viously[22] that the Au(I), Ag(I), and Cu(I) complexes $[M(eppe)_2]^+$ underwent an intramolecular inversion process in solution involving facile breaking of at least one M-P bond. The kinetic stability of the Cu(I) complex was slightly higher than that of the Ag(I) and Au(I) complexes.

The effect of exchanging phenyl substituents for ethyls in $[Cu(eppe)_2]Cl$ is to increase water solubility and decrease antitumor activity. A similar trend was observed for the Au(I) series, and the reduced activity was attributed partly to a greater reactivity of $[Au(eppe)_2]Cl$ toward protein disulfide bonds.[4] These were cleaved in model reactions with release of the phosphine oxide.

The cytotoxic potency of dppe in vitro and its toxicity in vivo are significantly increased when dppe is incubated in the presence of noncytotoxic concentrations of Cu(II) salts.[6] It is possible that complexation to the metal protects the ligand from oxidation and promotes its uptake into cells. In addition, delivery of Cu(I) into a cell, or its translocation within cells, could play an additional important role in the cytotoxicity of the copper(I) diphosphine complexes. Copper has been implicated in the anticancer activity of a number of potential chelating agents, such as thiosemicarbazones,[30] 2,9-dimethyl-1,10-phenanthroline,[31] 1,10-phenanthroline,[32] and 4'-(9-acridinylamino)methanesulfon-*m*-anisidide.[33] For the latter two agents there is evidence that DNA strand cleavage results from a Cu(II)-dependent production of oxygen free radicals. We have observed[34] by agarose gel electrophoresis that $(CuCl)_2(dppe)_3$, not $[Au(dppe)_2]Cl$, produced single-strand breaks in supercoiled plasmid-DNA. Further experiments are required to investigate whether molecular O_2 is involved in the strand scission.

There are clearly many problems in identifying whether copper(I) diphosphine complexes play a key role in the cytotoxicity and antitumor activity of diphosphine ligands. The Cu(I) dppe system is particularly complicated. The studies reported here have shown that Cu(II) will react with an excess of dppe to form the bischelated species $[Cu(dppe)_2]^+$. However, we have not investigated the effect of Cl^- ions on this reaction, and from the above discussion it is clear that chloride could successfully compete as a ligand and a number of diphosphine- and halide-bridged species may be present in solution. The position of equilibria involving Cl^- could change greatly on passage of the complex from outside cells, where the Cl^- concentration is high (ca. 104 mM), to inside cells, where the concentration is much lower (ca. 3 mM). In addition, copper(II) phosphine oxide complexes could be formed. Anderson et al. have reported[17] that $(CuNO_3)_2(dppe)_3$ readily converts into a copper(II) bis(phosphine oxide) complex in chlorinated solvents.

These studies have shown that the bischelated complex $[Cu(dppey)_2]Cl$ exhibits good antitumor activity in animal models, and in contrast to the Cu(I) dppe complexes, it has a well-defined structure in solution. These properties make it highly suitable as a probe for investigating the effect of copper(I) diphosphines on critical cellular processes, with the aim of elucidating the possible role of copper in the antitumor activity of diphosphine ligands and complexes. The mixed-ligand complex $[Cu(eppe)_2]Cl$ is less active in vivo, but it is still potently cytotoxic. It also has a well-defined solution chemistry, and its high water solubility may make it more suitable for some model studies.

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Supplementary Material Available: A figure showing 200-MHz ^1H NMR spectra of saturated solutions of $(CuCl)_2(dppe)_3$ at various temperatures and a table listing ^1H NMR data for $[Cu(dppey)_2]Cl$ and $[Cu(dppp)_2]Cl$ (2 pages). Ordering information is given on any current masthead page.

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Electron Transfer between Hemoglobin and Arenediazonium Salts. Mechanism of Heme Aryl-Iron Complex Formation

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Two distinct processes characterize the electron-transfer reactions between hemoglobin and arenediazonium tetrafluoroborate salts. In the first, oxidation of hemoglobin by arenediazonium ions that possess polar substituents (*p*-NO_2, *p*-CN, *p*-CH_2COO^-, and *p*-PhNH) results in the formation of methemoglobin and arene products obtained by hydrogen abstraction. Their reaction rate constants correlate with those from ferrocyanide oxidation and with Hammett σ values (ρ = 3.0). In the second, reactions of hemoglobin with arenediazonium ions whose substituents are more hydrophobic (*p*-Cl, *p*-F, *p*-CH_3, *p*-Et, *p*-Me_2CH, *p*-Me_3C, *p*-$CH_3(CH_2)_4CH_2O$, *p*-CH_3O) form σ-bonded aryliron(III) complexes. Their rate constants are greater than predicted from the Hammett plot, but there is good correlation with the hydrophobicity parameter π. These results are explained by a mechanism in which electron transfer either takes place in the aqueous medium surrounding the hemoprotein, where hydrogen atom abstraction from a hydrogen donor solvent is the preferred process, or at the hydrophobic surface of hemoglobin, after which the neutral aryl radical enters the heme pocket to form the σ-arylheme adduct.

Introduction

Arylhydrazines react with hemoglobin and myoglobin in the presence of dioxygen to form σ-bonded aryliron(III) complexes whose thorough characterization by ^1H NMR spectroscopy in the intact protein[1,2] and by X-ray crystallography of phenylmyoglobin[3] has recently been reported. Although stable within the protein, these heme adducts normally rearrange to their corresponding *N*-arylprotoporphyrin IX complexes when the prosthetic group is extracted aerobically from the inactivated hemoprotein,[2,4,5] and

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recent investigations with model compounds have begun to reveal the intermediates involved in this transformation.[6-9] However, anaerobic extraction provides protoporphyrin IX with the aryl group bound directly to iron.[5,10,11] These complexes are implicated as products from reactions of arylhydrazines with a variety of hemoproteins whose prosthetic group is accessible to the aryl moiety,[12,13] and we have recently reported that the same σ-bonded aryliron(III) complexes are formed in reactions of arenediazonium salts with hemoglobin under anaerobic conditions.[14]

The oxidative conversion of arylhydrazines to σ-bonded aryliron(III) adducts of hemoproteins is complex and not fully understood. However, there is general agreement that aryldiazene intermediates are involved in this transformation.[1,2,15,16] Superoxide and phenyl radicals have been observed[10,17] as has hydrogen peroxide,[18] which suggests the intervention of aryldiazenyl radicals.[19] By comparison, the reduction of arenediazonium ions by hemoproteins is a simpler and kinetically manageable approach to these same aryldiazenyl radicals.[14] Both processes result in the formation of aryl radicals, with consequent production of aryliron(III) hemoprotein complexes, and they would thus appear to intersect mechanistically.

The oxidation of hemoglobin by arenediazonium salts is uniquely suited to detailed kinetic investigations. Our previously reported studies have demonstrated first-order rate dependence on both hemoglobin and the arenediazonium salt and inverse first-order dependence on the concentration of dioxygen.[20] However, the reduction of certain diazo compounds, specifically *p*-nitro- and *p*-cyanobenzenediazonium tetrafluoroborate, resulted in the formation of hydrogen abstraction products rather than the σ-bonded aryliron(III) adduct of hemoglobin.[14,20] Furthermore, preliminary data have shown that rate constants for the reduction of those diazonium salts that form aryliron(III) adducts are greater than predicted from results with model compounds.[14] We now present the results of a thorough kinetic and product evaluation of this unusual redox transformation. This study was undertaken to develop a clearer understanding of the mechanistic events associated with the formation of these adducts and to define how a normally restrictive hemoprotein allows access of a large aryl group into the heme pocket.

Experimental Section

Materials. Human hemoglobin A was prepared from fresh normal human blood (Southwest Texas Regional Blood Bank) by the method of Gibson,[21] stripped of organic phosphates, and deoxygenated as previously described.[20] Sperm whale myoglobin (type II), obtained from Sigma Chemical Co., was reduced with excess sodium dithionite and further purified by passing the resulting aqueous solution through a G-25 Sephadex column using 0.050 M phosphate buffer at pH 7.0. Solutions of oxymyoglobin were degassed under reduced pressure (less than 0.5 Torr) without evidence of deoxygenation of the hemoprotein. Heme concentrations were calculated from the published molar absorptivities for deoxyhemoglobin[22] and oxymyoglobin.[23] Arenediazonium tetrafluoroborate salts were prepared from the corresponding anilines, *tert*-butyl nitrite, and boron trifluoride etherate,[24] recrystallized from acetone–hexane, and characterized by NMR and IR spectroscopy. The diazonium salt prepared from (*p*-aminophenyl)acetic acid was the acid tetrafluoroborate salt. Variamine Blue RT was obtained from the Aldrich Chemical Co. Acetonitrile was distilled from calcium hydride, and double-distilled water was employed. Stock solutions of the diazonium salts (0.02 M) were prepared in anhydrous acetonitrile and maintained under nitrogen. Stock solutions of deoxyhemoglobin were prepared in deoxygenated phosphate buffer (0.050 M, pH 7.0) and kept in sealed air-tight flasks under nitrogen.

Kinetic Measurements. Procedures for reactions with potassium ferrocyanide have been described.[25] Reactions with hemoglobin were initiated with the injection, using a gastight syringe, of 20–40 μL of the stock solution of the diazonium salt into 3.00 mL of hemoglobin solution containing 0.050 M phosphate buffer at pH 7.0 in a septum-fitted spectrophotometer cell thermostated at 25.0 °C. Initial reactant concentrations of deoxyhemoglobin were $(0.7–7) \times 10^{-5}$ M heme, and those for diazonium salts were $(1–7) \times 10^{-4}$ M. Rates of reaction were determined by monitoring the decrease in absorbance of deoxyhemoglobin at 552 nm with time[26] using a Cary 118 or Hewlett-Packard 8451A spectrophotometer. Rapid-mixing experiments were required for reactions with *p*-nitro- and *p*-cyanobenzenediazonium tetrafluoroborate salts, and they were performed in a Dionex Model 110 stopped-flow spectrophotometer interfaced to a high speed, 12-bit A/D converter (OLIS, Inc.) and a microcomputer system. Reactions were carried out under pseudo-first-order conditions. The resultant time courses were fitted to an integrated single exponential process from which the pseudo-first-order rate constants were calculated. Linearity in semilog plots was observed through a minimum of 2 half-lives. The kinetic dependence on the diazonium salt was established by varying the molar ratio of diazonium salt to hemoglobin. Averaged second-order rate constants from 3 to 12 kinetic determinations are reported with average deviations of ±6%. Spectral changes for the oxidation of hemoglobin by *p*-nitro- or *p*-cyanobenzenediazonium tetrafluoroborate were characteristic of the conversion of deoxyhemoglobin to methemoglobin.[27] Those for oxidations by other *p*-substituted diazonium salts were similar but not superimposable.

Homogeneity of the hemoglobin solution in the presence of arenediazonium salts did not remain throughout the entire reaction. Precipitation of hemoprotein occurred during the course of the reaction, and its onset was dependent on the diazonium ion substituent. *p*-Nitrobenzenediazonium tetrafluoroborate caused precipitation at approximately 75% reaction, whereas with *p*-methyl- or *p*-chlorobenzenediazonium tetrafluoroborate the reaction solution was homogeneous through more than 3 half-lives. Precipitation did not occur upon addition of the arenediazonium salt to deoxyhemoglobin even when a 20-fold molar excess of the diazonium salt was employed. The precipitate that formed during the course of reaction contained a form of methemoglobin, but its structure was not determined. Methemoglobin appeared to be involved since addition of either the *p*-methyl- or *p*-nitrobenzenediazonium salt to freshly prepared methemoglobin also resulted in precipitation of the hemoprotein. However, methemoglobin does not reduce this diazonium salt, and no evidence of either the reduced form of hemoglobin, its σ-bonded aryliron(III) adduct, or nitrogen evolution was obtained from control experiments with methemoglobin. Although myoglobin is more suitable to these investigations because of its relative structural simplicity,[2] recognized difficulties in the deoxygenation of this hemoprotein and maintenance of deoxygenated conditions precluded its use for these studies.

Analyses for arene products were obtained directly from the reaction solution by HPLC immediately following complete hemoglobin oxidation. Reactions were performed with hemoglobin at heme concentrations of 0.5–1.5 mM and 1–5 molar equiv of the diazonium salt. Product yields were determined by separations on a 5-μm C_{18}-column using a Waters Associates Model ALC/GPC-244 Liquid Chromatograph system with the use of calibration curves obtained from external standards. Yields were reproducible to within ±3% of their reported values. Control ex-

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periments using the diazonium salt without hemoglobin were employed to monitor arene formation from residual diazonium salt caused by the analytical procedure (<2%). Analyses for *m*-xylene (from reactions with 2,4-dimethylbenzenediazonium tetrafluoroborate) and mesitylene (from reactions with 2,4,6-trimethylbenzenediazonium tetrafluoroborate) were performed by capillary GC (Hewlett-Packard Model 5890A) following ether extraction of the reaction mixture; their yields increased from 19 and 25%, respectively, when 1.0 equiv of the diazonium salt was employed, to 73 and 82% with the use of 5.0 molar equiv of diazonium salt.

σ-Arylheme complexes from reactions of arenediazonium salts with hemoglobin were isolated by an adaption of the procedure formulated by Kunze and Ortiz de Montellano.[1] Hemoglobin heme (1.0 mM) and the arenediazonium salt (0.5–2.0 mM) in 3.0 mL of 0.050 M deoxygenated phosphate buffer (pH 7.0) were combined under argon in a 10-mL flask. After reaction was complete, 3.0 mL of argon-presaturated 2-butanone containing 0.025% butylated hydroxytoluene (BHT) was added, the mixture was shaken gently, and the red organic layer was separated from the aqueous layer, which contained a hemin residue. The electronic spectrum of an aliquot of this organic extract dissolved in deoxygenated tetrahydrofuran exhibited a Soret maximum at 410 nm and an additional characteristic absorption at 562 nm with a shoulder at 530 nm. The absorption maxima for these complexes, formed from *p*-chloro- through *p*-methoxybenzenediazonium salts, showed no apparent variation with the benzene substituent (<±3 nm). The solution containing the *p*-tolyl complex was transferred to a Schlenk tube, and 2-butanone was evaporated and replaced by acetone-d_6 for NMR analysis, which confirmed its assignment.[1] Mass analyses following solvent evaporation showed that 46 ● 8% of total heme was extracted into the 2-butanone layer; further extraction of the aqueous layer produced less than 3% additional arylheme complex. Residual hemin that precipitated from the aqueous layer amounted to approximately half of the total heme mass and was not extracted into the 2-butanone layer. Neither cysteine (1.0 molar equiv) nor 2-propanol (0.05 M) prevented the formation of the σ-arylheme adduct formed from reactions between deoxyhemoglobin and *p*-methylbenzenediazonium tetrafluoroborate. The presence of the red 2-butanone solution and its characteristic absorption spectrum for the σ-aryliron(III) adduct was confirmed for reactions of hemoglobin with *p*-chloro-, *p*-fluoro-, *p*-methyl-, *p*-ethyl-, *p*-isopropyl-, *p*-*tert*-butyl-, *p*-*n*-hexyloxy-, and *p*-methoxybenzenediazonium and benzenediazonium tetrafluoroborate salts. For those diazonium salts that did not form these complexes, control experiments were performed at the same time with one of those that did to certify the viability of the method. The red σ-aryliron(III) adducts were stable for prolonged periods (>7 days) in the absence of oxygen.

Reactions of *p*-methylbenzenediazonium tetrafluoroborate with oxymyoglobin were performed at dioxygen concentrations that were approximately 10 times that of the hemoprotein. Following complete oxidation, residual dioxygen was removed under vacuum (<0.5 Torr). Extraction of the red σ-tolylhemin complex was performed as described.

***N*-Arylhemin derivatives** were produced upon exposure of the σ-aryliron(III) adducts to air. Hemin reaction products were esterified with methanol and converted into their corresponding zinc(II) complexes by established procedures.[4,10] The mixture was chromatographed on silica gel (Kodak) preparative thin-layer plates developed with 5:1 (v/v) chloroform–acetone, and the green and brown porphyrin bands were analyzed separately. The Soret band for the green zinc(II) complex of *N*-(*p*-tolyl)protoporphyrin IX dimethyl ester was at 442 nm, and characteristic absorptions at 550 and 608 nm with a shoulder at 648 nm were evident. In contrast, the Soret band for the zinc(II) complex of protoporphyrin IX dimethyl ester is found at 400 nm. Equimolar amounts of the zinc(II) complexes of *N*-(*m*-tolyl)protoporphyrin IX and protoporphyrin IX dimethyl esters were produced in reactions between deoxyhemoglobin and *m*-methylbenzenediazonium tetrafluoroborate. Absorption maxima for these complexes did not vary (<±2 nm) with the benzene substituent.

Reaction of Oxyhemoglobin with (*p*-Nitrophenyl)hydrazine. Hemoglobin (0.65 mM heme) in 50 mL of 0.050 M phosphate buffer (pH 7.0) containing 0.1 mM EDTA was combined with 1.0 molar equiv of (*p*-nitrophenyl)hydrazine. The reaction was performed under aerobic conditions, and upon completion, the resulting solution was treated as previously described[10] to ascertain the presence of the zinc(II) complex of *N*-(*p*-nitrophenyl)protoporphyrin IX. Chromatographic separation and analysis showed a mixture of zinc(II) complexes of *N*-(*p*-nitrophenyl)-protoporphyrin IX (20%) and protoporphyrin IX (80%) dimethyl esters. Separate experiments with either *p*-nitrophenylhydrazine or *p*-nitrophenyldiazene[28] designed to isolate the σ-arylheme complex were unsuccessful.

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Table I. Second-Order Rate Constants for the Reduction of Arenediazonium Tetrafluoroborate Salts ($ArN_2^+BF_4^-$) by Potassium Ferrocyanide and by Hemoglobin[a]

Ar	$k_X^{Fe(CN)_6^{4-}}$, M^{-1} s^{-1}	$(k_X/k_{PhNH})^{Fe(CN)_6^{4-}}$	k_X^{Hb}, M^{-1} s^{-1}	$(k_X/k_{PhNH})^{Hb}$
p-$NO_2C_6H_4$	695×10^2	2.76×10^6	99.2×10^2	3.63×10^3
p-NCC_6H_4	103×10^2	4.09×10^5	36.8×10^2	1.34×10^3
p-ClC_6H_4	276	1.10×10^4	262	96.0
p-FC_6H_4	52.6	2.09×10^3	102	37.4
C_6H_5	22.7	9.01×10^2	56.2	20.6
m-CH_3	8.43	3.34×10^2	194	71.1
p-Me_2CH	2.44	96.8	132	48.4
p-CH_2COO^-	2.08	82.5	31.1	11.4
p-CH_3	2.06	81.7	113	41.4
o-CH_3	1.22	48.4	31.5	11.5
p-(*n*-HexO)	1.23	48.8	41.2	15.1
p-CH_3O	0.708	28.1	74.4	27.3
p-PhNH[b]	2.52×10^{-3}	1.00	2.73	1.00

[a] Reactions were performed under nitrogen in 0.050 M phosphate buffer at pH 7.0 and 25.0 °C. [b] Bisulfate salt (Variamine Blue RT salt).

Results

The reaction of arenediazonium tetrafluoroborate salts with potassium ferrocyanide was chosen as a model redox system for comparative evaluation of outer-sphere electron transfer in reductions of these same diazonium salts by hemoglobin. In both cases reactions were performed under nitrogen in aqueous phosphate-buffered solutions at pH 7.0 and 25.0 °C. Reaction rates were first order in both the diazonium salt and in the reducing agent, and their calculated second-order rate constants, spanning more than 6 orders of magnitude for reductions by ferrocyanide and more than 3 orders of magnitude for reductions by deoxyhemoglobin, are presented in Table I (X = benzene substituent).

The reduction of arenediazonium salts by ferrocyanide has been established as an outer-sphere electron-transfer process through comparative kinetic evaluations of diazonium salt reductions by decamethylferrocene together with application of the Marcus relationship to obtain their self-exchange rate constants.[25] Aryldiazenyl radicals, generated by electron transfer from ferrocyanide (eq 1), dissociate dinitrogen, and in the presence of a

$$ArN_2^+ + Fe(CN)_6^{4-} \rightarrow ArN_2^{\bullet} + Fe(CN)_6^{3-} \quad (1)$$

$$ArN_2^{\bullet} \xrightarrow{-N_2} Ar^{\bullet} \xrightarrow{SolH} ArH \quad (2)$$

hydrogen donor (SolH), the resulting aryl radicals undergo hydrogen abstraction (eq 2). Rate constants for reduction are dependent on arenediazonium ion substituents, and for reactions with $Fe(CN)_6^{4-}$ excellent correlation has been obtained with Hammett σ constants ($\rho = +4.7$).[14,25]

We expected that if outer-sphere electron transfer occurred between hemoglobin and arenediazonium salts, a linear correlation would exist between the rate constants for reduction by $Fe(CN)_6^{4-}$ and Hb. However, the actual results of this analysis (Figure 1) show considerable scatter of points. The rate constants for reduction of *p*-methyl-, *p*-isopropyl-, and *p*-methoxybenzenediazonium salts by hemoglobin are 2.7–3.1 times higher than predicted, while those of *p*-chlorobenzene-, *p*-fluorobenzene-, and benzenediazonium salts are 2.0–2.5 times lower. The point scatter in Figure 1 is significant when compared to the modest 20% maximum deviation of individual second-order rate constants for reduction by decamethylferrocene relative to those for reduction by ferrocyanide.[25] That these differences were not due to configurational changes in the structure of hemoglobin caused by the reversible binding of diazonium salts to redox inactive protein sites[29] was established by performing these reactions at constant hemoglobin concentration with increasing amounts of $ArN_2^+BF_4^-$ ranging from 5 to 20 times molar excess. The influence of the

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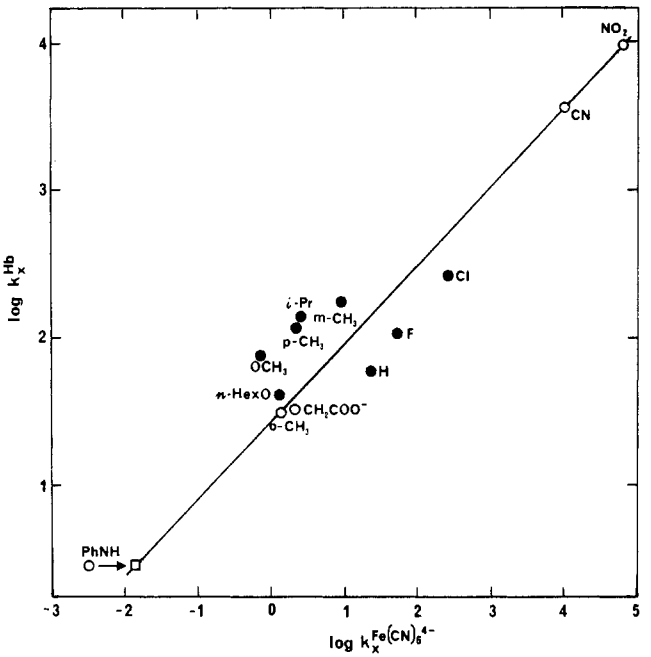

Figure 1. Plot of log k_X^{Hb} versus log $k_X^{Fe(CN)_6^{4-}}$ for reactions with substituted benzenediazonium ions in 0.050 M phosphate buffer (pH 7.0) at 25.0 °C: (●) diazonium ions that yielded σ-aryliron(III) adducts; (○) diazonium ions that yielded the hydrogen abstraction product.

$[ArN_2^+]/[Hb]$ molar ratio on the calculated second-order rate constants for hemoglobin oxidation was negligible.

Product analyses demonstrated a distinct dependence of the reaction course on diazonium ion substituents. Those diazonium salts whose rate constants lie on the line defined by (log k_X^{Hb} = 0.53 (log $k_X^{Fe(CN)_6^{4-}}$) + 1.43) in Figure 1 or close to it yielded the arene products expected from the sequence of reactions involving electron transfer, dissociation of dinitrogen, and hydrogen abstraction (eq 3). With the diazonium salt derived from (*p*-

$$X\text{–}C_6H_4N_2^+ + HbFe^{II} \xrightarrow{SolH} HbFe^{III} + X\text{–}C_6H_5 + N_2 \quad (3)$$

$$X = p\text{-}NO_2,\ p\text{-}CN,\ p\text{-}CH_2COO^-,\ o\text{-}CH_3,\ p\text{-}PhNH$$

aminophenyl)acetic acid, for example, an 82% yield of phenylacetic acid, based on the stoichiometry of eq 3, was realized when only a 2-fold molar excess of the diazonium salt was employed. Similar results were obtained with *p*-cyanobenzenediazonium tetrafluoroborate, and reactions of deoxyhemoglobin with *p*-nitrobenzenediazonium tetrafluoroborate provided a quantitative yield of nitrobenzene.[20] Acetonitrile is a hydrogen donor solvent[30] whose presence in the reaction medium increases the yield of the arene product. Control experiments showed that diazonium salts were stable in the presence of methemoglobin, $HbFe^{III}$.

In contrast, those diazonium salts whose rate constants for hemoglobin oxidation deviated from the line in Figure 1 resulted in σ-bonded aryliron(III) hemoprotein adducts. The red σ-arylheme complexes were separated from globin under anaerobic conditions and identified from their characteristic electronic spectra.[1,7] The separation procedure was specific for the σ-arylheme complex so that neither hemin nor *N*-arylprotoporphyrin IX provided interference. Exposure of these dioxygen-sensitive σ-arylheme complexes to air resulted in the formation of their corresponding *N*-arylhemin derivatives whose well-defined green zinc(II) complexes were further confirmed by chromatographic and spectroscopic characterization.[4,10] The only exception was *m*-methylbenzenediazonium tetrafluoroborate, which yielded *N*-(*m*-tolyl)protoporphyrin IX directly; the σ-arylheme complex could not be isolated under conditions that were successful with each of the para-substituted benzenediazonium salts, and toluene was not observed.

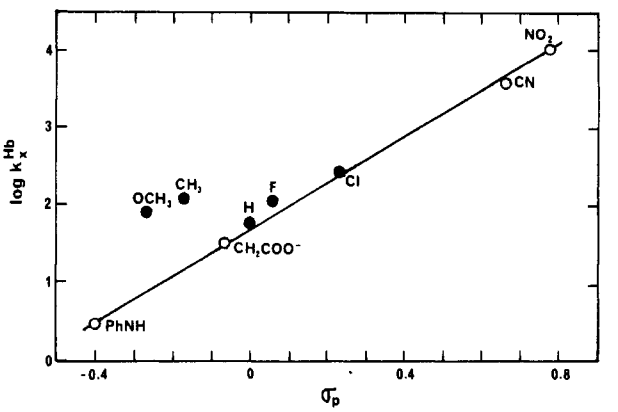

Figure 2. Hammett plot for reactions of para-substituted benzenediazonium ions with deoxyhemoglobin (ρ = +3.0).

The reaction stoichiometry was established to be that of eq 4 on the basis of extraction of identical amounts of the *p*-tolylhemin adduct when reactant $HbFe^{II}$:*p*-$CH_3C_6H_4N_2^+$ molar ratios of 2.0

$$X\text{–}C_6H_4N_2^+ + 2HbFe^{II} \rightarrow HbFe^{III}Ar + HbFe^{III} + N_2 \quad (4)$$

$$X = p\text{-Cl},\ p\text{-F},\ H,\ p\text{-}CH_3,\ m\text{-}CH_3,\ p\text{-}Me_2CH,\ p\text{-}CH_3O,\ p\text{-}(n\text{-HexO})$$

or less were employed and on the basis of the masses of the arylheme complex and hemin. Methemoglobin was identified by its characteristic absorption at 630 nm. Attempted detection of arene products failed to uncover these compounds, and even the use of hydrogen donors such as 2-propanol and cysteine did not inhibit formation of the σ-aryliron(III) adduct of hemoglobin from the reduction of *p*-methylbenzenediazonium tetrafluoroborate. The reaction of oxymyoglobin with *p*-methylbenzenediazonium tetrafluoroborate under controlled conditions also produced the σ-arylheme adduct. Although dioxygen is an inhibitor for these redox transformations,[20] its presence does not change the course of the reaction from that observed in its absence.

Of the diazonium salts that produce only arene products, *p*-anilinobenzenediazonium hydrogen sulfate is the only one that deviates from the line in Figure 1. The experimentally determined rate constant for its reduction by ferrocyanide does not fit the previously reported Hammett correlation.[14] However, calculation of the rate constant expected for reduction of this diazonium salt from the reaction ρ value of +4.7 and the σ value for the PhNH substituent[31] provides a number that gives an exact fit on the line in Figure 1. The cause for the deviation of this value from the calculated rate constant is unknown.

The marked differences between electron-transfer reactions that result in arene production and those that yield σ-arylheme adducts are even more evident in the Hammett plot for reactions of para-substituted benzenediazonium salts (Figure 2) than in Figure 1. Here, the values of log k_X^{Hb} for diazonium salts that undergo hydrogen abstraction are linearly related to σ values, whereas those for diazonium salts that produce σ-arylheme adducts all lie above this line. Each of the diazonium salts reacting according to eq 4 undergoes reduction at a faster rate than that predicted by the rate constants for those diazonium salts that result in arene production, and this deviation is greater than 1 order of magnitude for *p*-methoxybenzenediazonium tetrafluoroborate.

The data from Table I and corresponding product determinations pompted a more detailed analysis of the influence of arenediazonium ion substituents on reaction rate constants and on product formation from reactions with hemoglobin. To add to the series of *p*-alkyl-substituted benzenediazonium salts already consisting of methyl and isopropyl, rate and product analyses were performed with *p*-ethyl- and *p*-*tert*-butylbenzenediazonium tetrafluoroborate. Their respective rate constants for reactions with hemoglobin were 223 M^{-1} s^{-1} (*p*-Et) and 35.5 M^{-1} s^{-1} (*p*-*t*-Bu), and the σ-arylheme adduct was isolated in each case. In contrast,

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the diazonium salt derived from ethyl (*p*-aminophenyl)acetate oxidized hemoglobin with a rate constant (34.5 M^{-1} s^{-1}) that was nearly identical with the rate constant obtained from reduction of its carboxylate anion analogue (Table I), but the σ-arylheme adduct was not obtained in this case.

In order to obtain additional comparative results, *p*-nitrophenylhydrazine was subjected to oxyhemoglobin under a variety of conditions in attempts to isolate the corresponding σ-arylheme adduct. Although this complex was not obtained, the *N*-(*p*-nitrophenyl)protoporphyrin IX compound was isolated, and its structure was inferred from the electronic spectrum of its zinc(II) complex. A 20% yield of the *N*-arylprotoporphyrin IX complex was obtained when oxyhemoglobin was treated with 1 molar equiv of (*p*-nitrophenyl)hydrazine. By comparison, reduction of *p*-nitrobenzenediazonium tetrafluoroborate by hemoglobin under anaerobic conditions did not produce this adduct within the limits of our experimental detection (<5% yield).

Discussion

The mechanism for the oxidation of hemoglobin by arenediazonium salts that results in the production of methemoglobin and hydrogen abstraction products (eq 3) is straightforward. Electron transfer apparently occurs via the partially exposed heme edge without penetration of the oxidant into the heme pocket. Correlations between rate constants for hemoglobin oxidation by these diazonium salts with rate constants for ferrocyanide oxidation (Figure 1) and with Hammett σ values (Figure 2) suggest a uniformity of mechanism which parallels that of eq 1 and 2. Generation of the aryldiazenyl radical occurs in the aqueous environment that surrounds the protein. Following dissociation of dinitrogen, the resulting aryl radical abstracts a hydrogen atom from an available hydrogen donor molecule. That acetonitrile is effective as a hydrogen donor, even in the presence of dioxygen, has previously been demonstrated.[20] An alternative process in which the aryldiazenyl radical abstracts a hydrogen atom and then proceeds to products can be excluded from consideration since aryldiazenes are stable to further reduction by either deoxyhemoglobin[19] or ferrocyanide.[32] Similarly, electrophilic addition of the diazonium ion to the heme followed to homolytic cleavage[33] does not appear to be a viable alternative.

Oxidations of hemoglobin by arenediazonium ions that result in the production of σ-arylheme adducts are more complex. In these cases (eq 4) the rate constants for oxidation of hemoglobin do not correlate with those for oxidation of ferrocyanide or with Hammett σ values, as would be expected if electron transfer occurred in the same manner as, for example, hemoglobin reduction of the *p*-nitrobenzenediazonium ion. Precisely those arenediazonium ions whose rate constants deviate in these correlations are the ones that form σ-bonded aryliron(III) complexes, although rate constants for *p*-chlorobenzene-, *p*-fluorobenzene-, and benzenediazonium salts do approach predicted values (Figure 2).

We have previously suggested that those diazonium ions which react with hemoglobin to form σ-arylheme adducts undergo electron transfer in the heme pocket.[14] This explanation was based on the rationale that electron transfer produces a neutral aryldiazenyl radical that is more likely to expel dinitrogen and combine with iron(III) than to reenter the hydrophilic region outside of the heme pocket where hydrogen abstraction is the product-forming process. Accordingly, a crossover barrier for the diazonium ion should exist between the outer hydrophilic and inner hydrophobic[34] hemoglobin environments (eq 5). Since crossover

$$[HbFe^{II}]_{H_2O} + [ArN_2^+]_{H_2O} \rightarrow [HbFe^{II}(ArN_2^+)]_{H_2O} \rightarrow [HbFe^{III}(ArN_2^{\bullet})]_{H_2O} \quad (5)$$

into the hydrophobic region reduces the distance for electron transfer from the heme group, the rate for oxidation of hemoglobin becomes a function of the kinetic barrier for transport of the diazonium ion from the hydrophilic to the hydrophobic environment. Consistent with this explanation, a plot of log k_X for hemoglobin oxidations by those diazonium salts that produce σ-arylheme adducts (eq 4) versus the hydrophobic parameter π[35] (Figure 3) shows a good correlation (slope = 0.57) except for arenediazonium ions with bulky isopropyl and *tert*-butyl substituents. With the exclusion of the *o*-methylbenzenediazonium ion, the diazonium ions that apparently undergo electron transfer outside of the heme pocket possess substituents that are more polar, and are therefore capable of a higher order of solvent stabilization, than those of diazonium ions that form σ-arylheme complexes.

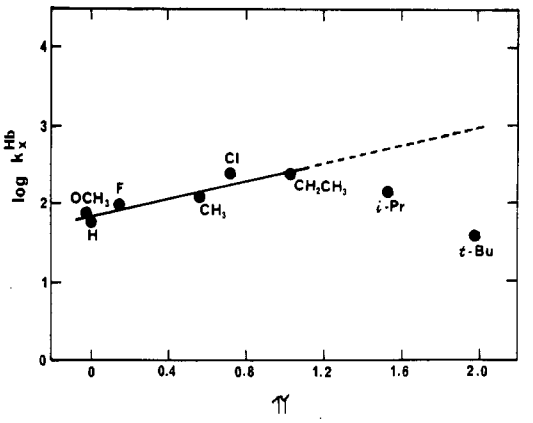

Figure 3. Plot of log k_X^{Hb} versus the hydrophobicity parameter π for para-substituted benzenediazonium ions whose oxidations of hemoglobin resulted in the formation of σ-arylheme adducts.

Although there is sufficient kinetic evidence to suggest that transport of diazonium ions occurs from a hydrophilic environment to a hydrophobic protein environment, evidence for our earlier suggestion that electron transfer takes place in the heme pocket is based solely on the formation of σ-bonded aryliron(III) adducts. However, actual entrance of an ionic diazonium ion into the hydrophobic heme pocket appears to be unlikely, particularly if the ionic diazonium group must be oriented toward iron. Hemoglobin is a closely packed structure that has no obvious static pathway for ligation of molecular entities as large as phenyl.[36] Instead, we suggest that *electron transfer takes place at the hydrophobic surface of the protein, and only then does the neutral radical enter the heme pocket.* Diazonium ions that come onto the protein surface can undergo electron transfer in closer proximity to the heme. Crossover of the diazonium ion onto the hydrophobic surface may be aided by interaction with basic protein residues such as that of histidine at the α-45 (CD3) position or at the β-63 and α-58 (E7) positions, but evidence for such a specific process is not available in this study. The energy associated with formation of the carbon–iron σ-bond may, in part, compensate for the energy expended in the configurational disruption of the heme pocket.

The variety of para substituents in the benzenediazonium ion that are effective in producing σ-bonded aryliron(III) adducts with hemoglobin is surprising. Previous reports have documented the formation of *p*-tolylheme complexes,[1,2] but not those with benzene substituents bulkier than methyl. Even very large substituents such as *tert*-butyl and *n*-hexyloxy do not prevent formation of the σ-arylheme complexes. Instead, once electron transfer has taken place, there is a greater driving force for formation of a carbon–iron σ-bond than for crossover of the neutral radical into the hydrophilic environment surrounding the protein. In contrast, reactions of three *o*-methyl-substituted arenediazonium salts with hemoglobin only resulted in the production of their respective arenes, despite large deviations of their rate constants for reduction

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Table II. Second-Order Rate Constants for the Reduction of Representative Di- and Trisubstituted Arenediazonium Salts ($ArN_2^+BF_4^-$) by Potassium Ferrocyanide and by Hemoglobin[a]

Ar	$k_X^{Fe(CN)_6^{4-}}$, M^{-1} s^{-1}	k_X^{Hb} (obsd), M^{-1} s^{-1}	k_X^{Hb} (calcd),[b] M^{-1} s^{-1}	k_X^{Hb} (obsd)/ k_X^{Hb} (calcd)
2-Cl-6-MeC_6H_3	24.0	194	145	1.34
2,4-$Me_2C_6H_3$	6.88	556	75.9	7.33
2,4,6-$Me_3C_6H_2$	4.07	19.8	56.2	0.35

[a] Same reaction conditions as described in Table I. [b] Calculated from the linear relationship of Figure 1: $\log k_X^{Hb} = 0.53(\log k_X^{Fe(CN)_6^{4-}}) + 1.43$.

from those predicted from ferrocyanide reductions (Table II). In the case of 2,4,6-trimethylbenzenediazonium tetrafluoroborate, steric factors apparently play a role in inhibiting electron transfer by hemoglobin. However, rate constants for both 2,4-dimethyl- and 2-chloro-6-methylbenzenediazonium tetrafluoroborate are higher than predicted if electron transfer had occurred without crossover onto the protein surface. One possible reason for the absence of σ-arylheme complexes in reactions of *o*-methylbenzenediazonium ions with hemoglobin is that the diazenyl radical undergoes intramolecular hydrogen abstraction from the *o*-methyl substituent.[37]

The formation of σ-bonded aryliron(III) adducts from reactions of hemoglobin with arenediazonium salts is limited relative to reactions of hemoglobin with arylhydrazines. In contrast to the corresponding hydrazine,[2] the *o*-methylbenzenediazonium ion does not form this adduct. In addition, *p*-nitrophenylhydrazine is effective in forming *N*-(*p*-nitrophenyl)protoporphyrin IX whereas the corresponding diazonium ion produces only the hydrogen abstraction product. The cause of these differences is apparently due to the existence of aryldiazene intermediates in reactions of hemoglobin with arylhydrazines.[15]

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Registry No. $Fe(CN)_6^{4-}$, 13408-63-4; *p*-$NO_2C_6H_4N_2^+BF_4^-$, 456-27-9; *p*-$NCC_6H_4N_2^+BF_4^-$, 2252-32-6; *p*-$ClC_6H_4N_2^+BF_4^-$, 673-41-6; *p*-$FC_6H_4N_2^+BF_4^-$, 459-45-0; $C_6H_5N_2^+BF_4^-$, 369-57-3; *m*-$CH_3C_6H_4N_2^+BF_4^-$, 1422-76-0; *p*-$Me_2CHC_6H_4N_2^+BF_4^-$, 403-48-5; *p*-$CH_2COO^-C_6H_4N_2^+$, 110118-02-0; *p*-$CH_3C_6H_4N_2^+BF_4^-$, 459-44-9; *o*-$CH_3C_6H_4N_2^+BF_4^-$, 2093-46-1; *p*-(*n*-HexO)$C_6H_4N_2^+BF_4^-$, 88360-98-9; *p*-$CH_3OC_6H_4N_2^+BF_4^-$, 459-64-3; *p*-$PhNHC_6H_4N_2^+BF_4^-$, 2367-19-3; 2-Cl-6-$MeC_6H_4N_2^+BF_4^-$, 85070-46-8; 2,4-$Me_2C_6H_3N_2^+BF_4^-$, 452-02-8; 2,4,6-$Me_3C_6H_2N_2^+BF_4^-$, 23755-18-2.

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Active-Site Chemistry of Hemerythrin: Mechanistic Routes in the Redox Interconversion of Deoxy and Met Forms

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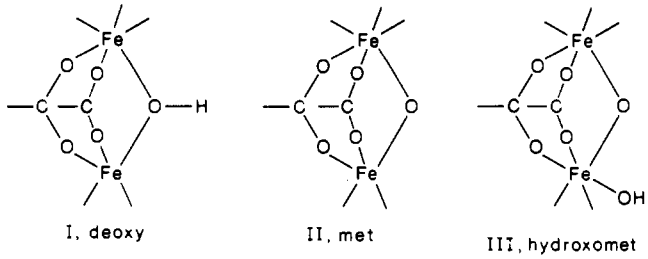
Two kinetic stages are observed in the oxidation of *Themiste zostericola* deoxyHr $Fe(II,II)_8$ to metHr $Fe(III,III)_8$ with $[Fe(CN)_6]^{3-}$, $[Co(dipic)_2]^-$, and $[Ru(NH_3)_5(CH_3CN)]^{3+}$ at 25 °C, pH 6.3–7.0 (Mes) and 7.4–9.0 (Tris), and I = 0.15 M (Na_2SO_4). With use of intensely colored spinach plastocyanin, PCu^{II}, as oxidant it has been shown that 1 equiv of oxidant is consumed in each stage. A feature of the reaction with $[Fe(CN)_6]^{3-}$ is the observation of saturation kinetics for the first stage at pH 6.3 (but not pH 8.2), consistent with association of $[Fe(CN)_6]^{3-}$ with deoxyHr (K = 4300 M^{-1}) prior to electron transfer. The second stage (k_2) is independent of the concentration and identity of oxidant, with rate constants 0.61 × 10^{-3} s^{-1} at pH 6.3 and 1.27 × 10^{-3} s^{-1} at pH 8.8. The product of this stage, corresponding to $(semi\text{-}met)_R$, reacts rapidly with excess oxidant to give metHr. With insufficient oxidant, however, the product disproportionates to a species (F) also in a rapid step. Studies on the reduction of the latter with $[Co(sep)]^{2+}$ are included. The reduction of metHr from *Phascolopsis gouldii* has also been investigated briefly and is shown to proceed in three stages by a route similar to that previously reported for *Themiste zostericola*.

Introduction

There have been significant advances recently in understanding the structure and properties of the binuclear Fe active site of hemerythrin, which appears to be the same in the monomer (myo) and octamer forms and to be independent of the source. From X-ray crystallography[1] and EXAFS,[2,3] structure I is indicated for the deoxy form, with histidine coordinated in the (five) terminal positions. The Fe(II)'s in I are only weakly antiferromagnetically coupled (−13 cm^{-1}),[4,5] which together with the evidence obtained from crystallography and EXAFS is consistent with a hydroxo-bridged structure. Strong antiferromagnetic coupling is however observed for the met form (J = −134 cm^{-1}), which indicates the presence of an oxo bridge. There are in addition μ-carboxylato ligands. Recent X-ray crystal studies[6] on metHr from *Themiste dyscritum* (pH <6.5) have indicated a structure in which one of the Fe(III) atoms is octahedral and the other trigonal bipyramidal as in II. It has been demonstrated that solvent OH^- coordinates to the five-coordinate Fe to give III in a relatively slow acid–base equilibrium ($t_{1/2} \approx$ 1 min at 25 °C),[7] the pK_a of which is ~7.8. At pH >9 resonance Raman studies have detected the hydroxomet form III.[8] X-ray crystallography,[6] EXAFS,[2,3] Mössbauer,[4] and



I, deoxy II, met III, hydroxomet

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