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Inactivation of Myoglobin by Ortho-Substituted Arylhydrazines. Formation of Prosthetic Heme Aryl–Iron but Not *N*-Aryl Adducts†

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ABSTRACT: Stable phenyl–iron complexes are known to form in the reactions of myoglobin, hemoglobin, and catalase with phenylhydrazine. The phenyl moiety in these complexes migrates from the iron to a nitrogen of the porphyrin upon denaturation of the hemoproteins. Complexes obtained from myoglobin and ortho-substituted phenylhydrazines, however, are much less stable, have distinct chromophores, and do not yield *N*-arylporphyrins. These abnormal properties imply that the complexes differ in structure (e.g., they are aryldiazenyl- rather than aryl–iron complexes) or that ortho substitution strongly alters the chemistry of aryl–iron complexes. The present NMR studies unambiguously demonstrate that ortho-substituted phenylhydrazines give normal aryl–iron complexes but that the aryl group in these complexes is conformationally locked and is unable to shift from iron to nitrogen.

The reaction of phenylhydrazine with hemoglobin, which irreversibly alters the hemoprotein chromophore (Itano & Robinson, 1961; Jandl et al., 1960), gives rise to superoxide and phenyl radicals (Goldberg et al., 1976, 1979; Hill & Thornalley, 1981; Augusto et al., 1982) and hydrogen peroxide (Rostorfer & Cormier, 1957; Cohen & Hochstein, 1964). Myoglobin reacts similarly with phenylhydrazine except that hemoglobin precipitates from the solution whereas myoglobin does not (French et al., 1978; Augusto et al., 1982). *N*-Phenylprotoporphyrin IX is obtained when the prosthetic group is extracted aerobically from the inactivated hemoproteins (Ortiz de Montellano & Kunze, 1981; Saito & Itano, 1981), but the phenyl group is bound to the iron rather than to one of the nitrogens when the prosthetic moiety is extracted anaerobically (Kunze & Ortiz de Montellano, 1983; Ortiz de Montellano & Kerr, 1983). Coordination of the phenyl to the iron in the intact myoglobin complex has been confirmed by an NMR study (Kunze & Ortiz de Montellano, 1983) and by X-ray crystallography (Ringe et al., 1984). The phenyl group thus shifts from the heme iron to one of its nitrogens as the heme complex separates from the protein matrix (Au-

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gusto et al., 1982; Ortiz de Montellano et al., 1982; Kunze & Ortiz de Montellano, 1983; Mansuy et al., 1982a).[1] Studies with model porphyrin complexes have confirmed the feasibility of this rearrangement (Ortiz de Montellano et al., 1982; Mansuy et al., 1982a).

The complexes of hemoglobin with meta- and para-substituted phenylhydrazines are similar to that of phenylhydrazine itself whereas the complexes of ortho-substituted phenylhydrazines have a different chromophore (Itano, 1970; Itano et al., 1975, 1977; Huang & Kosower, 1968), are relatively unstable, and do not give rise to *N*-arylporphyrins (Augusto et al., 1982). The divergent properties of the ortho-substituted phenylhydrazine complexes could reflect steric interactions of the ortho substituents with the heme face or could signal a basic structural difference. Ortho-substituted phenylhydrazines, for example, could give rise to iron–diazenyl rather than iron–aryl complexes (Itano, 1970; Huang & Kosower, 1968; Itano & Mannen, 1976; Battioni et al., 1983; Mansuy et al., 1982b). The present NMR study of the complexes of myoglobin with ortho-substituted phenylhydrazines was undertaken to resolve this question.

Experimental Procedures

Materials. Sperm whale metmyoglobin was obtained from Sigma; substituted phenylhydrazines, perdeuterated chlorobenzene, 98% deuterated water, and 37% $^2HCl/^2H_2O$ were from Aldrich Chemical Co.; perdeuterated toluene and 99.96% $^2H_2$ were from Stohler Isotope Chemicals.

Synthesis of Perdeuterated (2-Methylphenyl)hydrazine. A precooled mixture of concentrated nitric acid (5.5 mL) and acetic anhydride (6.0 mL) was added dropwise at 0 °C to a stirred solution of perdeuterated toluene (5 g, 55 mmol) in 15 mL of acetic anhydride. The reaction mixture was stirred 4 h, at which time the absence of toluene was established by gas chromatographic analysis on a 6-ft glass column packed with 3% OV-225 on 100/120-mesh Supelcoport programmed to rise from 50 to 200 °C at 20 °C/min [retention times (min): toluene, 2.50; 2-nitrotoluene, 11.17; 3-nitrotoluene, 11.74; 4-nitrotoluene, 12.22]. The reaction mixture was added to 500 mL of water and was stirred overnight before it was extracted with diethyl ether (3 times 100 mL). Gas chromatographic analysis of the ether extracts indicated the presence of a 60:5:35 ortho/meta/para mixture of nitrotoluene isomers. The residue obtained from the dried (Na_2SO_4) extracts by solvent removal (excess acetic anhydride, when present, was removed azeotropically with toluene) was catalytically hydrogenated in ethyl acetate (100 mL) over 300 mg of 5% (w/w) Pd/C in a Parr apparatus (55 psi) for 5 h. The product mixture was filtered, the solvent removed, and the residue fractionated by column chromatography (30 × 2.5 cm column of 70–230-mesh silica gel 60 eluted with CH_2Cl_2). Perdeuterated 2-methylaniline (200 mg, 1.9 mmol, λ_{max} 233 nm), the first product eluted from the column, was dissolved in 15 mL of 37% (v/v) $^2HCl/^2H_2O$. To the resulting mixture, stirred and cooled in an ice bath, was added a solution of $NaNO_2$ (140 mg, 2.0 mmol) in cold ^2H_2O (2 mL). Stannous chloride (1.5 g, 6 mmol) in 10 mL of concentrated ^2HCl was added 2 h later. Thin-layer chromatographic analysis (silica gel, 1:1 ether/hexane) of the mixture after a further 2 h established complete conversion of the amine (R_f 0.4) to the hydrazine (R_f 0.1). The mixture was slowly poured into 100 mL of *cold* concentrated NaOH, and the product was extracted with ether. The combined and dried (Na_2SO_4) extracts were concentrated to approximately 20 mL, and a solution of oxalic acid (200 mg) in ether (5 mL) was added. Recrystallization of the precipitated oxalate salt from methanol/ether yielded 90 mg (20%) of the desired [$^2H_7$](2-methylphenyl)hydrazine: mp 147 °C dec; ^1H NMR δ 9.0 (br d, 3 H, NH). The oxalate salt of undeuterated (2-methylphenyl)hydrazine, prepared from the commercial hydrochloride salt, melted at 151 °C dec. The perdeuterated compound gave a molecular ion at *m/e* 129 without a detectable peak at *m/e* 122, as expected for material with greater than 95% deuterium.

Synthesis of Perdeuterated (2-Chlorophenyl)hydrazine. This material was synthesized as described above except that the nitration reaction required 12 h at 0 °C and 48 h at 25 °C. The ortho/meta/para isomers of nitrochlorobenzene (retention times, respectively, of 5.49, 4.30, and 5.13 min with the column programmed to rise from 70 to 170 °C at 20 °C/min) were obtained in a 22:2:76 ratio. The ortho isomer, isolated by low-pressure silica gel chromatography on a column eluted with 1:1 hexane/diethyl ether, yielded on hydrogenation 170 mg of 2-chloro[$^2H_4$]aniline (λ_{max} 290 nm). The final yield of the recrystallized oxalate salt of [$^2H_4$](2-chlorophenyl)-hydrazine was 50 mg (16%): mp 143–145 °C dec (authentic mp 147–149 °C dec); ^1H NMR δ 10.2 (br s, 3 H, NH). Greater than 95% deuteration of the aromatic ring was confirmed by the presence in the mass spectrum of the molecular ion doublet at *m/e* 142 and 144 expected for a monochlorinated compound and by the absence of peaks at *m/e* 138 and 140.

Preparation of Myoglobin Complexes for NMR Studies. Sperm whale myoglobin (71 mg, 4 μmol) and 100 μL of concentrated phosphate buffer solution (0.5 M K_2PO_4, 0.5 M NaH_2PO_4, and 1 mM EDTA in deionized distilled water) were added to 5 mL of 99.96% deuterated water, and the resulting solution was lyophilized to dryness. The process was repeated 2 more times. The final residue, taken up in 1 mL of 99.8% deuterated water, yielded a solution (pD 6.9) with approximately a 4 mM heme concentration. To this was added 40 μmol (10-fold excess) of the desired arylhydrazine (as the hydrochloride or oxalate salt). The mixture then was allowed to sit 1–4 h in the dark until the Soret band shifted completely from 408 nm (native myoglobin) to 426–432 nm (aryl–iron complex). The incubation mixture was then centrifuged in a table-top centrifuge, and the supernatant was transferred to a 5-mm NMR tube for NMR analysis. The absorption spectrum was rechecked at the end of NMR experiments to confirm that the sample had not decomposed.

NMR Data Acquisition. The ^1H NMR spectra were recorded on a Nicolet 240-MHz instrument with quadrature phase detection. The signal due to residual water protons was presaturated with a 1-s decoupler pulse. Approximately 30 000–50 000 transients were normally acquired in blocks of 3000–5000 with a sweep width of 50 kHz and 32K memory points. NMR spectra were obtained at 25 °C with a deuterium lock on ^2H_2O.

Results and Discussion

Myoglobin Complexes with (2-Methylphenyl)-, (3-Methylphenyl)-, and (4-Methylphenyl)hydrazines. The proton signals of the tolyl moiety in the 240-MHz ^1H NMR spectrum of the complex obtained from sperm whale metmyoglobin and (2-methylphenyl)hydrazine are readily identified when this spectrum is compared to those of complexes obtained with perdeuterated ($^2H_7$) (2-methylphenyl)hydrazine (Figure 1) and with (3-methylphenyl)hydrazine and (4-methylphenyl)-hydrazine (Figure 2). The absence of the pairs of signals at

[1] The term "heme" is used to describe iron protoporphyrin IX regardless of the oxidation state of the iron or the porphyrin.

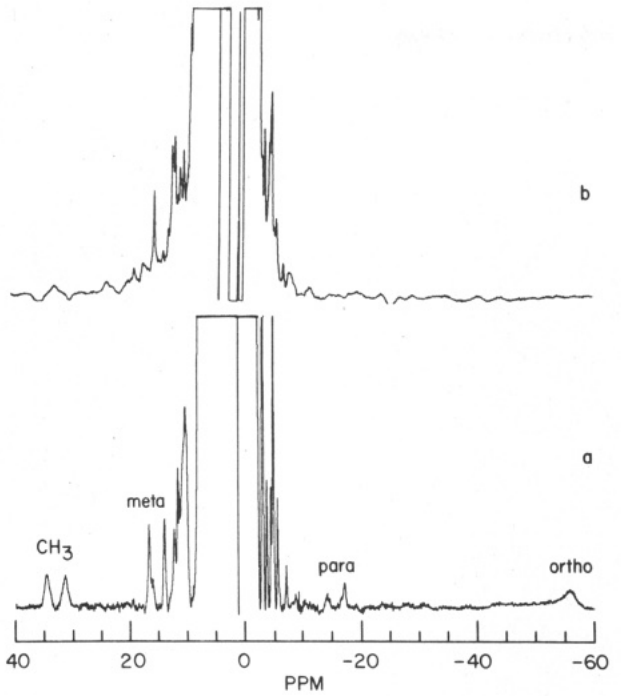

FIGURE 1: NMR spectra of the metmyoglobin complexes with (a) (2-methylphenyl)hydrazine and (b) perdeuterated (2-methylphenyl)hydrazine. The signal at approximately 18 ppm in the spectrum of the deuterated sample is seen as a shoulder to the right of the signal of one of the meta protons in the undeuterated material.

34.7 and 31.3, 16.6 and 13.9, and −14.4 and −17.3 ppm and of the broad singlet at −56 ppm in the NMR spectrum of the complex with the deuterated substrate (Figure 1) shows that these signals are due to protons of the 2-methylphenyl moiety. The exact proton assignments for the NMR signals can be deduced from our earlier studies with phenylhydrazine, [$^2H_5$]phenylhydrazine, and (4-methylphenyl)hydrazine (Kunze & Ortiz de Montellano, 1983). The highly unusual signal at −55 ppm in the spectrum of the 2-methyl isomer, which is essentially at the same position in the spectrum of the complexes with the 3-methyl (−56 ppm) and 4-methyl (−58 ppm) isomers, is clearly due to a proton ortho to the carbon–iron bond of the σ-bonded aryl–iron complex. The breadth of the ortho proton signal is presumably due to rapid relaxation caused by proximity to the unpaired iron electrons. The signals at −14.4 and −17.3 ppm fall in the region assigned to protons para to the carbon–iron bond in the unsubstituted (−8.1 ppm) and 3-methyl-substituted (−8.6 ppm) complexes. No signal is observed in this region for the 4-methylphenyl complex, as required for the complex of a substrate without a para proton. The pair of signals at 13.9 and 16.6 ppm are in the region assigned to protons meta to the carbon–iron bond in the complexes with phenylhydrazine (19 ppm) (Kunze & Ortiz de Montellano, 1983), (3-methylphenyl)hydrazine (16.2 ppm), and (4-methylphenyl)hydrazine (20 ppm) (Figure 2). The signals at 34.7 and 31.3 ppm, by exclusion, can be assigned to the 2-methyl group. This assignment is consistent with the observation that the methyl group of the 4-methylphenyl complex gives a signal at 47 ppm (Figure 2) and with the relative intensity of the signals determined by integration of the spectrum. The NMR spectrum of the complex obtained with (2-methylphenyl)hydrazine, however, is differentiated from those of the 3-methyl, 4-methyl, and unsubstituted complexes by the fact that all the signals occur in pairs except for that of the ortho proton at −56 ppm. Signal integration confirms that the two signals attributed to the 2-methyl groups account for a total of three protons, those assigned to the meta

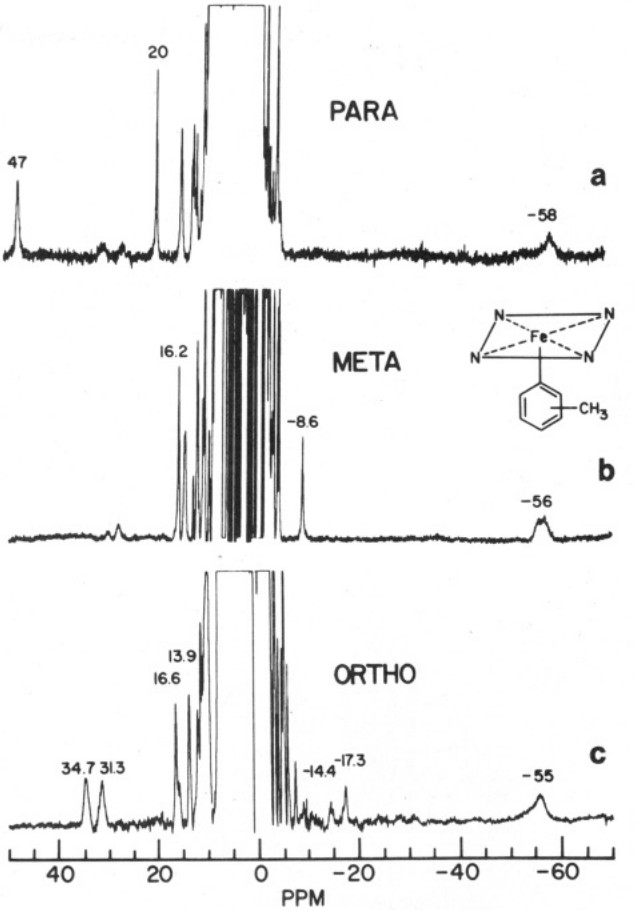

FIGURE 2: NMR spectra of the metmyoglobin complexes with (a) (4-methylphenyl)hydrazine, (b) (3-methylphenyl)hydrazine, and (c) (2-methylphenyl)hydrazine.

protons for two protons, those attributed to the para proton for one hydrogen, and the singlet attributed to the ortho proton for one hydrogen. Electronic integration indicates that the signals in each pair are of equal intensity. These results point to the existence of isomeric complexes in which the corresponding protons, with the exception of the ortho protons, have slightly different NMR chemical shifts.

Myoglobin Complexes with (2-Halophenyl)hydrazines. The 240-MHz ^1H NMR spectra of the complexes obtained with (2-fluorophenyl)hydrazine, (2-chlorophenyl)hydrazine, and (2-bromophenyl)hydrazine (Figure 3) directly establish that 2-halo analogues form aryl–iron complexes analogous to that obtained with phenylhydrazine. The 2-fluoro- and 2-chlorophenyl complexes are less stable than the complexes with meta or para substituents (Augusto et al., 1982) but are stable enough not to decompose significantly during the overnight NMR experiments required to acquire the spectral data in Figure 3. The NMR spectrum of the 2-bromophenyl complex, however, is relatively poor because this complex does decompose during the NMR experiments. The signals of the *o*-, *m*-, and *p*-aryl protons are nevertheless readily located in the spectra of all three complexes. The aryl protons in the complex formed with (2-chlorophenyl)hydrazine have been specifically identified by comparing its NMR spectrum with that of the complex with the perdeuterated substrate (Figure 4). The chemical shift of the ortho proton in the NMR spectrum correlates qualitatively with the electronegativity of the halogen atom (2-F, −55 ppm; 2-Cl, −44 ppm; 2-Br, −42 ppm). A smaller but similar trend is observed for the para proton in the complexes (2-F, −10.2 ppm; 2-Cl, −7.6 ppm; 2-Br, −6.9 ppm) whereas the meta protons (21–22 ppm) appear to be

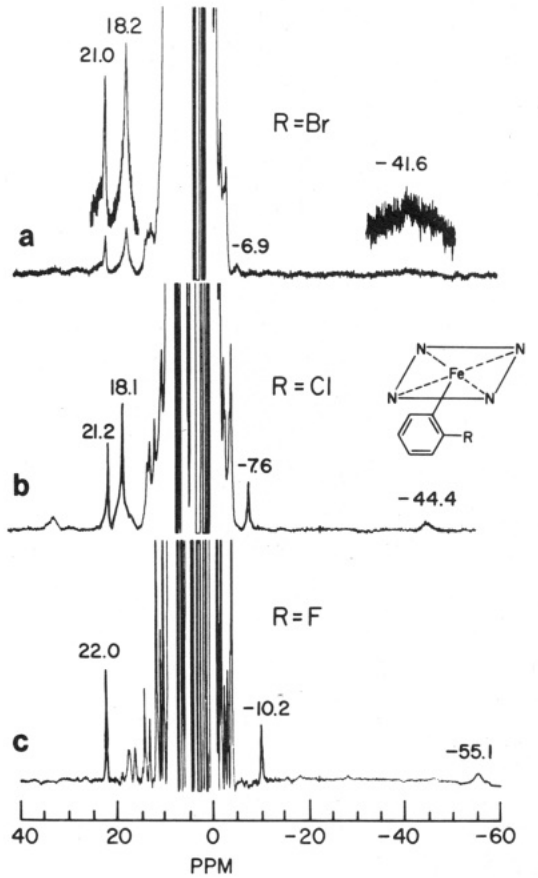

FIGURE 3: NMR spectra of the metmyoglobin complexes with (a) (2-bromophenyl)hydrazine, (b) (2-chlorophenyl)hydrazine, and (c) (2-fluorophenyl)hydrazine.

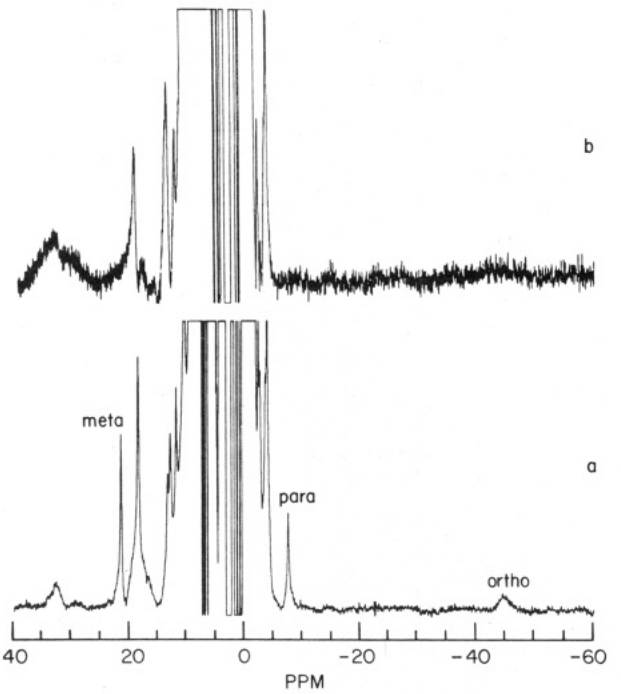

FIGURE 4: NMR spectra of the metmyoglobin complexes with (a) (2-chlorophenyl)hydrazine and (b) perdeuterated (2-chlorophenyl)-hydrazine.

relatively insensitive to the nature of the halogen atom. The peak at 18.1 ppm (Figure 3) is due to a proton of the parent hemoprotein because it remains in the NMR spectrum when the complex is prepared with perdeuterated (2-chlorophenyl)hydrazine (Figure 4). The dominant influence of the iron on the chemical shifts of the phenyl ring protons is emphasized by the fact that the chemical shifts of the two meta protons in each complex are equivalent regardless of whether a halogen or hydrogen is located at the intervening ortho position. It is somewhat surprising, in view of the pairs of peaks observed in the NMR spectrum of the (2-methylphenyl)hydrazine complex, that the aryl protons of the 2-halophenyl complexes give rise to single NMR signals.

A remarkable feature of the NMR spectra of the 2-chloro- and 2-bromophenyl, but not 2-methyl- or 2-fluorophenyl, complexes is the presence of a peak at approximately 18.1 ppm (Figures 2 and 3) due to a proton of the parent hemoprotein (see above). The magnitude (18 ppm) of this chemical shift places the proton responsible for the signal in the vicinity of the prosthetic group because only an interaction of the proton with the unpaired electron density of the heme can account for such a shift. The absence of this peak in the spectrum of the isomeric 2-methylphenyl complexes is to be noted because it indicates that a halogen atom, and not merely a group of approximately equal size (i.e., the methyl), is required to shift the proton out of the envelop of protein signals between 0 and 10 ppm.

Conclusions

The NMR data clearly identify the complexes formed with (2-methylphenyl)-, (2-fluorophenyl)-, (2-chlorophenyl)-, and (2-bromophenyl)hydrazine as structures in which the 2-substituted phenyl moiety is σ-bonded to the prosthetic heme iron atom. This conclusion rests on the approximate identity of the chemical shifts for the equivalent protons in complexes with the 2-substituted and with unsubstituted, or 3-methyl- or 4-methyl-substituted, substrates. This requires, particularly in view of the unusual chemical shifts of the protons, that the structures of all the complexes be very similar. Our earlier characterization of the phenylhydrazine complex as a phenyl–iron structure, confirmed recently by a high-resolution X-ray structure (Ringe et al., 1984), consequently firmly establishes that substituted phenylhydrazines also yield aryl–iron complexes. The NMR data specifically exclude complexes in which the two nitrogens of a diazene moiety separate the phenyl moiety from the iron atom because this would radically alter the chemical shifts of the phenyl ring protons.

The formation of isomeric aryl–iron complexes in the reaction of metmyoglobin with (2-methylphenyl)hydrazine is readily rationalized if the 2-methylphenyl moiety fits into a groove that prevents rotation about the carbon–iron bond (Figure 5). The X-ray structure of the phenyl–iron myoglobin complex shows that, at least in the crystalline state, the phenyl group is indeed tightly wedged into such a groove and is unable to rotate about the carbon–iron bond axis (Ringe et al., 1984). The phenyl group in the crystal structure lies in a plane perpendicular to the heme plane that passes through the α- and γ-meso carbons. One of the meta protons thus points toward the α-meso and the other toward the γ-meso position. The NMR data for the 2-methylphenyl complex suggest that rotation about the carbon–iron bond is as circumscribed in solution as it is in the crystal. The 2-methylphenyl group thus exists in distinct orientations that place the 2-methyl in the vicinity of (presumably) the α- and γ-meso positions. The distinct signals at 31.3 and 34.7 ppm (Figure 1) for the 2-methyl group then reflect the different environments at the isomeric sites. The chemical shift nonequivalence of the meta protons can be similarly explained. The observation of two signals for the para proton, however, requires the further postulate that substitution of a methyl group at one of the ortho

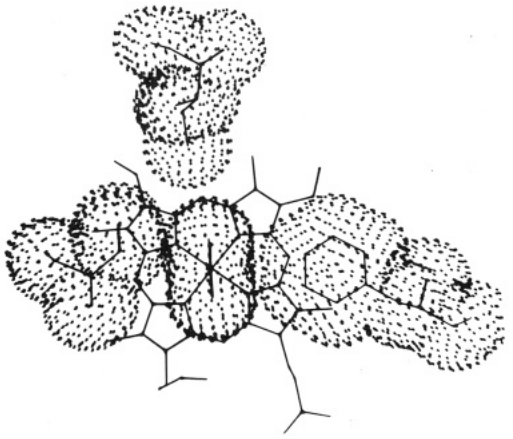

FIGURE 5: View of the myoglobin phenyl–iron complex constructed from the X-ray coordinates determined in an earlier study (Ringe et al., 1984). All the amino acid residues in the crystal structure except those that directly hinder rotation about the carbon–iron bond have been deleted for clarity. The phenyl group is seen edge on in the center with the heme group below it. The three amino acid residues in direct contact with the phenyl group are valine-68 (left), phenylalanine-43 (right), and isoleucine-107 (top). The volumes of the phenyl group and the amino acid residues, calculated by the method of Bash et al. (1982), are defined by the dotted surfaces.

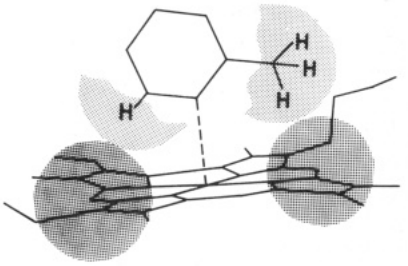

FIGURE 6: Tilt of the iron–aryl moiety required to accommodate the steric interaction of an ortho substituent with the porphyrin ring. The surface contours of the ortho proton, a 2-methyl substituent, and the edge of the porphyrin ring, calculated as in Figure 5, are indicated. Tilting of the ring and lengthening of the carbon–iron bond are required to prevent overlap of the surfaces. This structural extrapolation is based on the X-ray structure of the myoglobin complex but is hypothetical rather than empirical.

positions forces the phenyl to tilt away from an imaginary axis perpendicular to the heme in order to minimize the steric interaction between the ortho methyl group and the porphyrin ring. This displaces the para proton from a position directly above the iron atom and puts it, if the 2-phenylmethyl moiety exists in two orientations, in two possible different environments (Figure 6). Finally, the observation of a single signal for the ortho proton merely requires that it exist in both isomers in positions with comparable environments, a not unreasonable constraint given that the ortho proton will be thrust into the π system of the porphyrin ring. The data thus fully support the existence of two rotameric orientations for the 2-methylphenyl complex with the phenyl moiety tilted to relieve steric compression of the methyl group and the porphyrin ring.

It is surprising that the 2-chloro and 2-bromo analogues do not give rise to NMR-distinguishable isomers analogous to those observed with the 2-methylphenyl moiety. This requires the formation of only one isomer in the case of the 2-chloro and 2-bromophenyl complexes or the formation of isomeric complexes in which the corresponding protons have identical chemical shifts. The difference between the 2-methyl and 2-halo complexes, in any case, must stem from an electronic rather than steric difference because the chloro, bromo, and methyl substituents are of roughly comparable dimensions. The available data, however, do not allow us to distinguish between these two alternatives.

The identity of the proton responsible for the signal at 18 ppm in the NMR spectrum of the 2-chloro and 2-bromophenyl complexes remains ambiguous, but the proton at the α- or γ-meso position of the heme appears to be the most likely candidate. Structural models (see Figure 5) show that the 2-substituent is placed in close proximity to the α- or γ-meso positions if the phenyl ring is constrained to the groove observed in the crystal structure of the phenyl complex (Ringe et al., 1984). Any unpaired electron density that is delocalized from the iron into the 2-halogen substituent through the phenyl π system will also be localized in the vicinity of the meso proton. The unpaired electron density could shift the meso proton out of the region of the NMR spectrum occupied by most of the hemoprotein protons. The 2-methyl group, in constrast, is relatively insulated from the unpaired electron density in the phenyl π system and thus would not be expected to cause a similar NMR shift.

The finding that the 2-substituent interacts with the porphyrin ring strongly enough to tilt the phenyl ring away from an axis perpendicular to the heme iron rationalizes the failure of these complexes to undergo the iron-to-nitrogen shift required to give isolable *N*-arylheme adducts (Augusto et al., 1982). Only in the case of the 2-fluoro group, which is only slightly larger than a proton and thus does not interact strongly with the porphyrin face, have we been able to isolate an *N*-aryl adduct from the reaction of myoglobin with a 2-substituted phenylhydrazine.[2]

Registry No. [$^2H_7$]-(2-Methylphenyl)hydrazine oxalate, 94295-23-5; [$^2H_4$]-(2-chlorophenyl)hydrazine oxalate, 94295-25-7; [$^2H_8$]-toluene, 2037-26-5; [$^2H_7$]-2-nitrotoluene, 84344-04-7; [$^2H_7$]-2-methylaniline, 68408-22-0; [$^2H_4$]-*o*-nitrochlorobenzene, 94295-26-8; [$^2H_4$]-2-chloroaniline, 94295-27-9; iron, 7439-89-6.

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^1H and ^{31}P Relaxation Rate Studies of the Interaction of Phosphoenolpyruvate and Its Analogues with Avian Phosphoenolpyruvate Carboxykinase†

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ABSTRACT: The interactions of the substrate phosphoenolpyruvate and the substrate analogues (*Z*)-phosphoenol-α-ketobutyrate and (*E*)-phosphoenol-α-ketobutyrate with the enzyme–Mn complex of chicken liver phosphoenolpyruvate carboxykinase have been investigated by ^1H and by ^{31}P nuclear relaxation rate studies. Studies of the ^1H and the ^{31}P relaxation rates of the ligands in the binary Mn–ligand complexes show that these ligands interact with the metal ion via the phosphate group but not through the carboxylate. An inner sphere coordination complex is formed but the metal–ligand complex is not in the most extended conformation. In the relaxation rate studies of the ligands in the presence of the enzyme, conditions were adjusted so that all of the Mn^{2+} that was added resided in the ternary enzyme–Mn–ligand complex. The ^1H relaxation rates for each of the three ligands were measured at 100 and at 300 MHz. In each case the normalized paramagnetic effects showed that $1/(pT_{2p})$ was greater than $1/(pT_{1p})$. A frequency dependence of the $1/(pT_{1p})$ and $1/(pT_{2p})$ values was also measured. The correlation time, τ_c, for the Mn–^1H interaction was calculated from the frequency dependence of $1/(pT_{1p})$ assuming a maximal frequency dependence of τ_c and assuming no frequency dependence of τ_c and from the T_{1M}/T_{2M} ratios at each frequency. The τ_c values for all of the complexes, calculated at 100 MHz, varied from approximately 0.3 to 2.0 ns. These values were used to calculate the Mn–^1H distances in each of the ternary complexes. The relaxation rates of ^{31}P were also measured. The values of $1/(pT_{2p})$ were more than 1 order of magnitude larger than the respective values for $1/(pT_{1p})$ of ^1H and of ^{31}P for each ligand. A frequency dispersion, measured at 40.5 and at 121.5 MHz, was also observed for the $1/(pT_{1p})$ values. A calculation of the Mn–P distances shows that phosphoenolpyruvate forms an outer sphere complex with the bound Mn^{2+} ($r = 7.44 \pm 0.52$ Å). The structures of the ternary complexes with the *Z* and the *E* analogues ($r = 3.74 \pm 0.15$ Å and $r = 4.79 \pm 0.34$ Å, respectively, where *r* is the Mn–P distance) demonstrate that these inhibitors bind differently to the enzyme than does the substrate. The exchange rate of phosphoenolpyruvate ($k_{off} = 2.2 \times 10^4$ s^{-1}), measured from a temperature dependence of T_{2p}, is several orders of magnitude greater than the turnover number (30 s^{-1}) for the reaction. The activation energy for substrate exchange, 13 kcal/mol, suggests that this ligand exchange process is not a rate-determining step. These structural and kinetic results lead to a refinement of the proposed mechanism of this reaction and a clarification of the role of the Mn^{2+} activator.

Phosphoenolpyruvate carboxykinase (EC 4.1.1.32) catalyzes the reversible carboxylation of P-enolpyruvate[1] with a concomitant transfer of the phosphoryl group to a nucleotide acceptor, either IDP or GDP:

P-enolpyruvate + CO_2 + IDP (GDP) $\overset{Mn^{2+}}{\rightleftharpoons}$ oxalacetate + ITP (GTP)

The avian liver enzyme, which is mitochondrial in location, has been shown to require a divalent metal ion for activity, and Mn^{2+} gives optimal activity when used as the activator (Hebda & Nowak, 1982b). Kinetic, electron paramagnetic resonance, and proton relaxation rate studies have demonstrated a specific site on the enzyme for this cation, and the results have suggested that this metal is important in the

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[1] Abbreviations: P-enolpyruvate, phosphoenolpyruvate; PEB, phosphoenolbutyrate; FID, free-induction decay; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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