Specific Azidophenyldiazene Hemoprotein Active Site Probes. Cross-Linking of the Heme to His-64 in Myoglobin

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Abstract: The reaction of myoglobin (Mb) with (*meta*- and (*para*-azidophenyl)diazene yields the corresponding σ-bonded *meta*- or *para*-phenyl−iron complex. Aerobic denaturation of these complexes in the dark yields *N*-(azidophenyl)protoporphyrin IX adducts. Photolysis of either the *meta*- or *para*-azidophenyl−iron complex of Mb prior to denaturation results in cross-linking of the protein to the prosthetic group via a linker formally composed of a phenyl group and a nitrogen atom. Tryptic digestion of the modified protein and mass spectrometric analysis of the peptides identifies His-64 as the residue to which the heme is attached by both probes. Photolysis of the azidophenyl−iron complexes is postulated to produce an iron-bound arylnitrene that binds directly, or after rearrangement to a seven-membered cyclic ketenimine, to the protein. Subsequent shift of the aryl group from the iron to a porphyrin nitrogen generates the heme−protein cross-link. This approach unambiguously identifies hemoprotein active site residues and defines their location with respect to the heme iron atom. This approach should prove useful in characterizing the active sites of structurally undefined hemoproteins because aryl−iron complex formation is a general hemoprotein reaction.

The development of methodologies that can be used to characterize the active sites of hemoproteins for which no crystal structures are available is highly desirable because of the physiological importance of such proteins. Among the structurally uncharacterized hemoproteins are the membrane-bound P450 enzymes, including all the sterol biosynthetic and drug metabolizing mammalian isoforms, the nitric oxide synthases,[1] and guanylate cyclase. We describe here a novel approach that not only unambiguously identifies active site residues but makes it possible to define their location with respect to the heme iron atom in the intact protein.

The reaction of hemoproteins with aryldiazenes to give σ-bonded aryl−iron complexes is demonstrated by the reactions of several proteins, including hemoglobin,[2,3] myoglobin (Mb),[3] chloroperoxidase,[4] the nitric oxide synthases,[5] and a variety of cytochrome P450 enzymes,[6] with phenyl-, 2-naphthyl-, and *p*-biphenyldiazene. The high-resolution crystal structures of the phenyl−iron complexes formed with Mb and $P450_{cam}$ (CYP101) show that the phenyl moiety is bound to the iron edge-on, as required for a σ-bonded complex.[7,8] Evidence for a σ-bonded aryl−iron complex has also been obtained for the myoglobin complex by ^{1}H NMR.[3] The Mb and $P450_{cam}$ aryl−iron complexes, and most of the complexes that have been examined, are stable in the absence of protein denaturation, although one or two complexes have been found to be thermally unstable.[9]

If the hemoprotein aryl−iron complexes are denatured in acidic solution under aerobic conditions, the aryl group migrates from the iron to the nitrogens of the porphyrin ligand.[2,10] Due to asymmetric substitution of the porphyrin, the four pyrrole nitrogens are distinct and the corresponding *N*-aryl adducts can be separated by HPLC.[10] If the migration occurs when the aryl−iron complex is free in solution, there is little or no preference for any of the nitrogens and comparable amounts of all four *N*-phenylprotoporphyrin IX isomers are formed. However, it is possible to promote the aryl−iron shift within the intact active site by oxidizing the hemoprotein complex with ferricyanide.[9,11] The aryl group shift only occurs under these conditions if the fifth heme ligand is a thiolate, as it is in the cytochrome P450 enzymes and nitric oxide synthases.[1,12] Under these conditions, the regioselectivity of the aryl group migration is controlled by the protein active site topology. Analysis of the *N*-arylprotoporphyrin IX pattern when the shift occurs within the protein can be used to construct a rough model of the active site topology.[6] The topological information thus obtained is limited by the fact that it cannot be merged with protein sequence information to produce a sequence-linked active site

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(1) A crystal structure of the heme domain of eNOS has been reported, but the protein fragment does not bind the normal cofactors or substrate and is not catalytically active and further evidence relative to the solution structure is highly desirable: Crane, B. R.; Arvai, A. S.; Gachhiu, R.; Wu, C.; Ghosh, D. K.; Getzoff, E. D.; Stuehr, D. J.; Tainer, J. A. *Science* **1997**, *278*, 425−431.

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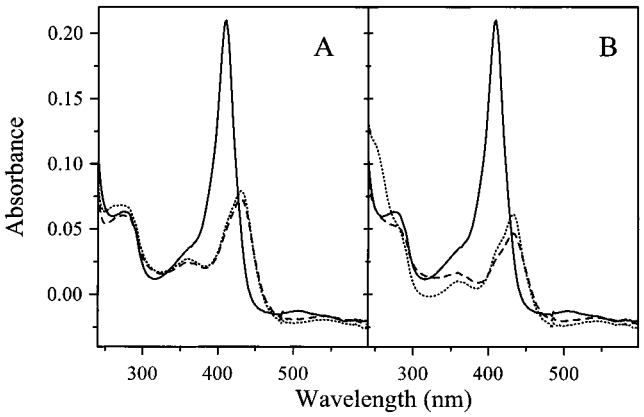

Figure 1. UV spectrum of native Mb (—), the Mb Fe—aryl complex (- - -), and the Mb Fe—aryl complex after photolysis (— —): (A) complex formed with (*meta*-azidophenyl)diazene and (B) complex formed with (*para*-azidophenyl)diazene.

model. This is a particularly vexing limitation in the case of the P450 enzymes because information on possible active site residues can be obtained by aligning the sequence of the target P450 with those of the soluble bacterial isoforms for which crystal structures are available.[13−15]

To overcome this limitation, we have sought to prepare aryldiazene probes that bear a photoactivatable group that could be used to cross-link the aryl group to the protein after formation of the aryl—iron complex. Subsequent shift of the aryl group from the iron to the porphyrin nitrogens would then attach the heme chromophore to the labeled protein residue, simplifying its isolation and identification. A major advantage of this approach over the simple use of reactive substrate analogues, whether the reactive function is unmasked by light or by catalytic turnover of the probe, is that the probe is fixed to the iron in a geometrically defined manner during the cross-linking process. This eliminates any ambiguity relative to whether the reaction occurs in the active site and, more importantly, makes it possible to define the approximate location of the alkylated residue with respect to the anchoring iron atom. The development of two such aryldiazene probes, and validation of the approach by their successful use with a hemoprotein (Mb) of known structure, are reported here.

Results

Iron—Aryl Complex Formation and Photolysis. Both (*meta*-azidophenyl)diazene and (*para*-azidophenyl)diazene react with ferric sperm whale Mb to form stable σ-bonded Fe—aryl complexes with absorbance maxima at 430 and 432 nm, respectively (Figure 1). Examination of the spectra of the complexes after photolysis with a mercury lamp showed at most a small loss of the absorbance at 430 nm, indicating that the carbon—iron bond in the complexes is stable under photolysis conditions (Figure 1). The photolyzed complexes were then denatured aerobically in acidic acetonitrile, conditions that promote migration of the carbon ligand from the iron to the porphyrin nitrogens.

Following the oxidative shift, the samples were analyzed by HPLC with the effluent monitored simultaneously at 214 and 416 nm (Figure 2). Panel A shows the HPLC profile of control

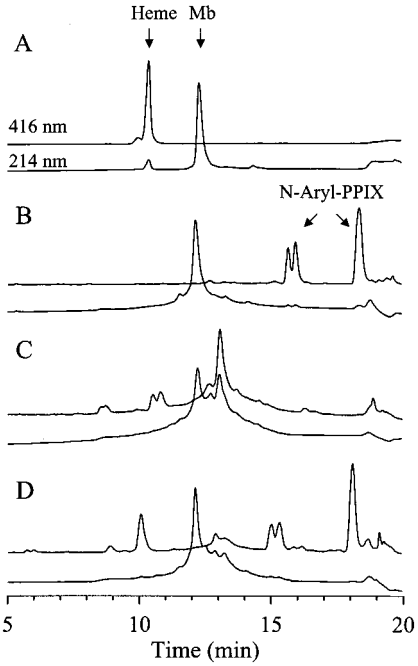

Figure 2. HPLC profile of (A) Mb, (B) Mb after reaction with (*meta*-azidophenyl)diazene but without photolysis, (C) Mb after reaction with (*meta*-azidophenyl)diazene and photolysis, and (D) Mb after reaction with (*para*-azidophenyl)diazene and photolysis. The HPLC separation was done on a Vydac C-4 reverse phase column eluted at 1.5 mL min^{-1} with a gradient of solvent B (acetonitrile/trifluoroacetic acid 0.1%) into solvent A (water/trifluoroacetic acid 0.1%): 0—1 min 35% B, 1—16 min 35—48% B, 16—17 min 48—80% B. The traces obtained at both 214 and 416 nm are shown. The peaks due to intact Mb, heme, and the *N*-arylprotoporphyrin IX adducts are labeled.

Mb, and panel B, that of the unphotolyzed iron *meta*-azidophenyl complex. The trace at 416 nm in these two panels shows that all of the heme of Mb, after formation of the *meta*-azidophenyl complex, is converted during the sample workup to the *N*-(*meta*-azidophenyl)protoporphyrin IX adducts. This is confirmed by the absorption maximum at 416 nm and retention times for the adducts similar to those for the authentic *N*-phenyl protoporphyrin IX adducts. Photolysis of the *meta*-azidophenyl complex prior to HPLC denaturation and HPLC analysis causes substantial modification of the protein and almost complete disappearance of "free" *N*-aryl PPIX adducts (panel C). The chromatogram monitored at 214 nm indicates that the modified protein, which appears as two distinct peaks, has a longer retention time than the unmodified protein. Correlation of the chromatograms monitored at 416 and 214 nm indicates that approximately 50% of the protein is labeled. The UV spectrum of the HPLC purified protein from panel C has an absorbance maximum at 416 nm, a value typical for an *N*-arylprotoporphyrin IX adduct (Figure 3).[10]

The HPLC profile obtained with the unphotolyzed iron—*para*-azidophenyl complex (not shown) is essentially identical to that of the unphotolyzed *meta*-azidophenyl complex (Figure 2, panel B). The only peaks with significant absorbance at 416 nm that are found in the chromatogram are those assigned to the isomeric *N*-(*para*-azidophenyl)protoporphyrin IX adducts. Photolysis of the iron complex again results in modification of the protein, but the *para*-azidophenyl ligand is a less effective myoglobin alkylating reagent and no more than 10% of the protein is covalently cross-linked to the heme chromophore upon photolysis of the iron complex (panel D). The modified protein is found in the chromatogram as a mixture of at least three peaks, of which only two have absorbance at 416 nm.

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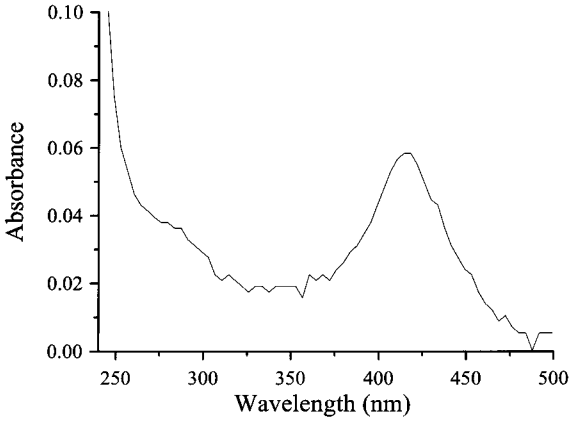

Figure 3. Absorption spectrum of Mb after photolysis of the complex formed with (*meta*-azidophenyl)diazene.

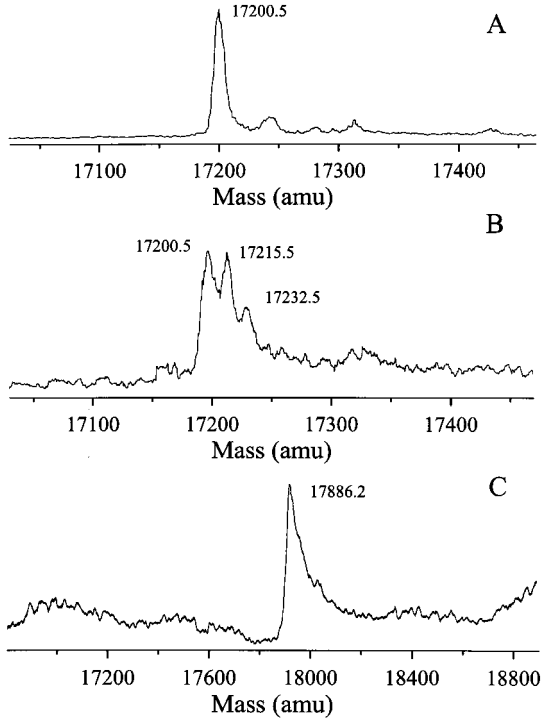

Figure 4. Mass spectrum of Mb before and after covalent modification with (*meta*-azidophenyl)diazene: (panel A) mass spectrum of unmodified Mb; (panel B) the deconvoluted mass spectrum of Mb after formation of the aryl−iron complex with (*meta*-azidophenyl)diazene but before photolysis to cross-link the heme to the protein; (panel C) the deconvoluted mass spectrum of Mb covalently modified by photolysis of the aryl−iron complex formed by reaction with (*meta*-azidophenyl)diazene.

Mass Spectrometric Analysis of (*meta*-Azidophenyl)diazene-Labeled Mb. Unmodified Mb and the HPLC-purified cross-linked protein were subjected to electrospray ionization mass spectrometric (ESIMS) analysis. The analysis of unmodified Mb, as expected from its calculated molecular mass of 17 200 Da, gave an average molecular mass of 17 200 (Figure 4, trace A). In contrast, the protein after reaction with (*meta*-azidophenyl)diazene and photolysis of the resulting aryl−iron complex exhibited a molecular mass that was 684 Da larger than that of the unmodified protein (Figure 4, trace C). Covalent modification of the protein by the heme plus the phenyl (C_6H_4) ring plus one nitrogen would increase the molecular weight by 651 Da (561 + 76 + 14 Da). Similar analysis of the protein after formation of the aryl−iron complex but *before* the aryl probe was photoactivated shows that the reaction with the aryldiazene yields a protein with a molecular mass 16 or 32 units higher than that of native Mb (Figure 4, trace B). These increases in the mass of the "unmodified" protein are tentatively assigned to the oxidation of one or two methionine residues, presumably by H_2O_2 generated by the autoxidation of the aryldiazene. The discrepancy of 32 Da in the mass of the protein after photolytic cross-linking of the heme is accounted for by the two methionines that are oxidized in forming the aryl−iron complex.

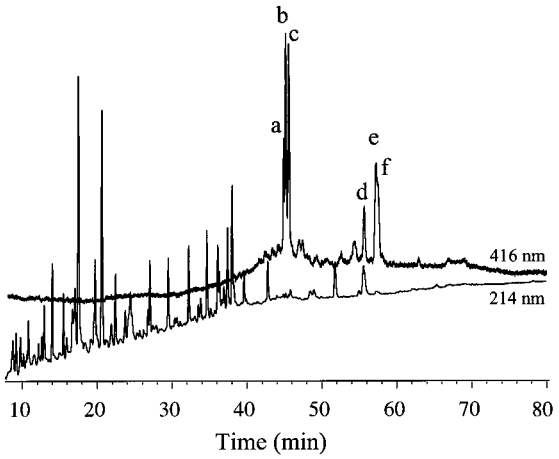

Figure 5. HPLC profile of the tryptic fragment of Mb modified by photolysis of the aryl−iron complex formed with (*meta*-azidophenyl)-diazene (trypsin 5/100 (w/w), reaction time 120 min). The peaks detected at 214 and 416 nm are shown.

HPLC Purification and Identification of Modified Tryptic Peptides. The tryptic digest of (*meta*-azidophenyl)diazene-cross-linked Mb was analyzed by HPLC while monitoring the column effluent at 214 and 416 nm (Figure 5). From the trace at 416 nm 6 major modified peptides (a−f) were isolated and subjected to MALDI mass spectrometry. Monoisotopic molecular weights determined in these experiments for the *meta*-substituted phenyl group were 2048.3 (a−c) and 2815.6 (d−f). This latter MH^+ represents the covalently modified tryptic peptide Ala^{57}-Lys^{77} with the attached probe fragment and porphyrin, while the lower molecular mass species corresponds to modified peptide Ala^{57}-Leu^{69}, which derives from the larger peptide via a nonspecific cleavage between Leu-69 and Thr-70. Both modified peptides were subjected to PSD analysis. Figure 6 shows the PSD spectrum of the longer peptide. The modified peptide gives abundant fragments containing the heme structure at *m*/*z* 564 (heme itself), 654 (heme + linker: Figure 7), 682, 721, 735, and 762. The values for these masses may be slightly off because the most abundant fragments are not focused even under PSD conditions. Some of these ions, such as 654 and 682, were also observed as prompt (formed upon ionization) fragments in the MALDI spectra of the fractions containing the modified species. Peptide backbone fragment $\mathbf{b_4}$ [for nomenclature, see ref 16] with charge retention at the N-terminus was detected at *m*/*z* 403 suggesting that the first four amino acids are not modified. Other N-terminal fragment ions were observed starting from $\mathbf{b_8}$ (formed via peptide bond cleavage after the His residue), and all of them showed the 561 Da shift in comparison to the nonmodified species. Similarly, ions with charge retention at the C-terminus were observed starting with $\mathbf{y_{14}}$ (formed via peptide bond cleavage at the N-terminus of the histidine; fragments are numbered from the

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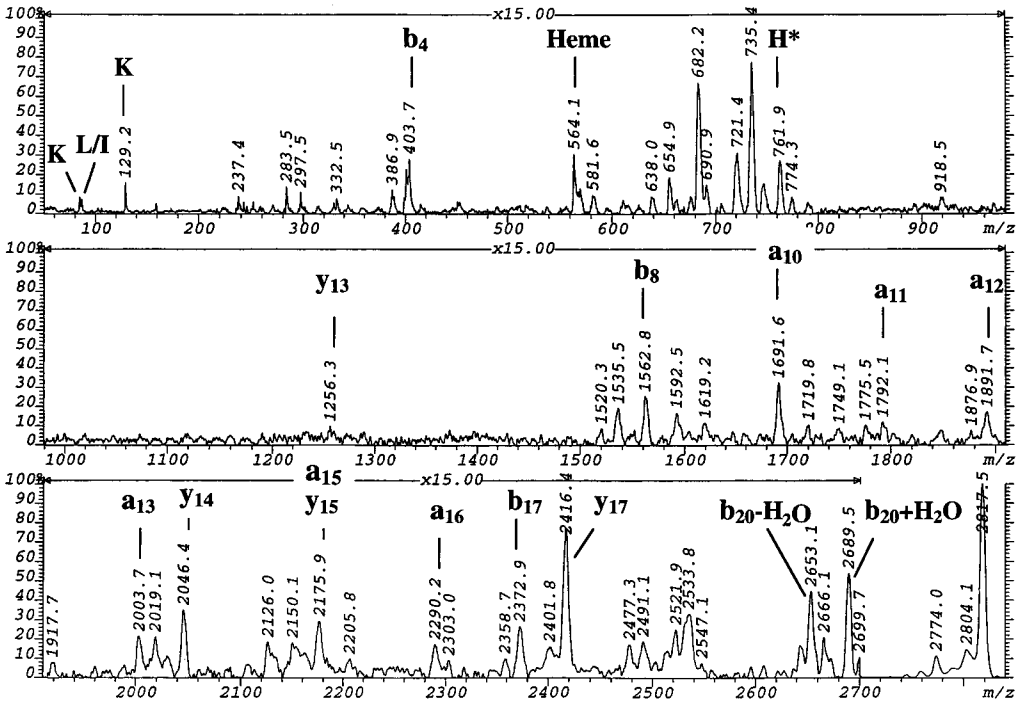

Figure 6. Postsource decay (PSD) mass spectrum of the labeled tryptic peptide Ala[57]-Lys[77]. The peak nomenclature is taken from the literature.[16] The peak labeled H* is due to the imminium ion from the His-64 plus the probe plus the porphyrin.

corresponding termini), and each of them indicated the presence of the covalent modification. Thus, fragmentation of the peptide backbone indicated that His-64 was covalently modified. In addition, His residues usually yield an abundant immonium ion at *m*/*z* 110 that was not observed in this spectrum. Instead, the modified His gave an immonium ion at *m*/*z* 762.

The tryptic digest of (*para*-azidophenyl)diazene-cross-linked Mb gave one principal modified peptide (a) (Figure 8). Partial tryptic digestion gave 3 additional peptides (b−d) (insert). The peptides were subjected to mass spectrometric analysis and gave MH^+ at *m*/*z* 1404.6 (a), 2944.0 (b), 2815.3 (c), and 2172.5 (d) (monoisotopic masses). These molecular masses correspond to the modified sequences Lys[63]-Leu[69], Ala[57]-Lys[78], Ala[57]-Lys[77], and either Lys[63]-Lys[77] or His[64]-Lys[78], respectively. The calculated MH^+ values for these species are 1404.8, 2943.7, 2815.6, and 2172.2 Da, respectively. These peptides were also subjected to PSD analysis, but the PSD spectra were much less informative than those obtained from peptides with the *meta*-substituted label. The most abundant fragments in each spectrum represent the heme itself (*m*/*z* 563) and the heme plus the linker (*m*/*z* 654). The peptide fragments are virtually absent from the spectra. Only the PSD spectrum of the longest peptide featured a few backbone fragments (**b$_4$** at *m*/*z* 403, **y$_{14}$** at *m*/*z* 1385, **a$_8$** at *m*/*z* 1533, and **y$_{18}$** at *m*/*z* 2543) that helped to identify His-64 as the site of modification. The PSD spectra of all the labeled peptides also included an ion at *m*/*z* 734 (see Figure 6) that was assigned to the modified His side-chain, cleaved between the α and β carbons of the amino acid (Figure 7).

All peptides resulting from modification with the *meta*-azido probe included ion source fragments at *m*/*z* 563.3, 654.3, 681.3, and 732.3, whereas the peptides from modification with the *para*-azido probe did not exhibit the fragments at *m*/*z* 563 and 681. The *m*/*z* 732 fragment indicates that all peptides are modified at His-64, but the absence of the *m*/*z* 681 fragment in the *para*-azido-modified peptides suggests structural differences in the nature of the cross-link formed with the two probes.

Discussion

(*meta*- and (*para*-azidophenyl)diazene have several advantages for the characterization of hemoprotein active sites. First, reaction of aryldiazenes with hemoprotein iron atoms is a general reaction that occurs with the P450 enzymes, the three nitric oxide synthase isoforms, and a diversity of other hemoproteins.[5,6] Aryl−iron complexes are not formed with hemoproteins that have two fixed iron ligands or have a highly constrained active site, but the reaction is sufficiently general that the approach described here has broad potential scope. Second, the aryldiazene reaction produces complexes with an aryl−iron bond that is stable to the conditions required to photoactivate the azide function. The azide group can therefore be photoactivated without breaking the carbon−iron bond, a feature of the reaction that ensures that the residues that are labeled are within the active site. Third, the geometry of the aryl ring with respect to the iron and its porphyrin ligand, as shown by the crystal structures of the Mb and $P450_{cam}$ phenyl−iron complexes,[7,8] is fixed. In the case of the symmetric *para*-azidophenyl ligand, the azide group is located directly above the iron atom at a distance of 6.8 Å. In the case of the *meta*-azidophenyl probe, the asymmetry of the ligand allows a greater latitude in the position of the azide function. If the *meta*-azidophenyl group is rotated about the carbon−iron bond, the azide group will describe a circle of 2.2 Å radius at an altitude of 5.7 Å above the heme plane. Thus, even with the *meta*-azidophenyl probe, the location of the azido function, and the nitrene derived from it, can be defined with some precision. Consequently, the location of the amino acids that react with the photoactivated azide function can be defined with respect to the heme plane. Finally, the resistance of the carbon−iron bond to the photolysis conditions means that the phenyl group is rigidly held during the photolysis process but can be shifted to the porphyrin nitrogens after the protein cross-linking event. This shift attaches a strong chromophore to the labeled amino acid that can be used to isolate and identify it.

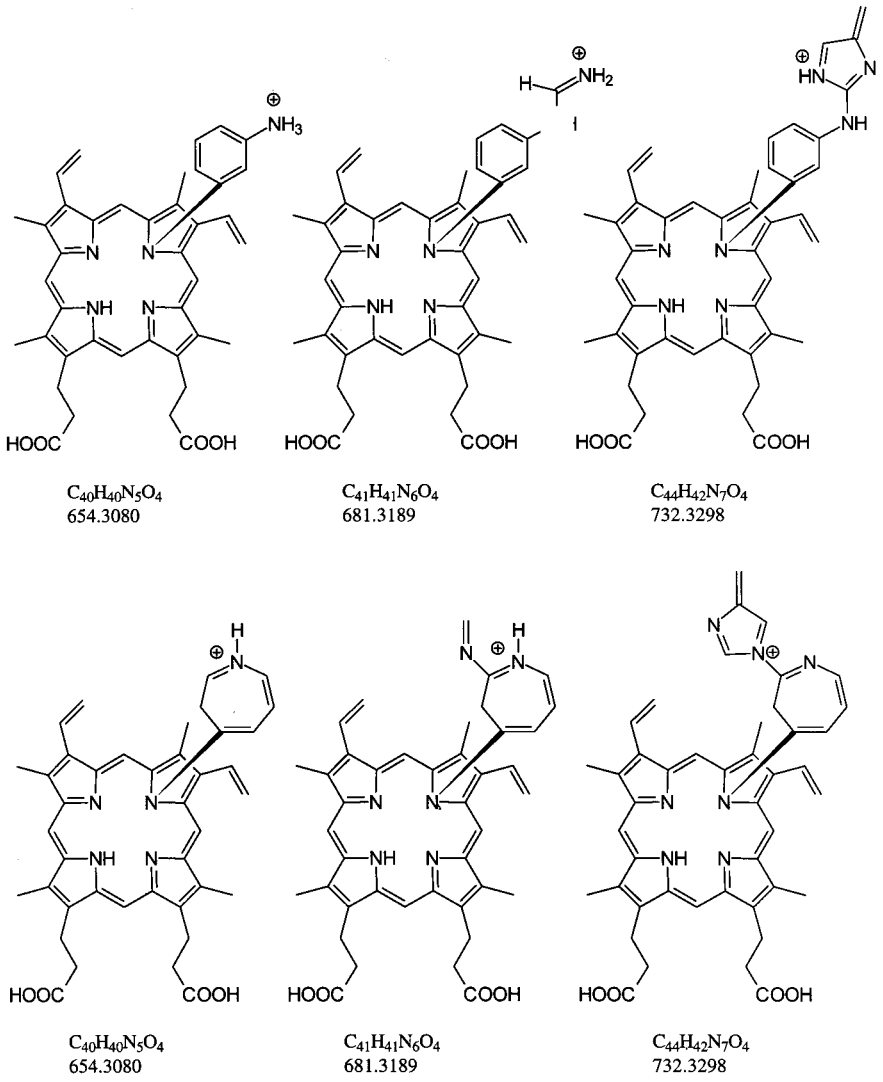

Figure 7. Possible structures of the heme-bearing fragments obtained in the mass spectrometric fragmentation of Mb cross-linked to the heme with the *meta*-azidophenyl probe.

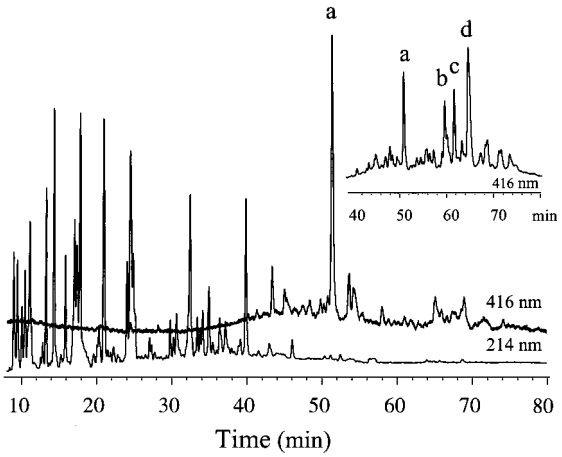

Figure 8. HPLC profile of the tryptic fragment of Mb modified by photolysis of the iron−*para*-azidophenyl complex (trypsin 3/100, reaction time 120 min). The peptide profiles detected at 214 and 416 nm are shown. In the insert, the tryptic peptide pattern detected at 416 nm after digestion of the modified Mb with trypsin 2/100 (reaction time of 90 min) is shown.

Photolysis of the *meta*- and *para*-azidophenyl−iron Mb complexes results in covalent attachment of the probe and the appended heme group to His-64. The covalent link may arise by direct reaction of His-64 with the photolytically generated nitrene function. However, arylnitrenes undergo a competing ring expansion to give a seven membered ring with a highly strained keteneimine function (Figure 9).[17] The observed alkylation reaction therefore could also result from addition of His-64 to the keteneimine (Figure 10). The relative rates of reaction of the nitrene with protein functional groups versus the ring expansion reaction will determine the nature of the bond between the probe and His-64. If the nitrene rearrangement is faster than reaction with protein residues, His-64 will be

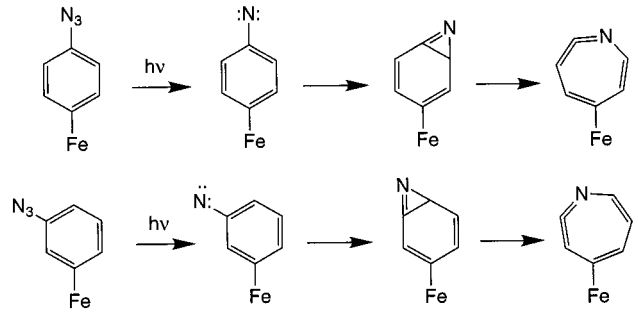

Figure 9. Reaction sequences that convert the azidophenyl iron-bound ligands to the corresponding seven-membered cyclic keteneimines.

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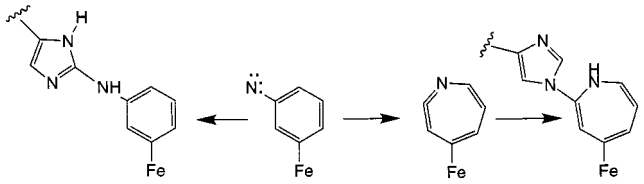

Figure 10. Alkylation of His-64 by the nitrene and keteneimine forms of the photolyzed *meta*-azidophenyl−iron probe.

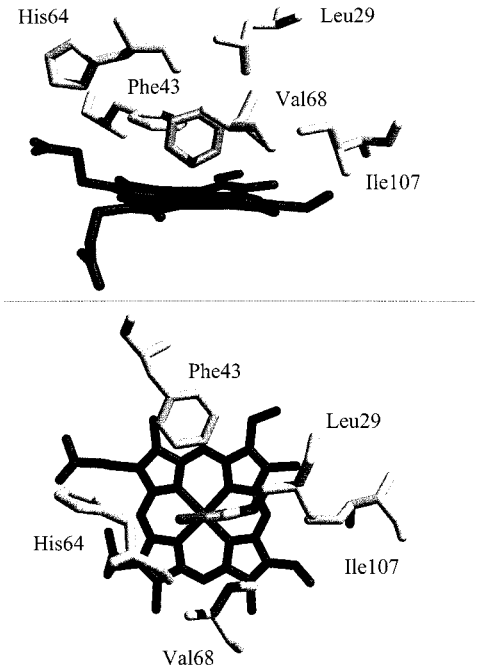

Figure 11. Side and top views of the active site of the Mb Fe−phenyl complex.[7] The heme group, iron-bound phenyl, and several active site residues are shown (side view and top view).

alkylated by the keteneimine. The nature of the bond between the probe and His-64 is not defined by this study because the mass spectrometric fragmentation patterns are consistent with both cross-linking mechanisms (Figure 7).

The finding that His-64 is the alkylated residue with both the *para*- and *meta*-azidophenyl probes is readily rationalized. The position of the reactive functionality, either the nitrene or the keteneimine, with respect to the His-64 imidazole ring does not differ greatly for the two probes. Reaction with the imidazole of His-64 is the only reaction consistent with the active site structure if the alkylation reaction occurs with the keteneimine because only a nucleophilic residue can participate in this reaction. The crystal structure of the phenyl−iron complex shows that the iron-bound phenyl group is in contact with the side chains of His-64, Phe-43, Val-68, Leu-29, and Ile-107 (Figure 11). Of these side chains, only that of His-64 has the required nucleophilic properties. In contrast, the unrearranged nitrene is sufficiently reactive to insert into activated C−H bonds or to add to aromatic π-bonds. Thus, the nitrene could potentially react with any of the residues in contact with it. His-64 in the crystal structure of the phenyl−iron complex is displaced outward, much like an open gate, to make room for the phenyl group (Figure 11).[7] However, in solution, the dynamic nature of the protein can be expected to swing the imidazole group of His-64 toward its closed position, a motion that will bring it into close contact with the iron-bound aryl ligand. Simple modeling experiments suggest that the imidazole could react with the reactive functionality generated from either the *para*- or *meta*-azidophenyl moiety (Figure 10). In the case of the *meta*-azidophenyl group, the nitrene and the corresponding keteneimines are optimally positioned for reaction with His-64. With the *para*-azidophenyl probe, the nitrene is suboptimally positioned, but rearrangement to the keteneimine improves the position of the reactive group. One explanation for the lower yield of cross-linked products obtained with the *para*-azido function is that the resulting activated group is suboptimally located for reaction with His-64. Predominant alkylation of His-64 is most consistent with, but does not require, a keteneimine mechanism.

The shift of a σ-bonded aryl group from the iron to the porphyrin nitrogens under acidic, aerobic conditions is well established.[6,10] However, if photolysis of the arylnitrene ligand results in its expansion to a seven member cyclic keteneimine, the iron-bound ligand is no longer an aromatic ring. There is little information on the shift of a vinylic carbon from the iron to the porphyrin nitrogens. The only direct precedent is the demonstration that the 2,2-diphenylvinyl ($Ph_2C{=}CH-$) group bound to the iron in a tetraphenylporphyrin complex readily shifts to a nitrogen of the porphyrin when the complex in organic solution is oxidized with ferric chloride.[18] The absorption spectrum of myoglobin cross-linked with the *meta*-azidophenyl probe has an absorption maximum similar characteristic of an *N*-arylporphyrin, but this spectrum is unlikely to differ significantly for the *N*-vinyl adduct.

The presence of three or more protein peaks after photolysis indicates that the protein is multiply modified (Figure 2), but heme labeling is only significantly associated with one or two of these peaks. This protein multiplicity may be due to intramolecular or intermolecular modifications independent of the probe, or the probe in solution may become attached to additional sites on the protein. However, a strength of this approach is that only the heme-labeled residues need to be identified. Digestion of the protein labeled with the *meta*-azido probe yields two sets of three peptides, whereas only one peptide is obtained from the protein labeled with the *para*-azido probe (Figures 5 and 8). Two sets of peptides are obtained from the *meta*-azido-labeled protein due to hydrolysis of the same protein sequence at two different sites. In view of the fact that the molecular ions and mass spectrometric fragmentation patterns are the same for the three peptides in each set, it is likely that the secondary peptide multiplicity is due to the formation of isomeric *N*-aryl- or *N*-alkylporphyrins during shift of the protein-linked aryl or heterocyclic vinyl group to the porphyrin nitrogens. These isomeric peptide adducts, like the protein-free (*N*-azidophenyl)protoporphyrin IX adducts (Figure 2), may be partially resolved by HPLC. An alternative but less likely explanation is that HPLC-separable porphyrin−protein adducts are obtained by reaction of the protein with both the nitrene and ketenimine reactive groups. In the case of the *para*-azido probe, the cross-linking process appears to yield a single adduct (Figure 8).

The present results demonstrate that photolysis of iron−azidophenyl hemoprotein complexes formed in the reaction with (*para*- or (*meta*-azidophenyl)diazene can be used to cross-link the heme to one or more specific active site residues. This approach, which not only ensures that the labeled residue is in the active site but defines its location with respect to the heme iron atom, should be of considerable utility in characterizing the active sites of hemoproteins for which crystal structures are not available.

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Experimental Procedures

Materials. Ethyl (*meta*-azidophenyl)diazenecarboxylate and ethyl (*para*-azidophenyl)diazenecarboxylate esters were prepared as described elsewhere (Tschirret-Guth, R. A.; Ortiz de Montellano, P. R. Manuscript in preparation). Sperm whale Mb and TPCK treated trypsin were purchased from Sigma.

Iron Complex Formation and Heme−Protein Cross-Linking. Typically, the probes (1 mg) were dissolved in 50 μL of methanol and hydrolyzed to the active diazene by addition of 5 μL of 2 N NaOH. The aryl−iron complexes were formed by adding 1−2 μL of the solution of the probe to 1 mL of a 2 mg/mL solution of Mb in 100 mM sodium phosphate, pH 7.4. Complex formation was monitored by UV−visible spectroscopy. Irradiation of the aryl−iron complexes was performed at 25 °C for 2 min using an Oriel 6035 low-pressure Hg (Ar) pen lamp. The sample was placed in a 0.4 mL (1 mm path length) quartz cuvette and clamped 3 cm from the lamp. Following irradiation 1 mL of the mixture was poured into 16 mL of acetonitrile/trifluoroacetic acid (95:5), and the resulting mixture was kept at 4 °C in the dark for 16 h. The mixture was then evaporated to dryness, and the residue was resuspended in 200 μL of water/acetonitrile/trifluoroacetic acid (70:30:1). The cross-linked Mb was separated from unmodified Mb by HPLC on a Vydac C-4 reverse phase column eluted at 1.5 mL min^{-1} with a gradient of solvent B (acetonitrile/trifluoroacetic acid 0.1%) into solvent A (water/trifluoroacetic acid 0.1%): 0−1 min 35% B, 1−86 min 35−48% B, 86−87 min 48−80% B.

Mass Spectrometric Analysis of Cross-Linked Mb. The modified Mb was analyzed by electrospray ionization mass spectrometry performed on a Sciex API 300 triple quadrupole mass spectrometer. A sample of Mb treated as above but, in the absence of the probe, was used as a control. The HPLC-purified proteins were analyzed in a 50:50 mixture of water/acetonitrile containing 1% acetic acid.

Tryptic Digestion and HPLC Purification of Peptides. HPLC purified modified Mb was lyophilized and resuspended in 200 μL of 50 mM NH_4HCO_3 containing 10% acetonitrile. Trypsin was then added to a final ratio of 1/100 to 5/100 (w/w) and the mixture incubated at 37 °C for 1−2 h. The digestion was stopped by injecting the mixture directly onto the HPLC for purification of the resulting peptides. HPLC purification of the tryptic peptides was performed on a Vydac C-18 reverse phase column eluted at a flow rate of 1.5 mL min^{-1} with a gradient of solvent B into solvent A: 0−1 min 5% B, 1−40 min 5−35% B, 40−80 min 35−45% B.

Mass Spectrometric Characterization of the Isolated Tryptic Peptides. MALDI (matrix-assisted laser desorption ionization) and MALDI-post-source decay (PSD) experiments were carried out on a Micromass TofSpec SE MALDI-TOF time-of-flight mass spectrometer, equipped with a nitrogen laser and operated in reflectron mode, or on a Perseptive Biosystems Voyager Elite mass spectrometer operated in linear or reflectron mode with delayed extraction. The matrix used for these experiments was α-cyano-4-hydroxycinnamic acid (Hewlett-Packard, Palo Alto, CA). The BioRad CZE standard peptide mixture was used for external two point calibration. Precursor ion gating was employed to selectively transmit an individual peptide and its metastable fragment ions to the reflectron for PSD sequencing. This experiment was performed by making 11 steps of the reflectron voltage; at each step the voltage was reduced to 75% of the previous step. Segments from each individual step were then stitched together to produce the complete spectrum. The spectra were smoothed so that partially resolved isotope peaks appear as single "average-mass" peaks. Calibration of each reflectron voltage step was done using the PSD spectrum of ACTH "clip"-peptide.

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