The Myoglobin Protein Radical

COUPLING OF TYR-103 TO TYR-151 IN THE H2O2-MEDIATED CROSS-LINKING OF SPERM WHALE MYOGLOBIN*

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Sperm whale metmyoglobin, which has tyrosine residues at positions 103, 146, and 151, dimerizes in the presence of H2O2. Equine metmyoglobin, which lacks Tyr-151, and red kangaroo metmyoglobin, which lacks Tyr-103 and Tyr-151, do not dimerize in the presence of H2O2. The dityrosine content of the sperm whale myoglobin dimer shows that it is primarily held together by dityrosine cross-links, although more tyrosine residues are lost than are accounted for by dityrosine formation. Digestion of the myoglobin dimer with chymotrypsin yields a peptide with the fluorescence spectrum of dityrosine. The amino acid composition, amino acid sequence, and mass spectrum of the peptide show that cross-linking involves covalent bond formation between Tyr-103 of one myoglobin chain and Tyr-151 of the other. Replacement of the prothetic group of sperm whale myoglobin with zinc protoporphyrin IX prevents H2O2-induced dimerization even when intact horse metmyoglobin is present in the incubation. This suggests that the tyrosine radicals required for the dimerization reaction are generated by intra-rather than intermolecular electron transfer to the ferryl heme. Rapid electron transfer from Tyr-103 to the ferryl heme followed by slower electron transfer from Tyr-151 to Tyr-103 is most consistent with the present results.

The reactions of metmyoglobin and methemoglobin with H2O2 give rise to a ferryl species with spectroscopic properties very similar to those of horseradish peroxidase Compound II (1, 2). Recent NMR (3), Raman (4) and x-ray edge absorption studies (5) have clearly demonstrated that an oxygen atom is bound to the heme iron atom in the ferryl myoglobin complex.

The complex is therefore best represented as PFe(IV) = 0, with P = protoporphyrin IX. George and Irvine (1) and Yonetani and Schleyer (6) established by titration with ferrocyanide that the myoglobin ferryl complex retains only 1 of the 2 oxidation eq expected from the reaction with H2O2. However, the 2nd oxidation eq is accounted for if the titrating reagent is present during the reaction with H2O2 rather than being added afterwards. One oxidation equivalent is thus rapidly dissipated by still poorly defined mechanisms. EPR studies show that a protein radical is engendered in the reactions of sperm whale metmyoglobin (7, 8), horse metmyoglobin (6, 9), horse methemoglobin (8), human methemoglobin (10, 11), and bovine methemoglobin (12) with H2O2. The intensity of the radical signal is observed accounts for less than 15% of the 2nd oxidation eq, but extrapolation to zero reaction time suggests that the protein radical accounts initially for 50% or more of the 2nd oxidation eq (9). Differences in the EPR spectra of the protein radicals obtained with the different hemoproteins suggest that the radical may exchange between 2 or more residues. The saturation coefficients of the protein radical EPR signals led King and co-workers (8, 13) to propose that the radicals are centered on aromatic residues, probably on tyrosines, but their evidence is not unambiguous and does not identify the specific tyrosine residues involved.

There are 3 tyrosine residues (Tyr-103, Tyr-146, Tyr-151) in sperm whale myoglobin, 2 (Tyr-103, Tyr-146) in horse myoglobin (14), and 1 (Tyr-146) in red kangaroo myoglobin (14, 15). Rice et al. (16) have shown that H2O2 causes covalent dimerization of sperm whale but not horse metmyoglobin and have detected significant losses of tyrosine, lysine, and histidine in the H2O2-treated myoglobin. We have recently demonstrated that red kangaroo myoglobin also does not dimerize in the presence of H2O2.2 Uyeda and Peisach (17) have shown, furthermore, that oxidation of sperm whale myoglobin to the ferryl complex followed by reduction to the ferric state results in loss of 85% of the absorbance of Tyr-151 and, by amino acid analysis, in net loss of 1 tyrosine residue. No such losses were observed when horse or red kangaroo myoglobin were similarly oxidized and reduced. These results suggest that Tyr-151 is modified in the reactions with H2O2 and is involved in the dimerization process.

We report here a detailed analysis of the dimerization of sperm whale myoglobin that identifies the specific residues involved in the dimerization reaction, establishes the nature of the bond between the myoglobin chains, partially clarifies the fate of the oxidation equivalent that is rapidly dissipated

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1 The abbreviations used are: heme, iron protoporphyrin IX regardless of oxidation and ligation state; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; LSIMS, liquid second ion mass spectrometry; zinc sperm whale myoglobin, zinc-protoporphyrin IX-reconstituted sperm whale myoglobin.

2 Red kangaroo (Macrofus rufus) myoglobin, kindly provided by Dr. Jack Peisach, has been shown by SDS-PAGE not to give dimeric species when incubated under the conditions described in this study (P. R. Ortiz de Montellano and C. E. Catalano, unpublished work).
in the reaction of myoglobin with \( \text{H}_2\text{O}_2 \), and sheds some light on the mechanisms by which radicals are generated, translocated, and quenched in hemoprotein framework.

**MATERIALS AND METHODS**

**Dityrosine Extinction Coefficient**—The extinction coefficient of dityrosine was estimated by measuring the absorbance at 340 nm after allowing the amino groups of four different concentrations of dityrosine to react with 2,4,6-trinitrobenzene-1-sulfonic acid. These reactions were run in duplicate, and control reactions were run in the absence of dityrosine. The extinction coefficient at 284 nm estimated by this method is \( 6.9 \times 10^4 \), but the reliability of this value is not high due to high background absorbance at 384 nm in the control reactions. A modified procedure was therefore adopted to determine the extinction coefficient. Three concentrations each of tyrosine and dityrosine, each in duplicate, were allowed to react with 2,4,6-trinitrobenzene-1-sulfonic acid. The 284:340-nm absorbance ratio was then determined for the dityrosine samples and the 274:340-nm ratio for the tyrosine samples. The ratios thus obtained were then used to calculate the extinction coefficient of dityrosine relative to that of tyrosine (1.4 \( \times 10^3 \)). The value obtained for dityrosine by this approach is 4.8 \( \times 10^3 \).

**H\(_2\)O\(_2\)-mediated Cross-linking of Sperm Whale Myoglobin**—SDS-PAGE analysis of sperm whale myoglobin after incubation with \( \text{H}_2\text{O}_2 \) shows, as previously reported by Rice et al. (16), that the monomeric protein \( (M_r = 17,000) \) is partially converted to dimeric products \( (M_r = 34,000) \) (Fig. 1). Comparison of the Soret band intensities of the monomer and dimer fractions of sperm whale myoglobin isolated from the incubation by column chromatography indicates that approximately 90% of the myoglobin is converted to the dimer under the present reaction conditions. Kangaroo myoglobin does not dimerize under the same conditions (Fig. 1).

**Dityrosine in Oxidized Sperm Whale Myoglobin**—The tyrosinedityrosine ratios in the monomer and dimer purified by column chromatography from incubations of sperm whale myoglobin with \( \text{H}_2\text{O}_2 \) have been determined by total acid hydrolysis and high pressure liquid chromatographic analysis (Table I). Control experiments with mixtures of authentic tyrosine and dityrosine show that the hydrolysis conditions cause no more than a small (e.g., approximately 7% increase) change in the tyrosine to dityrosine ratio. Tyrosinedityrosine ratios of 36 and 5 are thus obtained, respectively, for monomeric and dimeric \( \text{H}_2\text{O}_2 \)-treated myoglobin. A tyrosinedityrosine ratio of 4 is expected if one tyrosine of each myoglobin unit is quantitatively converted to a dityrosine cross-link (e.g., 6 tyrosines \( \rightarrow \) 4 tyrosines + 1 tyrosine dimer). The amino acid analyses of Rice et al. (16) indicate, however, that the monomer loses 1.4 and the dimer 1.8 tyrosines per myoglobin chain. The tyrosinedityrosine ratio should therefore be less than 4 if all the tyrosine loss is due to dityrosine formation. The fact that the dimer tyrosinedityrosine ratio is actually 5 thus clearly indicates that tyrosine is significantly converted to products other than dityrosine. Conversion of tyrosine to products other than dityrosine is clearly the dominant process in the case of the sperm whale myoglobin monomer recovered from incubations with \( \text{H}_2\text{O}_2 \) because it gives a tyrosinedityrosine ratio of 36. Furthermore, the amino acid data of Rice et al. (16) suggests that approximately 1 tyrosine is lost per myoglobin monomer. The fact that 2 tyrosine residues are lost per myoglobin chain in the dimer is therefore consistent with involvement of one tyrosine in the cross-linking reaction and 1 tyrosine in reactions analogous to those that take place in the recovered myoglobin monomer.

**Identity of the Cross-linked Residues in Dimeric Sperm Whale Myoglobin**—Chymotryptic digestion of the sperm whale myoglobin dimer provides, after a series of high pressure liquid chromatography purification steps (Figs. 2 and 3), a major peptide with a fluorescence spectrum identical to that of dityrosine (Fig. 4). Two minor peptides are also seen with a similar fluorescence spectrum (Table II), but it has not been possible to obtain them in pure form. Amino acid analyses of the major chymotryptic peptide from dimeric sperm whale myoglobin do not unambiguously identify the cross-linked amino acid sequences due to the presence of impurities, but the data are most consistent with cross-linking of Tyr-103 (16).

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**Table I**

| Protein drill | Molecular mass | Tyrosine-dityrosine ratio | Tyrosines
<table>
<thead>
<tr>
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<th></th>
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</thead>
<tbody>
<tr>
<td>----------------</td>
<td>-----------------</td>
<td>--------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Mb dimer</td>
<td>34</td>
<td>5</td>
<td>1.8</td>
</tr>
<tr>
<td>Mb monomer</td>
<td>17</td>
<td>36</td>
<td>1.4</td>
</tr>
</tbody>
</table>

\( ^{a} \) The hydrolysis and analytical conditions are described under "Materials and Methods." The tyrosine-dityrosine peak ratio is based on their chromatographic peak areas corrected for the differences in their 280 nm extinction coefficients (molar extinction coefficients: tyrosine, \( 1.4 \times 10^{3} \); dityrosine, \( 5 \times 10^{3} \)).

\( ^{b} \) The theoretical ratio is that expected if one tyrosine per hemo protein molecule is quantitatively and exclusively involved in dityrosine formation in the dimer.

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The "Materials and Methods" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
with Tyr-151 (Table II). This inference is strongly supported by the terminal amino acid sequence data for the cross-linked peptide (Table III). Two amino acid residues are expected in each sequencing cycle, except when the same residue is present at both positions, because the internal dityrosine cross-link yields a peptide with two amino and two carboxyl termini. Dityrosine formation between Tyr-103 and Tyr-151 should yield a peptide that contains Lys-98 to Phe-106 of one myoglobin chain and Lys-147 to Gly-153 of the other. The pairs of amino acids expected from the above sequences are actually found in each sequencing cycle. An arginine peak due to an impurity is also found in the second cycle. The lysine expected in the fifth cycle is not seen, but this is not unexpected because the bond between it and the cross-linked tyrosines may not be readily hydrolyzed. Final confirmation of the structure of the cross-linked peptide is provided by positive liquid second ion mass spectrometry, which gives peaks for the protonated peptide at \( m/z \) 1942 and for the monosodium complex of the peptide at \( m/z \) 1964 (Fig. 5). The calculated molecular mass for the peptide is 1941.06. The sequence of the cross-linked peptide is thus:

\[
\text{Lys}_{98}\text{-Ile-Pro-Ile-Lys-Tyr}_{103}\text{-Leu-Glu-Phe}_{106}
\]

\[
\text{Lys}_{147}\text{-Glu-Leu-Gly-Tyr}_{151}\text{-Gln-Gly}_{153}
\]

**Intra- Versus Intermolecular Tyrosine Radical Formation**

The structure of sperm whale myoglobin determined by x-ray

**FIG. 3.** The third and final high pressure liquid chromatographic step in the purification of the dityrosine-containing peptide. The peptide was eluted isocratically from a Whatman Partisil 5 ODS-3 column with 0.1% trifluoroacetic acid, 25% acetonitrile, and 75% water. The absorbances at 280 and 220 nm, and the fluorescence at 413 nm (excitation at 280 nm), are shown in traces A, B, and C, respectively.

**FIG. 4.** Fluorescence spectrum of A, the purified peptide isolated from the sperm whale myoglobin dimer and B, authentic dityrosine (excitation at 280 nm). The small peak at 310 nm is due to a tyrosine impurity in the isolated sample.
Tyrosine Dimerization in Myoglobin Cross-linking

Amino acid analysis of the major chymotryptic peptide from the sperm whale myoglobin dimer

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Run 1</th>
<th>Run 2</th>
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<tbody>
<tr>
<td>Arg</td>
<td>0.7</td>
<td>0.9</td>
</tr>
<tr>
<td>Gly</td>
<td>1.9</td>
<td>1.7</td>
</tr>
<tr>
<td>Glu</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Ala</td>
<td>0.8</td>
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</tr>
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<td>Ile</td>
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<tr>
<td>Leu</td>
<td>2.4</td>
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</tr>
<tr>
<td>Phe</td>
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<td>1.6</td>
</tr>
<tr>
<td>Lys</td>
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<td>3.0</td>
</tr>
<tr>
<td>Pro</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>Tyr</td>
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<td>0</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Theoretical relative amount*</th>
</tr>
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<tbody>
<tr>
<td>Tyr-103/ Tyr-103</td>
</tr>
<tr>
<td>Tyr-151/ Tyr-151</td>
</tr>
<tr>
<td>Tyr-151/ Tyr-151</td>
</tr>
</tbody>
</table>

*Theoretical values for the peptide expected if there is a Tyr-103/Tyr-103 cross-link (dimer of residues 102-107), Tyr-151/Tyr-151 cross-link (dimer of residues 147-153), or Tyr-103/Tyr-151 cross-link (residues 102-107 and 147-153).

Sequence of the major chymotryptic peptide from the sperm whale myoglobin dimer

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Amino acid (amount)</th>
<th>Expected*</th>
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<tbody>
<tr>
<td>1</td>
<td>Lys (28), Ile (25)</td>
<td>Lys</td>
</tr>
<tr>
<td>2</td>
<td>Ile (17), Glu (18), Arg (23)</td>
<td>Ile, Glu</td>
</tr>
<tr>
<td>3</td>
<td>Leu (18), Pro (13)</td>
<td>Leu, Pro</td>
</tr>
<tr>
<td>4</td>
<td>Ile (10), Glu (18)</td>
<td>Ile, Glu</td>
</tr>
<tr>
<td>5</td>
<td>Lys (Tyr)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Gln (5)</td>
<td>(Tyr) Gln</td>
</tr>
<tr>
<td>7</td>
<td>Gly (6), Leu (4)</td>
<td>Gly, Leu</td>
</tr>
<tr>
<td>8</td>
<td>Glu</td>
<td>Glu</td>
</tr>
</tbody>
</table>

*Residues expected for each sequencing cycle for the dimeric peptide composed of Lys-Ile-Pro-Ile-Tyr103-Leu-Glu-Phe and Lys-Glu-Leu-Gly-Tyr103-Gln-Gly cross-linked via the tyrosine residues.

The characterization of the reaction of myoglobin with H₂O₂ suggests that the hemoprotein undergoes a two-electron oxidation (1, 6). One of the 2 oxidation eq is accounted for by oxidation of Fe(III) to Fe(IV) of the ferryl species. The 2nd oxidation eq is rapidly dissipated but is associated, at least in part, with a protein-centered radical. The identities of the residues that carry the unpaired electron, and to some extent the quantitative importance of the protein radical, have remained unclear. We have addressed this question by examining the chemical fate of the protein radical engendered in the reaction of sperm whale myoglobin with H₂O₂.

The reaction of sperm whale metmyoglobin with H₂O₂ results in dimerization of the hemoprotein, but no dimerization is observed in the comparable reactions of horse or red kangaroo metmyoglobin (Fig. 1) (16). This suggests, as already noted, that Tyr-151, which is replaced by a phenylalanine in horse and red kangaroo myoglobins, is critical for the dimerization reaction. The present demonstration that dimerization results largely from the formation of a dityrosine cross-link (Table I) clearly confirms that tyrosine residues play a key role in the dimerization of myoglobin. The data also show, however, that a substantial fraction of the tyrosine residues lost from both the H₂O₂-treated monomer and dimer of sperm whale myoglobin are converted to products other than dityrosine (Table I).

The amino acid composition, amino acid sequence, and mass spectrum of the principal cross-linked peptide isolated from the myoglobin dimer establish that the dityrosine link between the myoglobin chains is forged by coupling of Tyr-151 of one chain with Tyr-103 of the other. This contrasts with the prediction that a Tyr-151/Tyr-151 cross-link would be responsible for the dimerization of myoglobin. This prediction is based on the absence of cross-linking with horse and red kangaroo myoglobin and on the fact that Tyr-151 is found in the crystal structure of sperm whale myoglobin in a relatively unhindered, solvent-accessible site. Evidence that Tyr-151 is, indeed, accessible to the medium is provided by the similarity of its pKₐ (pKₐ = 10.3) with that of tyrosine in water. In contrast, the pKₐ value of Tyr-146 (pKₐ = 12.8-12.9) indicates it is sequestered in a hydrophobic environment and that of Tyr-103 (pKₐ = 11.4-12.0) indicates that it is in a region of intermediate solvent accessibility (17). Computer graphics docking studies using the crystal structure of sperm whale myoglobin provide no clear evidence for steric or electrostatic barriers to the formation of a Tyr-151/Tyr-151 cross-link. The results unambiguously establish, nevertheless, that the dimer is primarily held by a link between Tyr-103 and Tyr-151. The possibility exists that a Tyr-151/Tyr-151 cross-link is occasionally formed because the

FIG. 5. Molecular ion region of the liquid second ion (thioglycerol matrix) mass spectrum of the cross-linked peptide isolated from the sperm whale myoglobin dimer. The protonated molecular ion (m/z 1942) and the molecular ion of the monosodium complex (m/z 1964) are indicated in the spectrum.
structures of two minor dityrosine-containing peptides have not been determined. The minor peptides, however, may reflect secondary oxidative modification of the Tyr-103/Tyr-151 peptide rather than minor formation of a Tyr-151/Tyr-151 cross-link.

Computer graphics docking experiments, again based on the crystal structure of sperm whale myoglobin, suggest that Tyr-151 of one chain has direct access to both Tyr-103 (Fig. 6) and Tyr-151 of the second chain. The formation of a Tyr-151/Tyr-103 rather than Tyr-151/Tyr-151 cross-link is therefore difficult to rationalize on simple steric grounds. The tyrosine specificity of the cross-linking reaction could be governed by charge interactions that disfavor cross-linking of the Tyr-151 residues, but visual analysis of the protein interactions after color coding the protein residues according to their charge does not provide convincing support for such a mechanism. The alternative, and most probable, explanation is that the population of Tyr-151 radicals is lower than the population of Tyr-103 radicals so that coupling of Tyr-151 with Tyr-103 is statistically favored over its coupling with Tyr-151. This assumes, as suggested by docking studies, that coupling of Tyr-103 with Tyr-151 is not favored. A higher population of Tyr-103 than Tyr-151 aryloxy radicals is consistent with the fact that Tyr-103 is in direct contact with the heme edge (Fig. 7) and thus is the most easily oxidized by the activated prosthetic heme group.

The failure of zinc sperm whale myoglobin to dimerize in the presence of H$_2$O$_2$ and ferric horse myoglobin confirms, first of all, that the redox-active prosthetic group is required for dimerization. This finding supports the argument that Tyr-103 is the first to be oxidized because it is the closest of the three tyrosines to the prosthetic heme group. Oxidation of Tyr-151 then requires intra- or intermolecular transfer of one of its electrons to a diffusible oxidizing species, to the activated heme, or to the Tyr-103 radical. Intermolecular electron transfer must be seriously considered here because the cross-linking data establish that Tyr-151 of one chain interacts directly with Tyr-103 of another. Electron transfer from Tyr-151 of sperm whale myoglobin to Tyr-103 of horse myoglobin should therefore be feasible if the structure of horse myoglobin is similar to that of sperm whale myoglobin. A recent crystal structure of horse myoglobin indicates, in fact, that both proteins are very similar in the Tyr-103 region (24). The absence of dimerization when zinc sperm whale myoglobin is coincubated with ferric horse myoglobin and H$_2$O$_2$, however, argues strongly against both reaction with a diffusible oxidant and intermolecular electron transfer. It appears, therefore, that Tyr-151 is oxidized by intramolecular electron transfer to the ferryl heme or to the Tyr-103 radical. The intramolecular electron transfer route is not defined by the present results, but the distances between the various residues (Fig. 7) suggest that the Tyr-151 electron is probably transferred to Tyr-103 rather than directly to the heme. The location of Tyr-146 suggests that it may play a role as a conduit for the electron transferred from Tyr-151 to Tyr-103, but no actual experimental data are available to support this hypothesis. This electron transfer sequence is consistent with the postulate that there is a higher population of Tyr-103 than Tyr-151 radicals and, therefore, with the mechanism invoked to explain the tyrosine cross-linking specificity.

The present results indicate that biradical coupling of Tyr-103 and Tyr-151 yields a dityrosine cross-link. The two tyrosine radicals therefore account for a significant proportion of the free radical density engendered in the reaction of sperm myoglobin with 

![Fig. 6. Computer graphics model of the docking of Tyr-103 of one molecule of sperm whale myoglobin with Tyr-151 of a second. The coordinates of Takano (23) have been used for this analysis. The backbone residues of one myoglobin are shown in purple and those of the other in blue. The prosthetic heme groups are shown in red. The Van der Waal surfaces of the two myoglobins have been calculated by the procedure of Connolly (32). For the sake of clarity, the two myoglobins are not shown at their closest point of approach. The ortho carbons of the tyrosines that are to be cross-linked are connected by a dotted line. The docking of the two myoglobins has been optimized visually rather than by computational methods.](image-url)
Tyrosine Dimerization in Myoglobin Cross-linking

the closest ring carbon of Tyr-103 is only 3.32 Å from the terminal

FIG. 7. Spatial relationships between Tyr-103, Tyr-146, Tyr-151, and the prosthetic heme group in the crystal structure of sperm whale myoglobin (23). Tyr-103 is the lowest and Tyr-151 the uppermost of the tyrosines in the figure. The distances between the centers of the atoms connected by dashed lines are, in angstroms, Tyr-151 to Tyr-146, 5.18; Tyr-146 to Tyr-103, 8.85; Tyr-103 to Tyr-151, 12.02. The closest ring carbon of Tyr-146 is 9.66 Å, and that of Tyr-151 12.03 Å, from the heme iron atom. In contrast, the closest ring carbon of Tyr-103 is only 3.32 Å from the terminal carbon atom of the heme vinyl group.

whale myoglobin with H2O2. Further evidence that Tyr-103 is the primary locus of the free radical density is provided by our recent demonstration that the prosthetic heme group is also cross-linked to Tyr-103 (25). Finally, experiments in progress indicate that Tyr-103 is oxidized to an unidentified product when metmyoglobin is incubated with H2O2 and styrene.4 The latter finding is consistent with our earlier postulate that the cooxidation of styrene by sperm whale myoglobin involves reaction of the Tyr-103 radical with molecular oxygen (26). These three distinct diradical reactions, all of which involve Tyr-103, account in the aggregate for most of the modification of Tyr-103 that occurs in the reaction with H2O2. The protein radical engendered by H2O2 therefore must reside largely on Tyr-103 or must be located on a residue that is in ready equilibrium with the Tyr-103 radical.

The sperm whale myoglobin protein radical differs from that in Compound I of cytochrome c peroxidase in that it is rapidly dissipated by a variety of reactions, including dimerization, heme-cross-linking, and reaction with molecular oxygen. It is not clear at this time why the protein radical is much less stable in sperm whale myoglobin than cytochrome c peroxidase, although the exact locus of the unpaired electron density in the latter enzyme remains ambiguous (27, 28). The fact that a tyrosine radical is a stable component of catalytically active ribonucleotide reductase clearly indicates that tyrosine radicals are not necessarily unstable radical centers in biology (29). A clearer understanding of the myoglobin system should provide useful insights into the mechanisms by which protein radicals are stabilized and utilized in enzymatic systems. A particularly interesting relationship exists between the tyrosine radical in myoglobin and that detected in the catalytic turnover of prostaglandin H synthase (30, 31) in that both tyrosine radicals are unstable, both are closely associated with a ferrous heme species, both are proposed to participate in the catalytic actions of their respective hemoproteins, and both are associated with modification or inactivation of the hemoproteins.

Acknowledgments—We thank Dr. Carlos E. Catalano for the kangaroo myoglobin SDS-PAGE data in Fig. 1.

REFERENCES

Continued on next page.

* Y. S. Cho, C. E. Catalano, and P. R. Ortiz de Montellano, unpublished work.
SUPPLEMENTARY MATERIAL TO

THE MYOGLOBIN PROTEIN RADICAL - COUPLING OF TYR-18 TO TYR-131

IN THE H2O2-MEDIATED CROSS-LINKING OF SPERM WHALE MYOGLOBIN

David Tew and Paul R. Ortiz de Montellano

METHODS AND MATERIALS

Materials. Sperm whale myoglobin (type I), equine myoglobin (type II), and alpha-chymotrypsinogen (Type VII) were purchased from Sigma and zinc protoporphrin IX from Porphyrin Products (Logan, Utah). All buffers were prepared with demineralized, double-distilled water.

Analytical Methods. Electronic absorption spectra were recorded on a Hewlett Packard Model 8450 diode array spectrophotometer. High pressure liquid chromatography was carried out on a system consisting of two Alliance Model 110A pumps and a pump controller coupled to a Hewlett Packard Model 1040A diode array UV/Vis detector and a Perkin Elmer Model PE 650-105 fluorescence detector. The same fluorescence unit was used for fluorescence spectroscopy. Peak integration was done with a Hewlett Packard Model 3900A integrator. Peptide mass spectra were obtained on a Kratos MS 50 instrument operating in the ESIMS mode. Amino acid analyses were done by the Waters "picoTage" method by the University of California, San Francisco Biomolecular Resource Center. SDS-PAGE was performed by Laemmli (10).

Synthesis of Authentic Dityrosine. Authentic dityrosine was prepared by the horseradish peroxidase catalyzed oxidation of N-acetylcysteine according to the method of Amado (12). N-Acetylcysteine (1.1 g) was dissolved in 200 ml of 0.1 M potassium borate buffer (pH 9.5). Horseradish peroxidase (12 mg) and 600 ml of a 0.1% aqueous solution of H2O2 were then sequentially added and the resulting mixture was incubated at 37°C for 24 hr. The solution was neutralized with concentrated HCl and a little caustic was added to drive excess periods. Water was removed on a rotary evaporator and the residue was extracted with 20 ml of ethanol. The concentrated extract was applied to two 20 x 20 cm preparative (1000 ml) silica gel thin layer plates (Analtech). The plates were developed with butanol-acetic acid-water (4:1:1 v/v/v). The fluorescent band due to the dityrosine was excised and the extract was extracted with methanol. The crude yellow material thus obtained was dissolved in 10 ml of 6 M HCl and was refluxed under nitrogen for 24 hr. The brown solid obtained when the solution was neutralized was chromatographed on a 2 x 50 cm silica gel column eluted with butanol-water-acetic acid 4:1:1 (v/v/v). The fluorescent fractions were pooled and the solvent removed on a rotary evaporator. The crude dityrosine thus obtained was further purified by high pressure liquid chromatography on a 10 x 250 mm semi-preparative C-18 (Type 2) column eluted isocratically with 0.1% acetic acid and 85:5% water containing 0.1% trifluoroacetic acid. Dityrosine (110 mg) was obtained as a white powder after removal of the solvent from the appropriate eluent fractions. Fluorescence, excitation 280 nm, emission, 415 nm; mass spectrum (negative ion LSIMS) m/z 349 [M+1]; 1H NMR 7.250 (dd, J = 8.5, 6.3 Hz, aryl-CH). and 3.14 ppm (dd, J = 8.3, 5.3 Hz, aryl-CH3). 3.29 ppm (d, J = 14.5, 5.3 Hz, aryl-CH2) and 3.14 ppm (d, J = 14.5, 7.8 Hz, aryl-CH3). The spectra are consistent with those in the literature (22).

Determination of the Excitation Coefficient of Dityrosine. Solutions of tyrosine and dityrosine in double distilled water were prepared such that dilutions of 30, 100, and 200 volumes to 2 ml gave reasonable absorbances at 274 and 284 nm, respectively. One ml was taken from each of these mixtures and the absorbance at 274 (tyrosine) and 284 nm (dityrosine) determined. To the remaining 1 ml of each solution was added 1 ml of 4% NaHCO3 and 0.1% 2,4-dinitrophenylglycine and the resulting solutions were incubated at 40°C for 2 hr. Control incubations were carried out on a mixture of tyrosine or dityrosine. The absorbance at 340 nm in the control was then measured after 5-fold dilution of the incubation mixtures with double distilled water as the solvent. The absorbance at 340 nm in the control was then measured after 5-fold dilution of the incubation mixtures with double distilled water as the solvent. The absorbance at 340 nm in the control was then measured after 5-fold dilution of the incubation mixtures with double distilled water as the solvent. The absorbance at 340 nm in the control was thus subtracted from that for the tyrosine and dityrosine samples before calculating the 284-340 nm (tyrosine) or 274-340 nm (dityrosine) ratio.