Intramolecular Translocation of the Protein Radical Formed in the Reaction of Recombinant Sperm Whale Myoglobin with H₂O₂*

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A sperm whale myoglobin gene containing multiple unique restriction sites has been constructed in pUC 18 by sequential assembly of chemically synthesized oligonucleotide fragments. Expression of the gene in *Escherichia coli* DH5α cells yields protein that is identical to native sperm whale myoglobin except that it retains the terminal methionine. Site-specific mutagenesis has been used to prepare all the possible tyrosine-phenylalanine mutants of the recombinant myoglobin, including the three single mutants at Tyr-103, -146, and -151, the three double mutants, and the triple mutant. All of the mutant proteins are stable except the Tyr-103 mutant. Introduction of a second mutation (Lys-102 → Gln) stabilizes the Tyr-103 mutant. Absorption spectroscopy shows that the active sites of the mutant proteins are intact. EPR and absorption spectroscopy show that all the proteins, including the triple mutant devoid of tyrosine residues, react with H₂O₂ to give a ferryl species and a protein radical. The presence of a protein radical in all the mutants suggests that the radical center is readily transferred from one amino acid to another. Cross-linking studies show, however, that protein dimers are only formed when Tyr-151 is present. Tyr-103, shown earlier to be the residue that primarily cross-links to Tyr-151 (Tew, D., and Ortiz de Montellano, P. R. (1988) *J. Biol. Chem.* 263, 17880-17886), is not essential for cross-linking. Electron transfer from Tyr-151 to the heme, which are 12 Å apart, occurs in the absence of the intervening tyrosines at positions 103 and 146. The present studies show that the peroxide-generated myoglobin radical readily exchanges between remote loci, including non-tyrosine residues, but protein cross-linking only occurs when radical density is located on Tyr-151.

The biochemistry of protein radicals has become of increasing interest as their pathological roles in lipid peroxidation (1, 2) and in the structural perturbations of proteins that cause their proteolytic degradation (3, 4) have become apparent. The interest in protein radicals also stems from their proposed roles in the normal catalytic mechanisms of a growing number of enzymes (5), including cytochrome c peroxidase (6), ribonucleotide reductase (7, 8), pyruvate lyase (9), galactose oxidase (10), and prostaglandin H synthase (11). A tyrosine-centered radical is specifically involved in some of these enzymatic reactions (7, 8, 10, 11).

Myoglobin is not ordinarily a catalytic protein but does support the H₂O₂-dependent oxidation of many substrates (12), including olefin epoxidation, a cytochrome P450-like reaction (13). Catalysis of these reactions is associated with the formation of a ferryl (Fe⁵⁺ = O) species (14, 15). This ferryl species retains one of the two oxidizing equivalents provided by reaction with H₂O₂, but the second oxidizing equivalent is dissipated by poorly understood mechanisms (14, 15). EPR studies indicate that a protein radical is formed in the reactions of H₂O₂ with sperm whale metmyoglobin (16, 17), horse metmyoglobin (14, 18), horse methemoglobin (17), human methemoglobin (19, 20), and bovine methemoglobin (21). Unlike the equimolar protein radical and ferryl complex produced in the reaction of cytochrome c peroxidase with H₂O₂ (6), the detectable radical signal in myoglobin accounts for no more than 50% of the second oxidation equivalent (15). EPR data suggest that the protein radical resides on an aromatic residue, most likely a tyrosine, although some evidence exists that the radical may first reside transiently on a histidine or other aromatic residue (17, 22). There are three tyrosine residues in sperm whale myoglobin (Tyr-103, Tyr-146, Tyr-151), two in horse myoglobin (Tyr-103, Tyr-146), and only one in red kangaroo myoglobin (Tyr-146) (23). Reaction with H₂O₂ results in covalent dimerization of sperm whale (24) but not horse (24) or red kangaroo (25) myoglobins. Analysis of the sperm whale reaction by tryptic digestion and peptide sequencing showed that dimerization primarily involves cross-linking of Tyr-103 of one myoglobin chain with Tyr-151 of the second (25). The heme group of H₂O₂-treated horse myoglobin is also covalently bound to the protein through Tyr-103 (26). Further evidence for dissipation of the second oxidizing equivalent through protein radical mechanisms comes from parallel work showing that a substantial proportion of the oxygen incorporated into the epoxide in the H₂O₂-dependent epoxidation of simple olefins comes from molecular oxygen (13). The proposed mechanism for these epoxidations involves co-oxidation by the peroxy radical generated when oxygen adds to the protein radical, a mechanism supported by the fact that 4-methylphenol, a stand-in for tyrosine, mediates the epoxidation of styrene by horseradish peroxidase and H₂O₂ (27).

Sperm whale myoglobin is ideal for the analysis of structure-function relationships pertinent to the biochemistry of protein radicals because it is a small, rugged protein and highly refined x-ray crystal structures are available for the native (28) and recombinant proteins (29). We report here chemical synthesis of a gene encoding sperm whale myoglobin, its expression in *Escherichia coli*, successful construction and

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expression of all the possible mutants in which the tyrosine residues are replaced by phenylalanine, and examination of the EPR properties and susceptibility to dimerization of the expressed proteins.

**MATERIALS AND METHODS**

**General Methods—**Plasmid purification, subcloning, and bacterial transformation were carried out as described (30). Detoxified, double glass-stained, Chelex-treated water was used for all biochemical experiments. Chelex was from Bio-Rad. Oligonucleotides (80-108 base pairs) were synthesized at the University of California, San Francisco, DNA Synthesis Resource Center using an Applied Biosystems 380B DNA synthesizer. Oligonucleotides were purified on PAGE, 12% acrylamide, 8 M urea gels (31). DNA was eluted from the gels in 0.5 M ammonium acetate and desalted by passage through a Pharmacia LKB Biotechnology Inc. NAP-25 column with water as the eluant. Oligonucleotides <60 nucleotides were desalted over a Waters Sep-Pak C18 cartridge using water/methanol (40:60) as eluant.

**Bacterial Strains—**E. coli strain TB-1 (φ80d lacZ AM15; ara, Δ(lac-proAB), rpsL, $80d$ lacZ AM15; ars, Δ(lac-proAB), rpsL, $80d$ lacZ AM15; hsdR) provided by T. O. Baldwin, Texas A&M University, was used as the host strain for plasmid-mediated transformation. The myoglobin gene, E. coli strain DH5α (F−, ara, Δ(lac-proAB) rpsL, $80d$ lacZ AM15 hsdR) was used for expression of the myoglobin protein.

**Assembly and Characterization of the Gene—**The myoglobin gene was constructed in five sections in the vectors pBR325, pUC18, pUC 18, and pYT Si46. The gene segments comprised of single pairs of oligonucleotides were annealed in 10-μl reaction mixtures containing 2.5 pmol of each of the oligonucleotides and ligase buffer (66 mM Tris-HCl, pH 7.5, 6.6 mM MgCl2, 10 mM dithiothreitol, and 0.4 mM ATP). The oligonucleotides were annealed by heating at 100 °C for 3 min and cooling slowly to room temperature over a period of 1 h. The annealed fragments were immediately ligated into 0.5 μg of a suitably digested vector, 5 μl ligation buffer, and 1 μl of 10 mM ATP in a final volume of 20 μl. Three units of T4 DNA ligase were added and the reaction allowed to proceed for 4 h at room temperature or overnight at 16 °C. One half of the ligation mix (10 μl) was used to transform E. coli strain TB-1, and the cells were plated on LB agar containing 20 μg of 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal)/ml, 0.1 mM isopropyl-β-D-thiogalactopyranoside/ml, and 100 μg/ml ampicillin (for plasmids pBR325 and pYT Si46 only ampicillin was used to screen for positive clones). Plasmid DNA was purified (32) from an average of 20 colonies, and glycerol stocks were made of the positive clones. Three or four of the clones were further analyzed by DNA sequencing (33, 34). Final assembly of the gene was carried out by subcloning the individual segments into pUC 18 to give plasmids pM b221 to pM b416 and ligation reactions were carried out as described for the initial gene construction. The ligated oligonucleotide concentration used in each of the reactions varied from 1.0 to 5.0 pmol. The mutants were sequenced to confirm the codon changes.

**Purification of the Mutant Proteins—**All of the mutant proteins were purified as described for the wild type protein with the exception of the pMb Y103F mutant. The purified proteins were essentially identical to the wild type with regard to their spectral characteristics and SDS-PAGE profiles. The pMb Y103F mutant showed no color in the cell pellets. Cell lysis was conducted as described for the wild type, but the myoglobin protein appeared to be aggregated and came down with the cell pellet. SDS-PAGE with the cell pellet, as judged by SDS-PAGE. The protein could be solubilized in the presence of detergent were unsuc-

**Protein Purification and Characterization—**The method of purification was based on that of Springer and Siger (35). Fresh overnight cultures of pMb221 (25 ml) were used to inoculate 2 liters of LB media containing 100 μg/ml ampicillin. Plasmid DNA was purified (32) from an average of 20 colonies, and glycerol stocks were made of the positive clones. Three or four of the clones were further analyzed by DNA sequencing (33, 34). Final assembly of the gene was carried out by subcloning the individual segments into pUC 18 to give pMb221. The DNA sequence of both strands of the gene was confirmed using M13 subclones (35, 34). The gene has the same ribosomal binding site and codons for the same protein sequence as that of Springer and Siger (35) but differs from it at approximately 10% of the nucleotide bases.

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**RESULTS**

**Design, Synthesis, and Expression of the Myoglobin Gene—**The design of the gene was based on the amino acid sequence of sperm whale myoglobin. A computer program, PROTORES (36), was used to reverse translate the amino acid sequence into a degenerate DNA sequence. The locations of restriction
sites with recognition motifs greater than 4 bases in length were noted. A DNA sequence was chosen with approximately 35 unique sites spaced approximately 20 base pairs apart. Restriction sites recognized by commercially available restriction enzymes were preferentially chosen to be retained within the gene. The codon usage of the resulting myoglobin gene was modified to include those triplets that are more frequently used in E. coli genes (37) while retaining the largest number of restriction sites possible. The ribosomal binding site AGGAGA was added 9 bases upstream of the coding region to direct the initiation of protein translation.

The gene was initially constructed as five segments: pBMb1 (EcoRI/NcoI, 105 bp), pCMb2 (NcoI/BglII, 107 bp), pYMb3 (BglII/SphI), pUMb4 (SphI/XmaI), and pUMb5 (XmaI/HindIII). Several isolates of each of the constructs were analyzed by DNA sequencing. Sections pUMb4 and pUMb5 were cloned into pUC18 and sequenced. The final step involved the simultaneous cloning of the remaining three segments in tandem with pUMb4 and pUMb5. The full-length gene carried a deletion at position 60 which was corrected by recloning the EcoR1/NcoI duplex into the gene construct. A map of the unique restriction sites is shown in Fig. 1.

Transformation of E. coli DH5α cells with pMb221 resulted in expression of myoglobin at a level of approximately 10% of the total protein, as judged by SDS-PAGE. Production of viable myoglobin was easily detected by the dark brown coloration of the cells and by whole cell UV/visible CO difference spectroscopy (not shown). The protein was purified by FPLC on a Mono S (HR 10/10) column. The yield of purified protein (>95% pure by SDS-PAGE) from 4 liters of cells ranged from 10 to 20 mg (Fig. 2). The recombinant protein appeared from the UV spectra of the oxidized and reduced complexes to be identical to sperm whale myoglobin (Fig. 3). N-terminal sequencing of the recombinant protein showed the first five amino acids to be identical to those of the native protein except that the initiating methionine is not processed during bacterial expression and is retained.

Expression, Purification, and Characterization of the Mutant Proteins—Difference spectroscopy showed that the mutant constructs, with the exception of pMBY103F, directed the expression of intact holoproteins (data not shown). The mutant proteins, with the exception of that produced by pMBY103F, were purified by the same protocol as, and in comparable yields to, the wild type protein. The UV/visible spectra of the purified proteins in the ferric (Mb3+), ferrous (Mbh+), ferrous dioxygen complex (MbO2), the ferrous carbon monoxide complex (MbCO), and the ferryl (Mb4+) states were essentially identical to those of the wild type protein. The ferric spectra of horse myoglobin (Sigma), recombinant sperm whale myoglobin, and the pMBK102Q/Y103F mutant are compared in Fig. 4. As already noted, pMBY103F did not give rise to colored cells, nor could a difference spectrum be obtained with the whole cells. Nevertheless, the presence of a band at 17,800 daltons on SDS-PAGE indicated that the protein was actually expressed by the pMBY103F construct (data not shown). The protein coded by pMBY103F was found in the pellet on cell lysis. The protein could be solubilized from the pellet in the presence of 2% n-octyl β-D-glucopyranoside. A small Soret band at 408 nm was observed on solubilization, but attempts to purify the protein in the presence of the detergent were unsuccessful. The Soret decreased following the Bio-Gel P-
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FIG. 4. Absorption spectra of (A) horse myoglobin, (B) recombinant sperm whale myoglobin, and (C) the K102Q/Y103F myoglobin mutant. The spectra were recorded in 50 mM potassium phosphate (pH 7.4) at a concentration of 15 mM.

100 column, and the protein eluted in an earlier fraction than the wild type protein, suggesting that the protein is unstable. Attempts to purify the protein further were discontinued when it was found that introduction of a second mutation at position 102 (Lys → Gln) yielded a stable protein that could be purified by the standard protocol and had absorption spectra identical to those of the other proteins.

EPR of the Recombinant Proteins—The EPR spectrum obtained for H₂O₂-treated, recombinant sperm whale myoglobin was essentially identical to that of native sperm whale myoglobin and exhibited a large peak at g = 2.003 and a small peak at g = 2.03 (Fig. 5) (17). A protein EPR spectrum was observed for each of the H₂O₂-treated myoglobin mutants. The EPR spectrum of the Y151F mutant resembles the spectrum of horse metmyoglobin, in which the peak at g = 2.03 accounts for a greater proportion of the total signal than in the spectrum of the wild type. This same pattern is observed in the EPR spectrum of each of the mutants lacking Tyr-151 (Figure 5, panels B, E, and F) but not in those that retain this tyrosine residue (Fig. 5, panels A, C, and D). These differences cannot yet be interpreted in detail but clearly require localization of the protein radical not only on Tyr-151 but also on other tyrosine residues or on other residues whose conformations are altered by the Tyr → Phe replacements.

Cross-linking of the Myoglobin Proteins in the Presence of H₂O₂—The results of the cross-linking experiments are shown in Fig. 6. Incubation of recombinant sperm whale myoglobin with H₂O₂, as reported for the native hemoprotein (25), results in the formation of dimeric products. In contrast, the Y151F mutant is not detectably cross-linked by H₂O₂. This strengthens the argument that horse myoglobin is not cross-linked because it lacks a tyrosine residue at position 151. Removal of Tyr-146 does not interfere with dimerization of the protein, as judged by SDS-PAGE. The Y103F mutant, in contrast to red kangaroo myoglobin (26), dimerizes on incubation with H₂O₂. Red kangaroo myoglobin has a Phe at position 103 but differs from the Y103F mutant in that it also has a Phe at position 151. On treatment of the double tyrosine mutants (K102Q/Y103F/Y146F, K102Q/Y103F/Y151F, and Y146F/Y151F) and the triple tyrosine mutant (K102Q/Y103F/Y146F/Y151F) with peroxide only the double mutant retaining tyrosine 151 exhibited the band on SDS-PAGE indicative of myoglobin cross-linking.

DISCUSSION

In order to elucidate the role(s) of protein radicals in the catalytic and pathological processes supported by myoglobin, we have constructed a gene into which we can readily introduce site-specific mutations. The gene, based on the amino acid sequence of sperm whale myoglobin, was designed with evenly spaced restriction sites to facilitate mutagenesis. The restriction sites retained within the gene code for commercially available enzymes whose recognition sequences are greater than four bases long. The flanking restriction sites were chosen to make the gene portable into any of the commonly available E. coli expression vectors. High level expression of the protein encoded by the gene has been achieved by optimizing the translation initiation signal and codon usage for bacterial expression.

Recombinant and native sperm whale myoglobin appear to be identical as judged by comparison of their absorption spectra. The spectra of the ferric (Fig. 4), deoxy (430 nm), oxy (418 nm), ferrous carbonmonoxy (423 nm), and ferryl (421 nm) states of the recombinant protein are identical to the corresponding states of native sperm whale myoglobin (38). This is consistent with the crystallographic integrity of the heme crevice (29), and the identical rate and equilibrium values for the binding of ligands (39) reported for a similarly expressed recombinant myoglobin. No more than slight changes are observed in the UV/visible spectra of the site-specific mutants, indicating that no major structural perturbations are created in the heme binding pocket by the amino acid substitutions. The maxima of the mutants in the ferric (408-409 nm), deoxy (430-433 nm), oxy (414-418 nm), carbonmonoxy (421-423 nm), and ferryl (~421 nm) states are very close to the corresponding maxima for recombinant sperm whale myoglobin and for the native protein itself. The Tyr-146 mutants, with the exception of K102Q/Y103F/Y146F, appear to be less stable than either the other tyrosine mutants or the parent protein because their R₅ (A₅₅₀/ A₄₃₀) values are generally lower (3.0-3.5) than that of the wild type protein (R₅ = 4.5). All the mammalian myoglobins so far sequenced conserve the tyrosine residue at position 146, suggesting that this residue has an important structural (or functional) role. The lower stability and slightly altered spectra of the Tyr-146 mutants, presumably caused by slight structural perturbations of the protein structure, are consistent with this view.

Mutation of Tyr-103 to Phe encodes a protein that is not soluble and remains with the cell debris when the cells are lysed. The crystal structure of sperm whale myoglobin shows that Tyr-103 is on the outside of the protein with the closest tyrosine ring carbon 3.3 Å from the heme iron atom (Fig. 7) and the phenolic hydroxyl group facing the solvent. It is possible that replacement of Tyr-103 with a phenylalanine, which cannot hydrogen bond with the solvent, forces the aromatic residue into the heme pocket where it interferes with heme binding and hence protein folding. Red kangaroo myoglobin, which has an overall sequence identity with sperm...
whale myoglobin of 79.5%, is one of the few myoglobins with a phenylalanine rather than a tyrosine at position 103. Analysis of the kangaroo myoglobin sequence shows that it has a glutamine at position 102, the residue adjacent to the tyrosine, rather than the lysine found in sperm whale myoglobin. Based on the hypothesis that the difference in the residue at position 102 might be responsible for the differential stabilities of kangaroo myoglobin and the Y103F mutant, we constructed a sperm whale myoglobin double mutant containing both Phe-103 and Gln-102. Indeed, the resulting protein is stable and can be purified by the same protocol as the wild type protein. Despite the success of the approach, it is not clear why the Lys→Gln substitution at position 102 stabilizes proteins with the Tyr-103 mutation. One obvious consequence of the substitution is loss of a positive charge and reduction of the basicity at position 102. It may be important that residues 102 and 103 lie at the interface of the FG helix transition. It is known that the FG helix moves away from the heme and slightly compresses the 13 residues of the E helix nearest the EF corner on going from deoxy- to metmyoglobin (40). Computer modeling and energy minimization studies are currently under way to analyze the structural consequences of the mutations at positions 102 and 103.

Surprisingly, a protein radical EPR spectrum is obtained not only in the reaction of recombinant sperm whale myoglobin with H2O2 but also in the corresponding reactions of all the tyrosine mutants (Fig. 5). The H2O2-treated proteins also exhibit a ferryl (FeIV = O) absorption spectrum. The EPR signal patterns appear to fall into two classes, depending on whether Tyr-151 is present in the protein or not. When Tyr-151 is present, a strong signal at g = 2.003 is accompanied by a relatively weak signal at g = 2.03, whereas in the absence of Tyr-151 the two signals are much closer to each other in intensity. The most notable observation, however, is that a protein radical is formed in the complete absence of tyrosine residues. The radical in the triple mutant has to be centered on an oxidizable amino acid other than a tyrosine, possibly an imidazole or tryptophan. One likely candidate is the active site imidazole because of its proximity to the oxygen binding site, although our present finding that the radical readily transfers between remote residues in the protein makes all oxidizable residues viable candidates for the radical center. Although the tyrosine replacements should make oxidation of the protein more difficult, we have not been able to detect a transient horseradish peroxidase-like compound I species with a porphyrin rather than protein radical by absorption spectroscopy.

Cross-linking experiments provide further information on the location of the protein radical. Recombinant sperm whale myoglobin, like the native protein (24), is cross-linked in the presence of H2O2. Replacement of Tyr-151 with a phenylalanine, however, prevents protein cross-linking, in agreement with the absence of cross-linking in the reaction of horse myoglobin with H2O2. In contrast, replacement of Tyr-146 with phenylalanine has no effect on the cross-linking ability of the protein. This is the first test of the role of Tyr-146, but its lack of involvement in the cross-linking process is consistent with the earlier finding that the primary cross-link in peroxide-treated sperm whale myoglobin is between tyrosines 103 and 151 (25). In contrast, the finding that the Y103F mutant is readily cross-linked by H2O2 despite the absence of tyrosine 103 appears to be at odds with the earlier finding. Computer graphics docking experiments suggest, however, that formation of a dityrosine cross-link between the Tyr-151 residues of two myoglobin chains is sterically allowed (24). The predominant formation of a cross-link between Tyr-103 and Tyr-151 therefore appears to be due to predominant location of the unpaired electron density on Tyr-103, the tyrosine vicinal to the heme group. Indeed, a Tyr-151/Tyr-151 cross-link is not excluded by the earlier work because the sequences of two minor dityrosine peptides could not be determined (25). The present observation that myoglobin with
a single tyrosine at position 151 is readily cross-linked confirms that a Tyr-151/Tyr-151 bond is, indeed, feasible. The specific role of this tyrosine in the cross-linking process is reinforced by the fact that the double mutant retaining Tyr-151 (Phe at 103 and 146) undergoes H$_2$O$_2$-dependent dimerization, whereas neither of the double mutants without Tyr-151, nor the triple mutant with no tyrosines, does so. Tyr-151 is therefore absolutely essential for myoglobin dimerization even though a protein radical is observed with all the mutants. Cross-linking is thus clearly a relatively specific process determined by the relative distribution and location of the unpaired electron density and by the steric environment of the residues that are involved.

The failure of zinc sperm whale myoglobin to dimerize when incubated with ferric horse myoglobin and H$_2$O$_2$ established that the heme group is required for dimerization and ruled out significant intermolecular electron transfer because the horse myoglobin protein radical was not transferred to zinc sperm whale myoglobin. This indicates that Tyr-151 is oxidized by intramolecular electron transfer to the heme and implies that Tyr-103, which is closest to the heme (25), is probably the first tyrosine residue to be oxidized. Although the electron transfer path for the oxidation of Tyr-151 has not been defined, the distances between the residues (Fig. 7) would seem to favor electron transfer from Tyr-151 to the Tyr-103 radical. Tyr-146 could also serve as a conduit for the electron transfer because it is located between Tyr-103 and Tyr-151. It is clear, however, that the electron transfer from Tyr-151 to the heme occurs readily in the absence of Tyr-103 and/or Tyr-146 because dimerization is observed with the Y103F and Y146F mutants, and with the double mutant lacking both Tyr-103 and Tyr-146. The electron transfer rate may be altered by the mutations but is nevertheless sufficiently rapid to permit the chemical behavior of the protein to remain unaltered.

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*Fig. 6.* SDS-PAGE analysis of the cross-linking of wild type and mutant sperm whale myoglobins. *Top panel* (lanes from left to right): 1, molecular weight standards; 2, ferric myoglobin (pMb221); 3, ferric myoglobin (pMb221) + H$_2$O$_2$; 4, ferric Tyr-103 mutant; 5, ferric Tyr-103 mutant + H$_2$O$_2$; 6, ferric Tyr-146 mutant; 7, ferric Tyr-146 mutant + H$_2$O$_2$; 8, ferric Tyr-151 mutant; 9, ferric Tyr-151 mutant + H$_2$O$_2$; 10, ferric Tyr-103/146 mutant; 11, ferric Tyr-103/151 mutant; 12, ferric Tyr-103/146/151 mutant + H$_2$O$_2$. Incubations were carried out as described under "Materials and Methods."

*Fig. 7.* Spatial relationships between Tyr-103, Tyr-146, Tyr-151, and the prosthetic heme group (40). 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 Å, 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. The closest ring atom of Tyr-103 is 3.32 Å from the terminal carbon atom of the heme vinyl group.
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