H$_2$O$_2$-mediated Cross-linking between LPO and MetMb—
Fig. 1 shows the profile of mixtures of LPO and MetMb on a
SDS-polyacrylamide gel after incubation with H$_2$O$_2$. Two types of
Mb, HoMb and SwMb, were utilized. As already known (12, 13, 27), H$_2$O$_2$ causes the covalent oligomerization of LPO (Fig.
Analysis of the cross-linking between SwMb and LPO. Lane 1, SwMb; lane 2, LPO + H2O2; lane 3, LPO + SwMb. Lanes 5 and 6, SwMb + LPO + H2O2. A, SDS-PAGE analysis of the cross-linking between SwMb and LPO. Lane 1, LPO; lane 2, LPO + H2O2; lane 3, SwMb; lane 4, SwMb + H2O2; lanes 5 and 6, SwMb + LPO + H2O2. The following concentrations were used: [H2O2] = 312 μM, [LPO] = 10 μM, [HoMb] = 375 μM, [SwMb] = 375 μM, and [polyacrylamide] = 20% (lanes 1–5) and 7.5% (lane 6).

1, A and B, lane 2) and SwMb (Fig. 1B, lane 4) but no more than a barely detectable trace on the gel with HoMb (Fig. 1A, lane 4). When the HoMb-LPO mixtures were exposed to H2O2, two new bands occurred at positions corresponding to Mr, 97,000 and Mr, 127,000 (Fig. 1A, lane 6), suggesting the formation of two types of heteromers, a dimer (LPO-Mb) and a trimer (LPO(Mb)2), respectively. Traces of two or three closely spaced bands of Mr, ~200,000, likely due to (LPO)2 homodimers and possibly also due to (LPO)2-Mb heterotrimers, were also present. One additional band was obtained with a molecular weight of ~153,000 using SwMb in place of HoMb (Fig. 1B, lane 6) suggesting the formation of a LPO(Mb)3 heterotetramer. These dimeric and oligomeric entities could not be attributed to S-S bond formation because (a) the protein samples were treated with 2-mercaptoethanol before electrophoresis, and (b) there are no cysteine residues in SwMb or HoMb. Inclusion of HoMb in the LPO/H2O2 system both decreased LPO homodimer and homotrimer formation and increased LPO-HoMb hetero-oligomerization in a concentration-dependent manner (Fig. 2). This suggests that HoMb competes with the two residues on the LPO surface involved in homotrimer formation. Heteromer formation increases to a maximum using reaction mixtures containing 1:40:40 stoichiometric concentrations of LPO, Mb, and H2O2, respectively (data not shown). No heteromer was observed by SDS-PAGE in incubations of apo-HoMb, LPO, and H2O2 (data not shown). Significant hetero-oligomerization only occurred when ferric HoMb or ferric SwMb was incubated with H2O2 in the presence of LPO.

**Isolation and Characterization of the LPO-HoMb Heteromers.—**The LPO-HoMb heteromers were separated by chromatography on a cationic exchange column (SP Sepharose Fast Flow). A typical elution pattern (Fig. 3A) indicates the presence of three major protein components (peaks 1–3) and several minor components. One of the minor components is the LPO homodimer, which elutes at ~170 ml (Fig. 3A). The fraction corresponding to the earliest eluting major peak (peak 1) contained only HoMb and was not investigated further. The fractions corresponding to peaks 2 and 3 were pooled individually and desalted and concentrated by ultrafiltration. The molecular weights and the purities of the resulting solutions were determined on a SDS-polyacrylamide gel (Fig. 3B). As shown, peak 2 yielded two bands with apparent molecular weights of 108,000 and 130,000, which are consistent with the LPO-HoMb heterodimer and the LPO(HoMb)2 heterotrimer, respectively. Peak 3 also yielded two bands, one of which was of higher molecular weight than that of native LPO and corresponded to the LPO-HoMb heterotrimer at Mr, 97,000.

The reaction of some hemoproteins with H2O2 are known to result in oligomerization of the proteins due to the formation of tyrosine-tyrosine cross-links (10, 34, 35). The dityrosine cross-links can be detected by their characteristic ultraviolet fluorescence (36, 37). To evaluate the presence of dityrosine cross-links between LPO and Mb, the fractions corresponding to peaks 2 and 3 were pooled individually, desalted, and concentrated by ultrafiltration as described above. They were then digested with Pronase and leucine aminopeptidase. Analysis of the hydrolysates of the two peaks by reverse phase HPLC (Fig. 4, A and B) showed in both cases a product eluting at 21 min with a strong fluorescence at 410 nm (excitation, 280 nm). The identity of this fluorescent compound was confirmed by demonstrating co-migration with authentic dityrosine on reverse phase HPLC (Fig. 4C). The fluorescence excitation and emission maxima of the fluorescent compounds were virtually identical to those of authentic, purified dityrosine at acid, neutral, and alkaline pH (data not shown), providing strong additional evidence that LPO-Mb hetero-oligomerization was due to the formation of tyrosine-tyrosine cross-links between the two proteins.

**Protein-Protein Radical Transfer Reactions.—**The possible occurrence of a radical transfer from Mb to LPO in the Mb/ H2O2/LPO system was investigated by sequentially adding catalase and then LPO to the Mb/H2O2 reaction mixture. Catalase and LPO were added 10 and 20 s after H2O2, respectively, to allow time for the catalase to remove unreacted H2O2 and thus to ensure that cross-linking was not due to LPO radicals.
formed by direct reaction with H$_2$O$_2$. As can be seen (compare Fig. 5 with Fig. 1), adding catalase before LPO had little effect on the type of cross-linked products that were formed. In particular, the dimeric and trimeric LPO-Mb forms were observable despite the addition of catalase to the HoMb incubation (Fig. 5A, lanes 1 and 6). Similar results were obtained with SwMb (Fig. 5B, lanes 1 and 6), except that additional bands are present due to the simultaneous formation of SwMb homodimers. Control experiments performed in the absence of Mb showed that (a) when catalase was omitted, monomeric LPO was partially converted to dimeric and trimeric products (Fig. 5, A and B, lane 5) and (b) addition of catalase 10 s before the addition of LPO prevented oligomerization of the enzyme (Fig. 5, A and B, lane 4). Thus, under the conditions used, catalase is effective in removing unreacted H$_2$O$_2$ before the addition of LPO. Thus, the oligomerization of LPO observed in the presence of Mb in this experimental protocol is the result of a radical transfer from Mb to LPO.

Essentially the same result was obtained using wild-type rLPO in place of native LPO (Fig. 6). rLPO displayed an enzymatic activity that was 50% lower than that of the native protein (data not shown) and, in unexpected contrast to the native form, exhibited little self-dimerization upon treatment with H$_2$O$_2$ (Fig. 6, lane 5). Nevertheless, ~50% of the monomeric rLPO migrates in the location of LPO-Mb heterodimers and LPO homodimers when treated with H$_2$O$_2$ in the presence of HoMb (Fig. 6, lanes 1 and 6).

An intense ESR spectrum characteristic of an immobilized nitroxide was detected when DBNBS was simultaneously added with LPO after preincubation of Mb/H$_2$O$_2$ with catalase to remove excess H$_2$O$_2$ (Fig. 7A). No signal could be detected in the absence of either SwMb (Fig. 7B) or H$_2$O$_2$ (Fig. 7C), indicating that formation of the EPR signal requires the presence of this protein and is not due to radicals formed by the direct reaction of LPO with H$_2$O$_2$. A much weaker signal was observed with the SwMb/H$_2$O$_2$/DBNBS system in the absence of LPO (Fig. 7D). This signal, which is reproducibly less than 30% of the intensity of the signal from the complete system, is believed to originate from direct reaction of H$_2$O$_2$-activated SwMb with DBNBS. Identical behavior and signals were observed when HoMb was used in place of SwMb (data not shown).

**Involvement of Specific Mb Residues**—To identify the resi-
cross-linking of the SwMb with LPO, and the triple tyrosine mutant gave no detectable cross-linking (Fig. 8A). This implicates these two tyrosine residues in the hetero-oligomerization process. In contrast, removal of Tyr-146, Trp-7, or Trp-14 had little or no effect. Spin trapping experiments showed the presence of radical species capable of reaction with DBNBS in the incubations of native LPO with all the recombinant Mb proteins that were investigated (Fig. 8B). Maximal ESR signal intensities were obtained with the single Y103F and Y151F mutants as well as with the triple tyrosine mutant (Fig. 8B). These intensities are approximately double the intensities seen with the other Mb mutants or with the wild-type enzyme. This may result from an increased efficiency of radical transfer from Mb to LPO caused by blocking the tyrosine radical to dityrosine decay pathway.

**DISCUSSION**

The formation of SwMb homodimers and the analogous formation of LPO homodimers on reaction of these hemoproteins with H2O2 were established by earlier studies (27, 38). However, the formation of hetero-oligomeric species involving the cross-linking of LPO to one, two, or possibly more Mb molecules (Fig. 1) is a new observation. As found earlier for the Mb and LPO homodimers, fluorescence spectroscopy indicates that the hetero-oligomeric LPO-Mb species, like the Mb and LPO homodimers, are held together by dityrosine cross-links (Fig. 3). Interestingly, LPO-Mb oligomers are formed with both SwMb and HoMb, even though HoMb does not self-dimerize because it lacks the Tyr-151 residue that is absolutely required for homodimer formation (Fig. 1). Earlier studies established that Tyr-151 was necessary for SwMb homodimerization, although the principal dityrosine cross-link was formed between Tyr-151 of one molecule and Tyr-103 of the other molecule (10). Tyr-146, which is buried in the protein, is not involved in dimerization reactions, but Tyr-151 can cross-link to itself. The dityrosine link between SwMb and LPO could therefore involve Tyr-151 or Tyr-103 of Mb. Because HoMb lacks Tyr-151, its dityrosine cross-link to LPO must be through Tyr-103. This inference is strongly supported by the finding that (a) mutation of either Tyr-103 or Tyr-151 to a phenylalanine in SwMb greatly decreases oligomerization and (b) mutation of both Tyr-103 and Tyr-151 suppresses oligomerization, whereas mutation of Tyr-146, Trp-7, or Trp-14 has no effect on the reaction (Fig. 8).

The LPO tyrosine residue linked to the tyrosine in Mb is more difficult to define. One possibility is that the link involves Tyr-289, which is the residue that is also involved in LPO self-dimerization (27). The fact that LPO dimer formation is attenuated in the presence of SwMb or HoMb supports this interpretation, but the evidence is inconclusive because trapping of a LPO radical at another site if the LPO radical is able to exchange among several sites on the protein would still diminish the formation of LPO dimers. Furthermore, the formation of LPO(HoMb)2 trimers involving dityrosine bonds virtually requires that at least two tyrosines in LPO must be able to cross-link to Mb to generate the HoMb-LPO-HoMb structure. In the case of SwMb, formation of the LPO(HoMb)2 hetero-oligomeric LPO-Mb species, like the Mb and LPO homodimers, fluorescence spectroscopy indicates that the tyrosine radical at another site if the LPO radical is able to exchange among several sites on the protein would still diminish the formation of LPO dimers. Furthermore, the formation of LPO(HoMb)2 trimers involving dityrosine bonds virtually requires that at least two tyrosines in LPO must be able to cross-link to Mb to generate the HoMb-LPO-HoMb structure. In the case of SwMb, formation of the LPO(HoMb)2 hetero-oligomeric LPO-Mb species, like the Mb and LPO homodimers, fluorescence spectroscopy indicates that the tyrosine radical at another site if the LPO radical is able to exchange among several sites on the protein would still diminish the formation of LPO dimers. Furthermore, the formation of LPO(HoMb)2 trimers involving dityrosine bonds virtually requires that at least two tyrosines in LPO must be able to cross-link to Mb to generate the HoMb-LPO-HoMb structure. In the case of SwMb, formation of the LPO(HoMb)2 trimer does not absolutely require the involvement of two LPO tyrosine residues because SwMb has two tyrosines that can undergo cross-linking reactions. It is therefore theoretically possible that the trimer has the structure LPO-SwMb-SwMb, so that there is only one bond to the LPO unit. However, the formation of a LPO(HoMb)2 trimer indicates that at least two tyrosines in LPO are available for cross-linking to HoMb.

Dityrosine cross-links are formed by the coupling of two tyrosine radicals in a radical recombination process, hence radicals must be present on both tyrosine residues for efficient coupling. To determine whether a protein radical present on a Mb molecule can be transferred to a tyrosine residue in LPO, Mb was exposed to a limited excess of H2O2, and catalase was then added to quench the remaining H2O2 before LPO was...
added to scavenge the excess peroxide. Under conditions in which the excess H₂O₂ is removed efficiently, cross-linking of LPO is still observed. This finding requires transfer of the Mb radical to LPO, followed by condensation with an Mb radical (Fig. 5). The radical transfer could involve specific residues, for example, transfer of the radical from Tyr-151 to the recipient site on LPO, or could result from a more general and undefined process that does not require the interaction of specific Mb residues with receptor residues in LPO. The finding that mutation of Tyr-103 and/or Tyr-151, but not Tyr-146, Trp-7, or Trp-14, inhibits or suppresses oligomerization clearly establishes that the electron transfer is not mandatorily mediated by Tyr-146, Trp-7, or Trp-14, although it is not possible to determine whether it is mediated by Tyr-103 or Tyr-151 because these two residues are required to form the dityrosine link. However, the finding that the trapping of LPO radicals by DBNBS is unimpaired when the triple tyrosine mutant of SwMb is used as the radical donor (Fig. 8) and is observed even in the absence of oligomerization indicates that radical transfer occurs in the absence of any of the residues that have been demonstrated to act as radical sites in Mb. Thus, although dimerization requires the participation of specific tyrosines in Mb, the radical transfer to LPO does not.

One very interesting finding is that rLPO is much less susceptible to self-dimerization than the native protein in the absence of Mb, but the two proteins form equal amounts of homodimers in the presence of Mb. Previous work with the heterologously expressed protein has shown that the primary difference between rLPO and LPO is that the heme group is susceptible to self-dimerization than the native protein in the absence of oligomerization indicates that radical transfer occurs in the absence of any of the residues that have been demonstrated to act as radical sites in Mb. Thus, although dimerization requires the participation of specific tyrosines in Mb, the radical transfer to LPO does not.

The present results establish that (a) the HoMb and SwMb protein radicals can be transferred to LPO, (b) none of the residues known to bear unpaired electron density in SwMb is specifically required for the radical transfer, and (c) the radical transferred to rLPO can support dimerization with Mb, even though the radicals generated by direct reaction of rLPO with H₂O₂ only very poorly yield the usual rLPO homodimer. This demonstration that radical transfer occurs between Mb and LPO complements recent reports that incubation of Mb with H₂O₂ in the presence of serum albumin produces EPR-detectable serum albumin-derived radicals through a radical transfer mechanism (39–41). However, it was concluded from the serum albumin experiments that a peroxo radical centered on Trp-14 was responsible for electron abstraction from, and therefore radical transfer to, serum albumin (40). Our finding that both the W7F and W14F SwMb mutant are fully able to transfer a radical to LPO clearly shows that neither tryptophan is essential for this reaction. It is possible that the Trp-14-peroxy radical participates in the reaction when present, but it is not essential for the radical transfer. The radical transfer thus appears to occur directly from the amino acid radicals in Mb. This finding is more closely mimicked by the finding that free tyrosine facilitates the oxidation of serum albumin by horseradish peroxidase (41), a reaction that presumably occurs by direct radical transfer from the free tyrosine radical to the protein.

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