Structure and Absolute Stereochemistry of the Four *N*-Phenylprotoporphyrin IX Regioisomers Isolated from Phenylhydrazine-Treated Myoglobin

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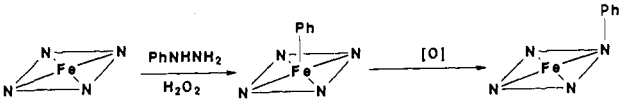
Abstract: The phenyl-iron complex formed in the reaction of phenylhydrazine with equine myoglobin rearranges, in the presence of acid and oxygen, to a mixture of the four possible *N*-phenylprotoporphyrin IX regioisomers. The four isomers have been separated by HPLC, and the pyrrole ring bearing the *N*-phenyl group in each of them has been identified by ^{1}H NMR spectroscopy. The isomers are chiral, and their circular dichroism spectra have been determined. The four *N*-phenyl isomers are formed in equal amounts when the phenyl-iron complex is anaerobically extracted from the protein before the iron-nitrogen shift is promoted by exposure to oxygen and acid. In contrast, when the extraction is carried out aerobically, two of the regioisomers are produced in higher amounts due to steric constraints imposed on the iron-nitrogen shift by the denaturing protein. The two isomers that are preferentially formed are those with the *N*-phenyl on pyrrole rings C and D, the pyrrole rings that face the entry to the heme crevice. The ability to separate and identify the individual regioisomers of *N*-phenylprotoporphyrin IX makes the regiochemistry and chirality of iron-nitrogen migration a powerful probe of the topology of hemoprotein active sites.

Relatively little is known about the active site structures of many hemoproteins, including most cytochrome P450 monooxygenases and dioxygenases like prostaglandin synthase, although spectroscopic studies provide some information on the nature of their active site residues.[1] In some instances, sequence alignments with hemoproteins for which crystal structures are available make possible the construction of very rough three-dimensional models of the undefined proteins.[2-4] The only crystallographic templates available for the construction of cytochrome P450 and peroxidase models, respectively, are cytochrome $P450_{cam}$[5] and cytochrome c peroxidase.[6] These two proteins are sufficiently atypical, however, that caution must be exercised in their use as structural templates.[7,8] The technical difficulties inherent in the crystallization of membrane-bound proteins make it unlikely that crystal structures of the mammalian cytochrome P450 enzymes, prostaglandin synthase, and other membrane-bound hemoproteins will become available in the near future. Alternative methods are therefore required to define their active site structures.

N-alkylporphyrins derived from the prosthetic heme of cytochrome P450 were isolated some time ago from rats to which linear olefins and acetylenes had been administered.[9,10] The regiochemistry of the *N*-alkylation products led to a proposed topological model for the active site(s) of the inactivated cytochrome P450 isozymes.[11] Unfortunately, the isozymes from which the heme adducts derived in these in vivo experiments cannot be precisely defined. Similarly, administration of ethylhydrazine to rats yielded *N*-ethylprotoporphyrin IX with the same circular dichroism spectrum as the material obtained from ethylhydrazine-treated myoglobin.[12] This identity demonstrates that the heme of at least one microsomal cytochrome P450 enzyme has the same absolute stereochemistry relative to the fifth iron ligand as the heme in myoglobin. In addition to the isozyme ambiguities inherent in the in vivo approach, the application of these methods to other hemoproteins is limited because heme alkylation by olefins and acetylenes is restricted to a small subset of cytochrome P450 isozymes. Furthermore, ethylhydrazine is a very poor N-alkylating agent for heme groups, as illustrated by the fact that alkylhydrazines primarily give meso- rather than N-alkylation products with myoglobin.[13] In contrast, phenylhydrazine reacts with nearly all the catalytic hemoproteins so far examined, including several cytochrome P450 isozymes,[14-16,17] catalase,[18] myoglobin,[19] hemoglobin,[20] and chloroperoxidase[21] to give an iron-phenyl complex. On exposure to strong acid and oxygen, the protein denatures and the phenyl group migrates from the iron to one of the four porphyrin nitrogens (Scheme I). The exception is horseradish peroxidase, which gives a meso adduct

Scheme I. Schematic Outline of the Reaction of a Prosthetic Heme Group with Phenylhydrazine[a]



[a] Heme is abbreviated as a square of pyrrole nitrogens.

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rather than an iron–phenyl complex.[22]

Preliminary studies with mammalian cytochrome P450 enzymes indicate that ferricyanide promotes migration of the phenyl group from the iron to the nitrogens within the *intact* hemoprotein complex. The regiospecificity of the migration would therefore be a highly useful probe of the active site topologies of these and other hemoproteins if the *N*-phenylprotoporphyrin IX regioisomers could be separated and the pyrrole ring bearing the *N*-phenyl group in each one identified. We describe here the chromatographic separation of the four *N*-phenylprotoporphyrin IX regioisomers, identification of the arylated pyrrole nitrogen in each isomer, the chiral properties of the isomers obtained from phenylhydrazine-treated myoglobin, and the regioselectivity of heme N-arylation in equine myoglobin.

Experimental Section

Materials. Horse heart myoglobin was obtained from Sigma. Phenylhydrazine·HCl (recrystallized from ethanol before use) and deuterated (99.96%) $CHCl_3$ were purchased from Aldrich. Zinc acetate dihydrate was purchased from Mallinckrodt. High-pressure liquid chromatography was performed with a Hewlett-Packard 9153C controller, a Hewlett-Packard diode-array detector, and two Beckman Model 110A pumps. Absorption spectra were recorded on a Hewlett-Packard 8450A diode-array spectrophotometer. Mass spectra were obtained on a Kratos MS 50 instrument operating in the positive liquid matrix secondary ion mode using 1% trifluoroacetic acid/thioglycerol as the ionization matrix. Circular dichroism spectra were obtained in $CHCl_3$ on a Jasco 500A instrument interfaced with an IBM XT computer.

Formation of *N*-Phenylprotoporphyrin IX. The four *N*-phenylprotoporphyrin IX regioisomers were obtained by extracting the iron–phenyl complex from phenylhydrazine-treated myoglobin under two different sets of conditions. In one set of experiments, the extraction was carried out aerobically in the presence of strong acid. Typically, 300 mg of myoglobin in 60 mL of water was incubated with 5 mM phenylhydrazine, an amount that results in complete shift of the Soret band from 408 to 430 nm. The solution was then added to 600 mL of 5% (v/v) H_2SO_4 in acetonitrile or water, and the mixture was allowed to sit for 2 h at room temperature or overnight at 4 °C. The resulting green solution (in the case of acetonitrile, after concentration on a rotary evaporator) was combined with 600 mL of 5% (v/v) H_2SO_4 in water, and the final mixture was extracted with ethyl acetate. The combined organic extracts were evaporated to dryness on a rotary evaporator or, if an oil formed, were redissolved in 30 mL of 5% aqueous H_2SO_4, extracted into CH_2Cl_2, and then taken to dryness.

In the second set of experiments, the prosthetic group was extracted anaerobically and was then, in a distinct step, exposed to air and acid. A solution of 500 mg of myoglobin in 300 mL of water was allowed to react with 5 mM phenylhydrazine until the shift in the Soret band was complete.[23] The solution was then made anaerobic by blowing argon over it for 1.5 h before it was acidified to pH 4 with 600 μL of glacial acetic acid. The reddish heme complex was extracted by using a total of 200 mL of argon-saturated butanone containing 0.025% butylated hydroxytoluene. Acetonitrile (200 mL) was then added to the butanone extract followed immediately by 8 mL of 5% aqueous H_2SO_4. The resulting mixture was aerated and was allowed to sit for 2 h at room temperature or overnight at 4 °C. It was then concentrated on a rotary evaporator, and the concentrate was extracted with CH_2Cl_2. Solvent removal yielded the crude *N*-phenylporphyrin mixture.

Separation and Characterization of the *N*-Phenylprotoporphyrin IX Isomers. The *N*-phenylprotoporphyrin IX isomers were purified by chromatography on a Whatman semipreparative (25 × 1 cm) Partisil 10 ODS-3 column. The column effluent was monitored at 410 nm. The column was eluted isocratically for 30 min at 4 mL/min with 85% solvent A (6:4:1 methanol/water/glacial acetic acid) and 15% solvent B (10:1 methanol/glacial acetic acid). The column was flushed at the end of each run with 100% solvent B. Each of the four unesterified isomers was collected separately, and the unresolved areas between the peaks were also collected separately and were rechromatographed on the same system. The purified isomers were rechromatographed a second time to ensure high purity.

Each of the purified *N*-phenylprotoporphyrin IX isomers obtained from a total of 900 mg of myoglobin was esterified by allowing it to sit overnight at 25 °C in 900 mL of 5% (v/v) H_2SO_4 in methanol. Two volumes of water were then added, and the mixture was immediately extracted with CH_2Cl_2. The solvent was removed from the combined extracts at a rotary evaporator. The zinc complexes were made by dissolving the adducts in 2 mL of CH_2Cl_2, adding approximately 100 μL of a 90 mM solution of zinc acetate in methanol, and monitoring the progress of the reaction at 440 nm. The esterified, zinc-complexed adducts were chromatographed on a Whatman Partisil 10 ODS-3 column eluted with 80% solvent A and 20% solvent B at a flow rate of 3.5 mL/min. The detector was set at 430 nm. Each isomer was immediately evaporated to dryness because the zinc complexes are acid labile. The extinction coefficient for the esterified, zinc-complexed *N*-phenylprotoporphyrin IX isomers (in $CHCl_3$) at 442 nm, determined by weighing on a Mettler UM3 microbalance, is 95 000 M^{-1} cm^{-1}.

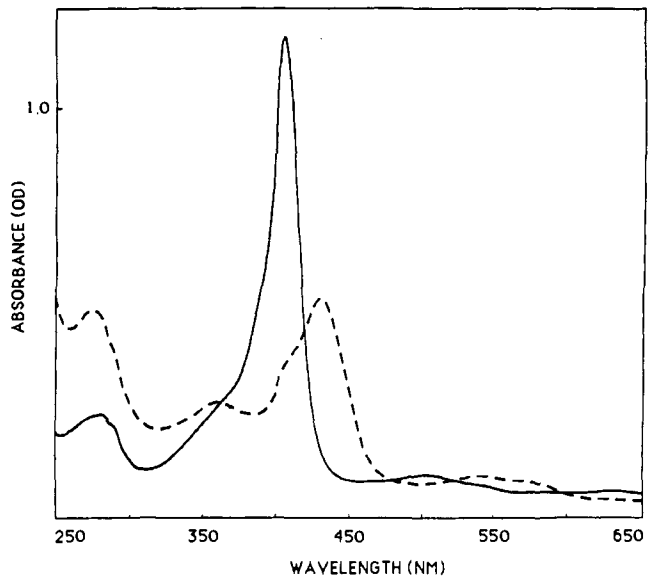

Figure 1. Absorption spectrum in water of equine metmyoglobin before (solid line) and after (dashed line) reaction with phenylhydrazine.

NMR. Two different General Electric GN 500-MHz spectrometers were used for NMR studies. Argon was blown over the samples dissolved in deuterated $CHCl_3$, but the solutions were not degassed.[24] Spectra were obtained with a sweep width of ±3000–3500 Hz in a 16 K block. A 1-Hz line-broadening function was applied to the FID. The conditions required for the nuclear Overhauser experiments varied between the two instruments and with time. Typically, methyl saturation was obtained with a 66-dB decoupler pulse (3 s) and a 10-μs 90° pulse. Internal methylene saturation was achieved with a 69-dB decoupler pulse (4.5–5 s) and a 10- or 12-μs 90° pulse. The internal vinyl protons were saturated with a 68–70-dB pulse (4–4.5 s) and a 12-μs 90° pulse. There was a 1-s delay between each acquisition. The decoupler was set off-resonance at about 9.1 ppm and was not changed within a session. Typically, 64 scans were taken for each of the two NOE spectra in interweaving 8 scan blocks; occasionally up to 192 scans were necessary. The NOE data are the difference between the transformed spectrum and the appropriate reference spectrum. Decoupling experiments were performed with the decoupler set around 75 dB, and used a 12-μs 90° pulse and a 1-ms delay time. Chemical shift values were measured relative to the residual undeuterated $CHCl_3$ signal at 7.25 ppm and are reported in parts per million relative to tetramethylsilane.

Circular Dichroism. Solutions of the dimethyl-esterified, zinc-complexed *N*-phenylprotoporphyrin IX isomers in 2 mL of $CHCl_3$ were washed three times with saturated NaCl solution and were prepared with an optical density of 1.50 at 440 or 442 nm. Circular dichroism spectra were obtained on a Jasco 500A instrument at room temperature in a quartz cell with a 1-cm path length. The spectrum was scanned from 320 to 500 nm with the following instrument parameters: time constant, 8 s; sensitivity, 1 m°/cm; wave expansion, 10 nm/cm.

Results

The aerobic reaction of horse myoglobin with phenylhydrazine results, as previously reported,[19,20,23] in the formation of a phenyl–iron complex that is stable in the absence of protein dena-

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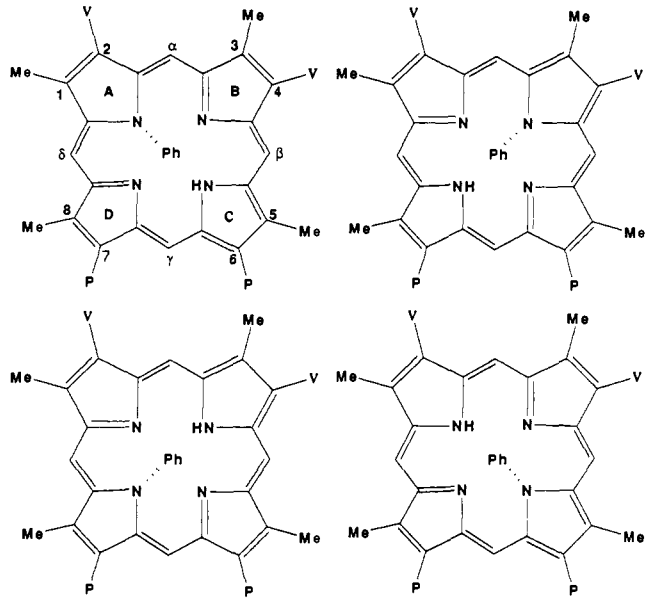

Figure 2. Structures of the four possible regioisomers of *N*-phenylprotoporphyrin IX. The pyrrole rings and the peripheral carbons are labeled in the first structure. The abbreviations for the substituents are V = $-CH{=}CH_2$ and P = $-CH_2CH_2CO_2H$. The correct absolute stereochemistry is shown.

turation (Scheme I). The formation of this complex has been demonstrated by several means, including NMR[23] and X-ray crystallography,[19] to be signalled by a shift of the Soret band from 408 to 430 nm (Figure 1). Extraction of the phenyl–iron complex from the protein with acidic methanol yields, as previously shown for the hemoglobin complex,[20] an unresolvable mixture of the methyl-esterified *N*-phenylprotoporphyrin IX regioisomers (not shown). Extensive efforts to separate the esterified *N*-phenylprotoporphyrin IX regioisomers proved unsuccessful. We have found, however, that the four regioisomers can be completely separated by high-pressure liquid chromatography if the carboxyl groups are not esterified. Thus, acidification and extraction of the prosthetic group from phenylhydrazine-treated myoglobin with butanone under an argon atmosphere in the presence of an antioxidant yield a heme complex with a spectrum identical with that of the iron–phenyl complex previously extracted by the same procedure from phenylhydrazine-treated hemoglobin (not shown).[20] Exposure of the isolated iron–phenyl complex to strong acid and air gives a mixture of the four possible *N*-phenylprotoporphyrin IX regioisomers (Figure 2) that are readily separated by reverse-phase high-pressure liquid chromatography (Figure 3a). As expected,[20] if the anaerobically extracted complex is exposed to air without acidification, the iron–phenyl complex reverts to heme rather than giving the *N*-phenylporphyrin products. The iron–nitrogen shift that takes place with the isolated phenyl–iron complex gives an essentially equimolar mixture of the four possible *N*-phenyl regioisomers (Figure 3a). It is clear from this result that there is no inherent regiochemical preference for the iron–nitrogen migration when it takes place in the absence of a protein matrix.

In contrast, HPLC analysis shows that two of the *N*-phenylprotoporphyrin IX regioisomers are formed in higher amounts than the other two isomers when they are obtained by direct aerobic extraction of phenylhydrazine-treated myoglobin with 5% H_2SO_4 in water (Figure 3b). The proportion of isomers III and IV is higher when the extraction is carried out with this solution than with 5% H_2SO_4 in acetonitrile, but the same pair of regioisomers predominates in both cases. In view of the demonstration that the iron–nitrogen migration of the phenyl group exhibits no inherent regioselectivity, the regioselectivity observed when the heme complex is directly extracted from the protein under aerobic conditions must reflect constraints imposed on the migration by the protein structure. The iron–phenyl shift thus occurs within the protein and, presumably, within the heme crevice

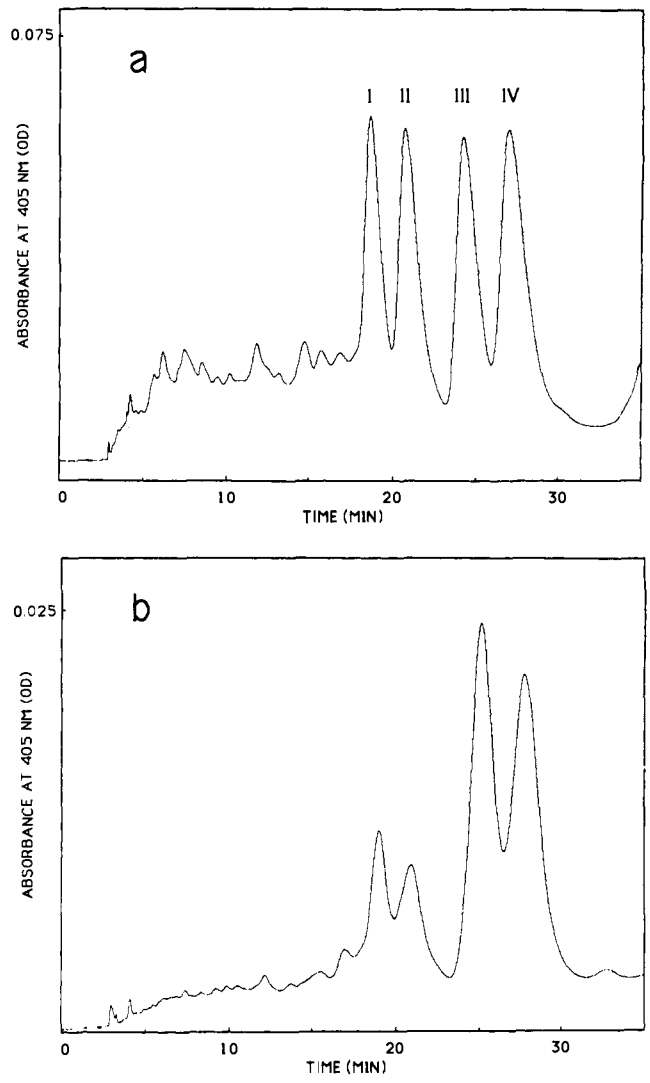

Figure 3. High-pressure liquid chromatogram of the *N*-phenylprotoporphyrin IX regioisomers obtained from (a) anaerobic extraction of the iron–phenyl heme complex from phenylhydrazine-treated myoglobin with acetic acid and argon-saturated butanone prior to aerobic treatment with H_2SO_4/acetonitrile, and (b) direct aerobic extraction of phenylhydrazine-treated myoglobin with 5% aqueous H_2SO_4. Chromatographic conditions are given in the Experimental Section. The isomers are designated I–IV in order of their elution from the column.

of the protein. However, the observation that a variety of oxidizing agents, including $K_3Fe(CN)_6$, H_2O_2, K_2IrCl_6, and Co(III)(phenanthroline)$_3$, do not promote the iron–nitrogen migration in the absence of the protein denaturation caused by strong acid suggests that partial denaturation of the protein matrix is required for the rearrangement to occur. Myoglobin resembles cytochrome $P450_{cam}$[17] in that its iron–phenyl complex is stable so long as the protein matrix is not denatured. In contrast, the iron–phenyl complexes of membrane-bound cytochrome P450 enzymes have been found to readily rearrange to the *N*-phenyl products when treated with ferricyanide under conditions that do not result in denaturation of the protein.[16]

In order to identify the pyrrole nitrogen in each isomer that bears the *N*-phenyl group, the detailed structures of the individual regioisomers have been determined by spectroscopic methods. The mass spectrometric molecular ion of a mixture of the four *N*-phenylprotoporphyrin IX isomers, after methylation of the carboxyl groups to improve their mass spectrometric properties, is at m/z 667. This value corresponds to the sum of protoporphyrin IX dimethyl ester, a phenyl group, and a proton, as expected for the *N*-phenyl adducts. The individual unesterified isomers exhibit nearly identical absorption spectra in chloroform with a Soret band

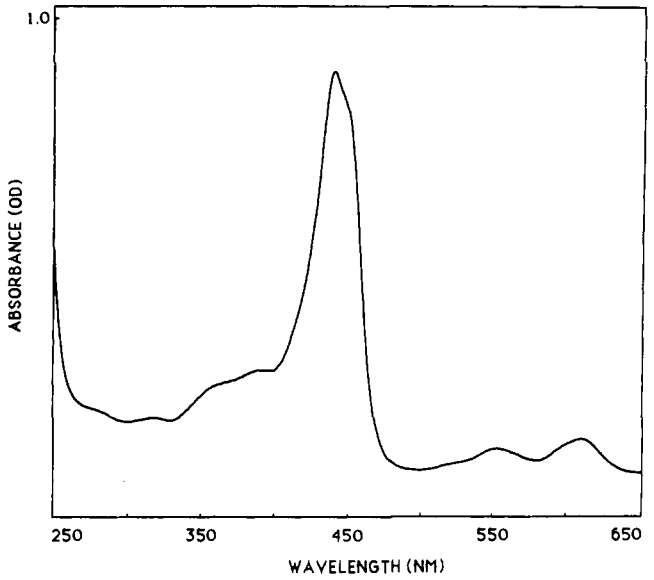

Figure 4. Spectrum in $CHCl_3$ of the zinc complex of the dimethyl ester of isomer II. The slight shoulder on the long-wavelength side of the peak in this isomer is also seen in the spectrum of isomer I but not in those of isomers III and IV.

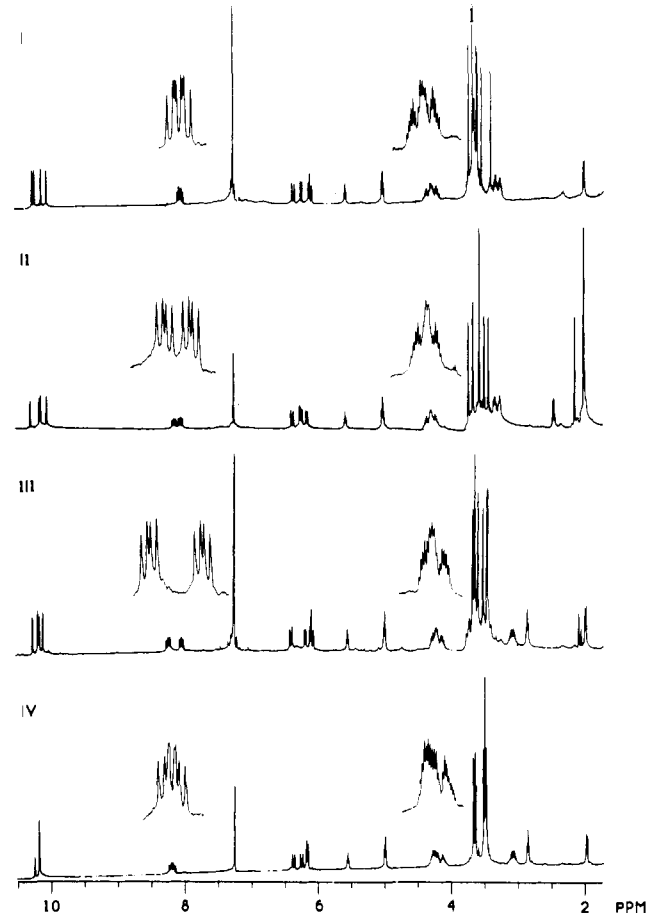

Figure 5. 500-MHz ^1H spectra in deuterated $CHCl_3$ of the four methyl-esterified, zinc-complexed *N*-phenylprotoporphyrin IX regioisomers. The spectra, from top to bottom, are those for isomers I–IV. The 8.0–8.5 and 4.0–4.5 ppm regions of the NMR spectra are shown in expanded form in the insets.

at 412 nm and long-wavelength maxima at 556, 578, 598, and 620 nm. The spectra in $CHCl_3$ of the zinc-complexed *N*-phenylprotoporphyrin IX regioisomers are similar but not identical: isomers I and II, 440, 552, 610, and 656 nm; isomer III, 442, 550, 606, and 650 nm; isomer IV, 444, 550, 606, and 650 nm. The

Table I. NMR Chemical Shifts and Proton Assignments for the Four *N*-Phenylprotoporphyrin IX Regioisomers

	regioisomer			
proton	I	II	III	IV
meso				
α	10.245	10.293	10.269	10.244
β	10.211	10.140	10.190	10.178
γ	10.039	10.051	10.166	10.178
δ	10.111	10.160	10.112	10.178
methyl				
M	3.715	3.725	3.641	3.636
M	3.654	3.657	3.459	3.475
1	3.598	3.560[a]	3.669[b]	3.515
3	3.523[a]	3.488	3.589	3.667[b]
5	3.389	3.560[b]	3.447[a]	3.493
8	3.585[b]	3.423	3.515	3.493[a]
internal vinyl				
2	8.046	8.143	8.236	8.209
4	8.015	8.044	8.036	8.168
external vinyl				
2	6.348	6.368	6.395	6.369
2	6.228	6.248	6.180	6.249
4	6.111	6.235	6.103	6.164
4	6.085	6.148	6.075	6.164
internal methylene				
	4.364	4.364	4.280	4.297
	4.283	4.291	4.218	4.241
	4.273	4.283	4.215	4.209
	4.191	4.209	4.132	4.118
external methylene				
	3.324	3.328	3.098	3.100
	3.314	3.317	3.050	3.050
	3.250	3.252	2.850	2.852
	3.242	3.247	2.850	2.852
phenyl ortho	2.001	2.000	1.977	1.971
phenyl meta	5.009	5.008	4.987	4.989
phenyl para	5.569	5.569	5.547	5.551

[a] Indicates methyl on the N-arylated ring. [b] Indicates methyl on the ring opposite to the N-arylated ring.

Soret bands of the zinc complexes of isomers I and II (isomer numbering in order of elution from the HPLC column) have slight shoulders on the long-wavelength side that are not present in the Soret bands of isomers III and IV (Figure 4). Previous work with the more easily separated *N*-methyl- and *N*-ethylprotoporphyrin IX isomers established that a long-wavelength shoulder on the Soret band of the zinc complexes is only observed when the *N*-alkyl group is borne by the vinyl-substituted pyrrole rings A and B.[25,26] The presence of an analogous shoulder in the spectra of only the first two *N*-phenyl isomers suggests, therefore, that the *N*-phenyl moiety is on pyrrole rings A and B in isomers I and II.

^1H NMR has been used to definitively identify the pyrrole ring arylated in each of the four regioisomers. This has proven much more difficult, and has required a different approach, than our earlier identification of the four *N*-methylprotoporphyrin IX isomers.[25] The first step in identification of the arylated ring is specific assignment of the signals in the NMR spectra of the four isomers (Figure 5). In general, the protons at the following positions are responsible for the signals in the indicated regions of the NMR spectrum: (a) meso (10.0–10.3 ppm), (b) internal vinyl (8.0–8.3 ppm), (c) external vinyl (6.0–6.4 ppm), (d) para phenyl (5.5–5.6 ppm), (e) meta phenyl (5.0 ppm), (f) internal propionic methylenes (4.0–4.4 ppm), (g) ring and ester methyls (3.3–3.8 ppm), (h) external propionic methylenes (2.8–3.4 ppm), and (i) ortho phenyl (2.0 ppm).

More specific proton assignments have been obtained by means of proton–proton decoupling and nuclear Overhauser experiments. In order to assign the four meso protons and the methyl groups to specific positions, use was made of the fact that irradiation of

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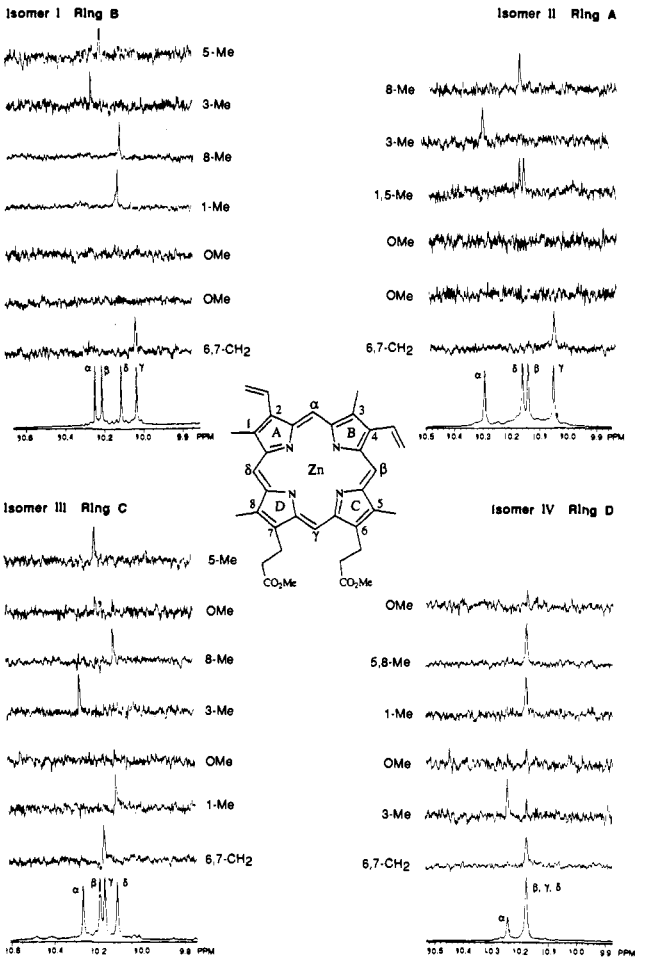

Figure 6. Nuclear Overhauser correlations between the meso protons and the methyl and propionic acid internal methylene protons. The irradiated group is indicated for each NOE tracing. The lettering and numbering schemes for zinc protoporphyrin IX are shown in the inset. The *N*-phenyl group is attached to a different pyrrole nitrogen in the four different isomers. The meso protons of each isomer are labeled.

a methyl group adjacent to a meso position causes a nuclear Overhauser enhancement (NOE) of the vicinal meso proton.[24,25,27] Thus, for isomer III, irradiation of the methyl groups at 3.641 and 3.459 ppm does not enhance the signal of any of the meso protons (Figure 6, Table I). The two methyls can therefore be assigned to the propionic acid methyl ester groups since they are the only methyls not adjacent to a meso proton. Irradiation of the methyls at 3.669 and 3.515 ppm causes nuclear Overhauser enhancement of the *same* meso proton. These two methyls are therefore at positions 1 and 8 because only those two methyls flank a common meso proton. Conversely, the signal at 10.112 ppm must be due to the δ-meso proton, since it is the only meso proton flanked by two methyl groups (Figure 6). The γ-meso proton is uniquely identified by the fact that it is not enhanced by irradiation of any methyl group but is enhanced when the internal propionic acid methylene protons are irradiated (Figure 6). The methyl at 3.589 is similarly linked by a NOE to the meso proton at 10.269, and the methyl at 3.447 to the meso proton at 10.190 ppm. However, specific attribution of these signals to the 3-methyl/α-meso protons or the 5-methyl/β-meso protons, and specific assignment of the 1- and 8-methyls, requires additional information. Irradiation of the internal methylene protons enhances not only the γ-meso proton signal but also the methyl signals at 3.447 and 3.515 ppm (not shown). The latter signal, already attributed to a methyl that flanks the δ-meso proton, must be that of the 8-methyl. The signal at 3.669 ppm is therefore, by exclusion, that of the 1-methyl. This is confirmed by the observation that it is enhanced by irradiation of the vinyl signal at 8.236 ppm. Enhancement of the methyl signal at 3.447 by irradiation of the propionic acid internal methylene protons identifies it as the 5-methyl, and therefore the meso signal at 10.190 ppm is that of the β-meso proton. Again, by exclusion, the signal at 3.589 ppm must be due to the 3-methyl and the meso signal at 10.269 ppm to the α-meso proton. This is confirmed by the fact that irradiation of the second vinyl signal, that at 8.036 ppm, enhances the methyl signal at 3.589 ppm. The NOE effects between the methyls and the vinyl internal protons uniquely assign the proton at 8.236 to the 2-vinyl and that at 8.036 to the 4-vinyl substituent. Specific assignment of the internal vinyl protons makes possible specific attribution of the external vinyl protons (Table I) to the individual vinyl groups by decoupling experiments.

All the porphyrin ring and substituent proton signals of isomer III are specifically assigned above except for the protons of the two propionic acid side chains. Three of the four internal methylene protons have overlapping resonances, but the furthest upfield proton at 4.132 ppm is relatively isolated. Since a nuclear Overhauser effect to the 8-methyl is seen when the upfield internal methylene proton is saturated, this signal and the downfield external methylene resonances coupled to it must be those of the propionic acid side chain at position 7 of pyrrole ring D. Irradiation of the upfield internal methylene proton at 4.132 ppm causes partial collapse of the downfield external methylene protons at 3.098 and 3.050 ppm. Conversely, irradiation of the same downfield external methylene proton collapses the two most upfield internal methylene signals at 4.215 and 4.132 ppm to two doublets. Irradiation of the upfield external methylene protons at 2.850 ppm decouples the two most downfield internal methylene protons at 4.280 and 4.218 ppm. By exclusion, the other propionic acid signals belong to the side chain at position 6 of pyrrole ring C.

The *N*-phenyl proton assignments indicated in Table I are confirmed by decoupling experiments. Irradiation of the para proton of isomer III at 5.547 ppm results in decoupling of the two meta protons at 4.987 ppm. Irradiation at 4.987 ppm decouples both the para proton at 5.547 ppm and the ortho protons at 1.977 ppm. Integration shows that the signals, from low to high field, are present in the ratio 1:2:2, in agreement with their identification as the para, meta, and ortho protons, respectively.[28]

The proton assignments for the other three isomers have been made by similar procedures. No difficulties were encountered in making these assignments, even though some of the resonances overlap, except for isomer IV. The analysis of the NMR of isomer IV was complicated by superposition of two of the methyl group resonances critical for the proton assignments. A systematic analysis of the temperature (−40 to +45 °C) and solvent dependence of the signals showed that they could be resolved in approximately a 20:80 mixture of deuterated acetone/chloroform at 15 °C. The critical assignments were made in this system. The combined decoupling and NOE results (Figure 6) result in the proton assignments made in Table I.

The absorption and NMR spectra of the four zinc *N*-phenylprotoporphyrin IX isomers readily identify isomers 1 and 2 as those substituted on pyrrole rings A and B and isomers 3 and 4 as those substituted on pyrrole rings C and D. Thus, the external propionic acid methylene protons of isomers III and IV are found at higher field (2.850–3.100 ppm) than the corresponding protons of isomers I and II (3.242–3.328 ppm). This is consistent with the fact that tilting of the C and D rings by the *N*-phenyl moiety moves the propionic acid protons into a more shielded region of the ring current and lowers their NMR chemical shifts. The proton signals are also more widely separated because only one of the two rings in substantially tilted by nitrogen substitution. The same pattern is observed in the *N*-alkylprotoporphyrin IX regioisomers.[25,26] Supporting evidence is provided by the observation that, of all the methyls, the methoxy methyls are at lowest field in isomers 1 and II whereas one methoxy methyl remains at low field and

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one moves to high field in isomers III and IV. This is consistent with movement of one ester methyl group into the shielding region of the ring current due to tilting of pyrrole ring C or D. In agreement with this is the fact that the propionic acid methylene protons adjacent to the porphyrin ring are at higher field in isomers III and IV (4.118–4.297 ppm) than in isomers I and II (4.191–4.364 ppm). Finally, the internal vinyl protons of isomers III and IV are downfield (8.236–8.036 ppm) of the corresponding protons for isomers I and II (8.143–8.015 ppm). Interestingly, the clean separation of the internal vinyl proton signals of rings A and B found to be diagnostic of N-*alkylation* of these two rings is not found with the *N*-phenyl isomers, presumably because the phenyl ring current alters the clear pattern obtained with the *N*-alkyl groups.[25] All of these NMR differences are consistent with each other and with the finding that isomers I and II have a shoulder in the Soret band of their zinc complexes. Isomers I and II therefore bear the *N*-phenyl group on pyrrole rings A and B and isomers III and IV on pyrrole rings C and D.

The remaining, nontrivial task is to determine whether pyrrole ring A or B is arylated in isomer I and pyrrole ring C or D in isomer III. By exclusion, this will also identify the pyrrole rings arylated in isomers II and IV. The approach taken in the case of the *N*-alkylporphyrins,[25] based on the observation that the methyl group of the N-alkylated pyrrole ring appears at highest field, is unfortunately invalid in the case of the *N*-phenylporphyrin isomers. The methyl group and the second substituent on the pyrrole ring bearing the *N*-phenyl group not only sense the ring current effect of the porphyrin ring but also that of the *N*-phenyl group. Because of the confounding effect of the phenyl ring current, no trend is immediately apparent in the relative chemical shifts of the protons on carbons directly attached to the porphyrin ring. This includes the internal vinyl, internal propionic acid methylene, and methyl protons. It has therefore been necessary to obtain some of the information necessary for isomer assignment by analysis of the chemical shift differences of protons further removed from the pyrrole ring nitrogens and the associated *N*-phenyl substituent. The propionic acid external methylene protons and the protons of the ester methoxy groups have been particularly useful in determining which ring is alkylated in isomers III and IV. Thus, as already noted, the two methoxy groups in the isomers substituted on pyrrole rings A and B have similar chemical shifts (isomer I, 3.715 and 3.654 ppm; isomer II, 3.725 and 3.657 ppm) and are in both cases the methyl resonances at lowest field (Table I). In contrast, the C/D ring substituted isomers retain one methoxy signal at low field (isomer III, 3.641 ppm; isomer IV, 3.636 ppm) but have one methoxy signal shifted to highest (isomer IV, 3.475 ppm) or next to highest (isomer III, 3.459 ppm) field (Table I). This is entirely consistent with the fact that a propionic acid substituted pyrrole ring (C or D) is tilted into the shielding region of the ring current in isomers III and IV. This tilting would be expected to shift one of the two propionic acid methyl ester groups into the deshielding region and thus to higher field than the normal position. The methoxy signals cannot be used for actual isomer assignment because it is not possible to independently attribute them to the ring C or D propionic acid moiety. A similar chemical shift pattern is discernible, however, for the external propionic acid methylene group protons for which an independent assignment can be made. Thus, isomers I and II show two barely resolved multiplets just upfield of the methyl resonances. Isomers III and IV, however, have two very well resolved external methylene multiplets that are 0.4–0.6 ppm upfield of the methyl resonances. This same pattern is observed in the already assigned *N*-methylprotoporphyrin IX regioisomers.[25] The signal at highest field should belong to the substituent on the ring with the *N*-phenyl group since tilting of that ring moves its propionic acid methylene group into the shielded region of the porphyrin ring current. The isomer assignments for these two rings can therefore be made by identifying to which propionic acid group the shifted protons belong. To do this, we have irradiated the most upfield internal methylene proton in each isomer. This produces a NOE to the vicinal methyl group that unambiguously identifies the propionic acid to which the internal methylene proton belongs. Decoupling experiments have then been used to determine to which set of external methylene protons the upfield internal methylene protons are coupled. The results of these experiments show that the two most upfield internal methylene protons in isomer III, those at 4.215 and 4.132 ppm, are on the propionic acid group of ring D. These protons are coupled, in turn, to the downfield external methylene protons at 3.098 and 3.050 ppm, so that these protons are on the propionic acid group of ring D. The upfield external methylene protons therefore belong to the propionic acid group of ring C. This indicates that the *N*-phenyl group is borne by the nitrogen of pyrrole ring C, since it is the tilting caused by the *N*-phenyl moiety that moves the external methylenes into a more shielded region of the ring current and causes them to appear at high field.

Independent determination of the *N*-phenyl-substituted pyrrole ring in isomer IV was complicated by the fact that the 5- and 8-methyl groups, the methyl groups on the two propionic acid substituted pyrrole rings, had identical chemical shifts. Since the key to isomer assignment is the NOE between the propionic acid internal methylene protons and one of these two methyl groups, it is essential for these two methyl groups to be clearly resolved. This was achieved, after some experimentation, by obtaining the NMR data in approximately 20% deuterated acetone–80% deuterated chloroform at 15 °C. The desired concentration of acetone was obtained by adding it in aliquots of 5% of the final solution so that the other chemical shifts caused by the solvent change, none of which was very large, could be monitored. The methyl resonances were then reassigned by the sequence of NOE experiments already described. The 8-methyl is separated from the 5-methyl under these conditions but is superimposed on the 1-methyl. This does not interfere with the required NOE studies since the 1-methyl is not on a propionic acid substituted pyrrole ring. The solvent change gave rise to two upfield internal methylene signals, both of which were assigned to the propionic acid group of ring C by NOE experiments. The decoupling experiments then showed that the internal methylene protons of the ring C propionic acid group were coupled to the downfield external methylene protons. By inference, the upfield external methylene protons in isomer IV are on the propionic acid moiety of ring D, and the *N*-phenyl group is borne by ring D. This provides independent confirmation of the assignment made by NMR analysis of isomer III.

The methyl and vinyl shift patterns were employed to determine the regiochemistry of the *N*-phenyl group in isomers I and II. Inspection of the ring methyl group resonances (Table I) shows that one of the methyls at any given position is significantly shifted downfield in one isomer relative to the corresponding methyls in the other three isomers. Each isomer contains only one of these downfield-shifted methyls. In isomers III and IV, for which the regiochemistry is assigned above, the downfield-shifted methyl group is on the pyrrole ring opposite to that which bears the *N*-phenyl substituent. Thus, the 1-methyl at lowest field is that of pyrrole ring A of isomer III (3.669 ppm), while the 3-methyl at lowest field is that of pyrrole ring B of isomer IV (3.667 ppm). The crystal structure of zinc *N*-phenyltetraphenylporphyrin shows that the ring bearing the *N*-phenyl ring tilts out of the heme plane by 42°. The ring opposite to that bearing the *N*-phenyl group tilts in the opposite direction to a lesser degree. Models show that tilting the juxtaposed pyrrole rings in opposite directions places the *N*-phenyl group closer to the substituents on the diagonally opposite pyrrole ring. The magnitudes of chemical shifts in simple aromatic systems have been calculated and shown to depend on both the distance from the center of the aromatic ring and the angle from the ring plane.[29] The ring current effect of benzene has been calculated to extend 6–7 Å from the center of the benzene ring, so that a proton 5 Å from the center and in the plane of the aromatic ring is predicted to be shifted downfield by approximately 0.3 ppm.[29] Inspection of the crystal structure of zinc *N*-phenyltetraphenylporphyrin suggests that the phenyl group and

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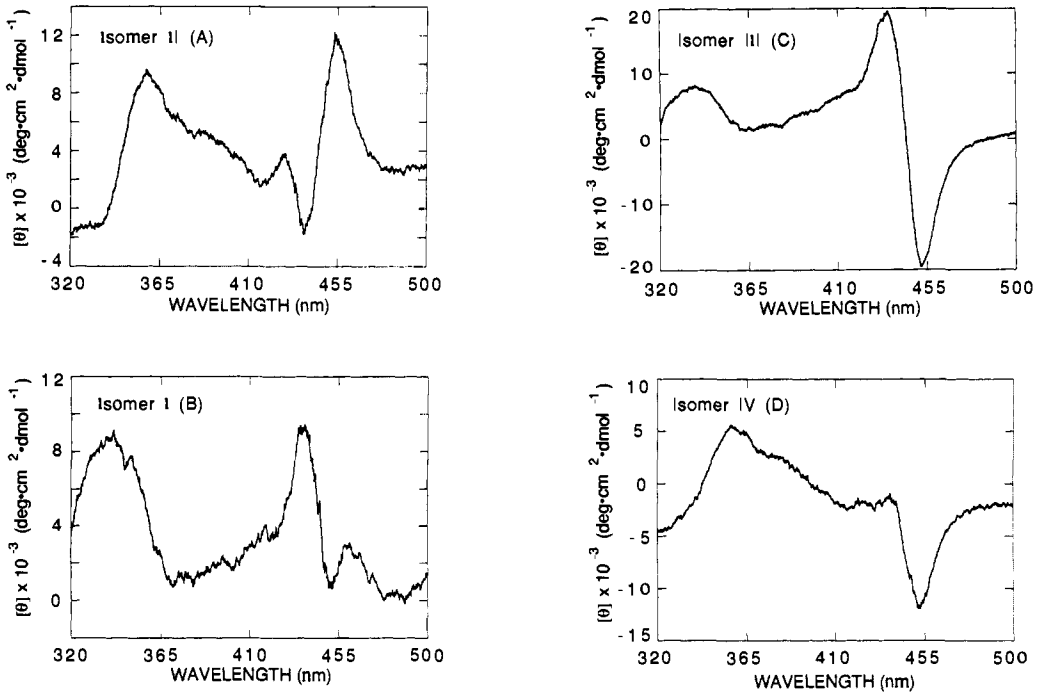

Figure 7. Circular dichroism spectra (in $CHCl_3$) of the zinc complexes of the methyl esters of the four regioisomers of *N*-phenylprotoporphyrin IX. The values of the rotation θ have been multiplied by 10^{-3}. The isomer number and the pyrrole ring substituted in that isomer are indicated on each spectrum.

the substituents on the opposite pyrrole ring should be within 7 Å of each other, so that the phenyl ring current should affect the chemical shifts of those substituents. For each set of methyl groups (e.g., the 3-methyls), one methyl is roughly 0.1 ppm downfield from the positions of the corresponding methyl in the other three isomers (Table I). This net relative downfield shift can be rationalized as the result of (a) deshielding by the *N*-phenyl group on the pyrrole ring opposite to that bearing the methyl group and (b) shielding by slight tilting of the pyrrole ring bearing the methyl into the porphyrin ring current. The earlier studies of *N*-methylprotoporphyrin IX indicated that tilting of the ring opposite to that bearing the *N*-methyl group resulted in a 0.03 ppm upfield shift of the methyl group attached to it.[25] This analysis indicates that the *N*-phenyl moiety is on pyrrole ring B in isomer I because the 8-methyl on pyrrole ring D, of all the 8-methyl groups, is at lowest field, and on pyrrole ring A in isomer II because the 5-methyl of pyrrole ring A, of all the 5-methyl groups, is at lowest field in this isomer. In agreement with these assignments, the internal and external vinyl resonances at lowest field in isomer III are those of ring A, which is opposite to ring C, the *N*-phenyl-substituted ring in this isomer. The 1-methyl of ring A is also, as already noted, at lower field than the 1-methyl of the other three isomers. The structures of the four isomers are given in Figure 2.

The four *N*-phenyl isomers obtained from phenylhydrazine-treated equine myoglobin are optically active. The circular dichroism spectra of the zinc complexes of the four dimethyl-esterified isomers are given in Figure 7. Isomers I and II, the isomers with the *N*-phenyl group on pyrrole rings B and A, respectively, show a predominantly positive rotation. Isomer III, with the phenyl group on pyrrole ring C, displays both positive and negative rotation whereas isomer IV, which is substituted on pyrrole ring D, primarily exhibits negative rotation. The spectra are those of the enantiomers shown in Figure 1 because the crystal structure of the myoglobin iron–phenyl complex indicates that the phenyl group occupies the oxygen binding site.[18] Migration of the phenyl group from the iron to the nitrogens therefore must yield the indicated *N*-phenyl enantiomers.

Conclusions

The final assignments for the ring bearing the *N*-phenyl group established by these studies are isomer I, B ring; isomer II, A ring; isomer III, C ring; and isomer IV, D ring (Figure 2). The order of elution from the HPLC column of the *unesterified* *N*-phenyl isomers is thus exactly the same as the order of elution of the methyl *esterified* *N*-methyl and *N*-ethyl isomers.[25,30] This identity in the elution order was unexpected, not only because of the difference in esterification state but also because of the substantial differences in the physical properties of the *N*-phenyl and *N*-alkyl adducts. The results with the three sets of N-substituted regioisomers suggest that the HPLC elution order B, A, C, D has a strong physicochemical basis. This should help, if used with caution, in identifying other sets of N-substituted protoporphyrin IX regioisomers.

Extraction of the iron–phenyl complex from the myoglobin matrix prior to promotion of the iron–nitrogen shift by exposure to acid and oxygen results in essentially equimolar formation of the four *N*-phenyl isomers (Figure 3). This clearly establishes that there is no inherent regiochemical preference in the migration reaction. Regioselective formation of the *N*-phenyl adducts in biological systems is therefore a consequence of the constraints imposed by the protein matrix on the migration reaction. This is confirmed by the finding that the four isomers are not formed in equal amounts when the myoglobin iron–phenyl complex is extracted with acid under aerobic conditions. Analysis of the isomer mixture shows that two isomers, those with the *N*-phenyl group on the propionic acid substituted pyrrole rings C and D, are formed in higher amounts than the ring A and B isomers. This is consistent with the fact that the propionic acid groups point toward the exterior of the protein, and with the demonstration by X-ray crystallography that the histidine residue that blocks the entry to the heme crevice is displaced by the phenyl group in the myoglobin iron–phenyl complex.[19] Migration toward the C and D rings thus appears to be favored when the migration occurs before the integrity of the heme crevice is seriously disrupted. The present results suggest that this is, indeed, the case. It is of some interest, however, that the iron–phenyl shift cannot be forced to take place within the intact myoglobin complex by treatment with oxidizing agents even though the mammalian cytochrome P450 phenyl–iron complexes can be made to shift by such treatment (see below). It is possible that the phenyl group

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is too tightly wedged in the heme crevice to undergo the migration reaction in the absence of some degree of protein denaturation. Alternatively, the oxidation potential of the myoglobin complex may be such that it is not readily accessible to exogenous oxidizing agents.

Recent work with several rat liver cytochrome P450 isozymes has shown that they form iron-phenyl complexes and, more importantly, that potassium ferricyanide promotes the iron-nitrogen shift within the undenatured protein complexes to give only two of the four possible *N*-phenyl adducts.[16] The isomer separation and identification reported here provide the key to the use of this N-arylation regiospecificity to define the topologies of the active sites of membrane-bound cytochrome P450 enzymes and possibly other hemoproteins with open (substrate-accessible) heme binding sites.

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Registry No. Isomer 1, 136006-23-0; isomer 2, 136006-24-1; isomer 3, 136006-25-2; isomer 4, 136006-26-3; heme, 14875-96-8; phenylhydrazine, 100-63-0.

Photochemistry of Intercalated Quaternary Diazaaromatic Salts

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Abstract: Certain quaternary diazaaromatic salts intercalate between base pairs in calf thymus DNA and in synthetic polynucleotides. The intense fluorescence of these salts, as observed in dilute aqueous solution, is quenched upon intercalation due to electron abstraction from an adjacent base. This reaction is not sequence-specific. The resultant radical cation of the quaternary salt decays within 100 ps. Individual nucleotide monophosphates also transfer an electron to the excited singlet state of the salts, both in fluid solution and after complexation in the ground state. Illumination of a salt/nucleotide ground-state complex generates the corresponding radical ion pair, which decays within 100 ns. These results indicate that photoinduced electron-transfer processes involving intercalated dye molecules occur on faster (i.e., 2500-fold) time scales than for the corresponding complexed reactants and are highly reversible.

Flat, cationic molecules can interact strongly with polynucleotides and, provided the cation is of the appropriate dimensions, intercalate into the DNA strand.[1] Many such cations have been reported, including metalloporphyrins,[2-4] ethidium bromide,[5] methylene blue,[6] and heterocycles,[7-10] and several have been shown to induce strand scission upon light or electrical stimulation. Of particular interest is the observation that certain diazapyrenium dications associate with polynucleotides and cause their photocleavage under anaerobic irradiation with visible light.[11-13] These compounds show intense fluorescence in solution that is quenched upon binding to certain polynucleotides.[11-13] In view of the fact that such quaternary salts are easily reduced,[14] it can be speculated that fluorescence quenching is due to electron abstraction from a nucleic acid base or ribose by the singlet excited state of the dye.[13,15] We have studied the photochemistry of this system using laser flash photolysis techniques and found that quaternary diazaaromatic dyes are able to abstract an electron from nucleotides and polynucleotides, under illumination with visible light, but the process is reversible.

Experimental Section

N,N'-Dimethyl-2,7-diazapyrene bis(tetrafluoroborate) (DAP^{2+}) and *N,N'*-dimethylanthra[2,1,9-*def*:6,5,10-*d'e'f'*]diisoquinoline dichloride

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