Oxidation of Hemoglobin by Arenediazonium Salts. The Influence of Dioxygen

MICHAEL P. DOYLE*, SURENDRA N. MAHAPATRO and SAT VAN TRAN
Department of Chemistry, Hope College, Holland, Mich. 49423, U.S.A.
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The reactions of human hemoglobin with p-nitro- and p-chlorobenzenediazonium tetrafluoroborates in the presence and absence of molecular oxygen have been investigated in kinetic detail. The oxidation of iron(II) occurs with first order rate dependence on both the hemoglobin and diazonium salt concentrations, but inverse first order dependence on the concentration of molecular oxygen characterizes reactions performed in the presence of O_2. In the absence of O_2, nitrobenzene is the only product observed from hemoglobin oxidation by $p\text{-}NO_2C_6H_4N_2^+BF_4^-$, and a 1:1 stoichiometry exists between nitrobenzene produced and Fe(II) oxidized. In the presence of O_2, p-nitrophenol is the dominant product, but product yield is dependent on the ratio of reactants. Electron transfer to the diazonium salt rather than its corresponding diazohydroxide or diazoate is inferred from the relative absence of pH dependence on the rate of oxidation. The composite results are consistent with a mechanism for hemoglobin oxidation that requires molecular oxygen dissociation from oxyhemoglobin prior to oxidation by the diazonium salt. Implications of this investigation for the mechanism of arylhydrazine reactions with hemoglobin are discussed.

Introduction

The oxidation of hydrazines, particularly arylhydrazines, by hemoproteins is a subject of considerable interest [1–13]. Arylhydrazines produce partial inhibition or destruction of hemoproteins [3–6] and with hemoglobin cause its oxidation and precipitation in the form of Heinz bodies [7–9]. The formation of carbon-centered free radicals has been shown to occur during hemoprotein-catalyzed oxidation of monosubstituted hydrazines $RNHNH_2$ [10, 11], and σ-aryliron(III) complexes have been identified as products from reactions of an arylhydrazine with hemoglobin and myoglobin [12]. The phenyliron(III) complex $(C_6H_5)Fe^{III}TPP$ (TPP = mesotetraphenylporphyrin dianion) has been obtained from phenyldiazene ($C_6H_5N{=}NH$) upon reaction with either $Fe^{III}TPP$ or $Fe^{III}TPP(Cl)$ or from phenylhydrazine in nearly quantitative yield upon reaction with $Fe^{III}TPP(Cl)$ and dioxygen [13]. However, the detailed mechanism of this complex transformation remains elusive.

Evaluation of the hydrazine oxidation is complicated by the number of potential intermediates in the transformation. The involvement of diazene intermediates is becoming increasingly evident [13] and, since carbon-centered free radicals have been observed and arenes (ArH) are the principal reaction products, aryldiazo radicals ($ArN_2\cdot$) should be considered as likely intermediates in hemoprotein-catalyzed oxidations of arylhydrazines. However, independent generation of aryldiazo radicals in the presence of hemoproteins has not been reported.

Arenediazonium salts are well-known precursors of aryldiazo radicals through electron transfer processes [14–16]. Surprisingly, the reactions of arenediazonium salts with hemoproteins have not been examined. In fact, despite the extensive literature on the synthetic utilization of diazonium salts [17, 18] and their known high electron affinities [16, 19, 20], there has been no report of their electron transfer characteristics with any iron(II) complex. Arenediazonium salts can be considered to be the final stage of arylhydrazine oxidations and, by electron transfer, they are convenient sources of aryldiazo radicals. We now report kinetic and product studies of the reactions of two arenediazonium salts with oxyhemoglobin and deoxyhemoglobin and their implications for the mechanism of arylhydrazine oxidations.

Experimental

Materials

Human hemoglobin A (type IV), 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 0.05 *M* phosphate buffer at pH 7.0. Concentrated solutions of oxyhemoglobin (approximate-

*Author to whom correspondence should be addressed.

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ly 1.0 m*M* heme) were degassed under reduced pressure (less than 0.5 torr), and the resultant deoxyhemoglobin was maintained in a sealed air-tight flask at atmospheric pressure under nitrogen. Treatment of hemoglobin with a 10-fold molar excess of iodoacetamide, relative to heme, at pH 7.8 [21] resulted in the selective conversion of β-93 cysteine residues to the corresponding sulfide. The absence of accessible sulfhydryl groups was determined with 5,5′-dithio-bis(2-nitrobenzoic acid) [22]. Heme concentrations for deoxyhemoglobin [23] and for oxyhemoglobin [24] were determined from their respective molar absorptivities. Oxygen concentrations in the buffered solutions were measured and varied as previously described [25]. Diazonium salts were prepared by the standard procedure [26].

Kinetic Measurements and Analysis

Reactions were initiated with the injection, using a gas-tight syringe, of a concentrated solution of the diazonium salt (2×10^{-2} *M*) in anhydrous acetonitrile into the hemoglobin sample (usually 20–40 μM) contained in 0.5 *M* phosphate buffer at pH 7.0. Rates for oxyhemoglobin oxidations were determined by monitoring the decrease in absorbance at 576 nm with time using a Pye Unicam SP8-200 spectrophotometer. With deoxyhemoglobin, oxidation rates were determined by monitoring the decrease in absorbance at 552 nm with time. Rapid mixing experiments with *p*-nitrobenzenediazonium tetrafluoroborate and deoxyhemoglobin were carried out in a Dionex model 110 stopped flow spectrophotometer interfaced to a high speed, 12-bit A/D converter (OLIS, Inc., Athens, Ga.) and a Northstar microcomputer system. In all kinetic experiments, reactions were carried out under pseudo-first order conditions where diazonium salt concentrations were generally in 5–10-fold molar excess with respect to the total heme groups present. The resultant time courses were fitted to an integrated single exponential process from which the pseudo-first order rate constants were calculated. Linearity in these plots was observed through 60–70% of the complete conversion of hemoglobin to methemoglobin. Averaged rate constants from a minimum of 4 kinetic determinations, or 20 in stopped-flow measurements, are reported with average deviations of ±5% of the reported values.

Product Analyses

Product yields were determined by HPLC on a 5μ C_{18}-column using a Waters Associates Model ALC/GPC-244 Liquid Chromatograph System interfaced with a Waters Intelligent Information Processor. Efficient separations were obtained in CH_3CN:H_2O:AcOH (26:68:6) at a flow rate of 2 ml/min. Yields were calculated with the use of calibration curves obtained from external standards and were reproducible to within ±4% of their reported values. Reaction solutions were analyzed directly. Control experiments using the diazonium salt without hemoglobin were employed to monitor product formation from residual diazonium salt caused by the analytical procedure. Products were identified by spectral analysis following their isolation by extraction as well as from HPLC analysis through peak enhancement and comparative retention times.

Results

Oxidation of Deoxyhemoglobin

Treatment of deoxyhemoglobin A (Hb) with diazonium tetrafluoroborates results in a facile and uniform conversion to methemoglobin (Hb^+). Figure 1 describes the spectral changes observed during the kinetically homogeneous course of the reaction of *p*-chlorobenzenediazonium tetrafluoroborate with deoxyhemoglobin. Although the production of Hb^+ is evident from the characteristic increase in absorbance at 630 nm with time, at approximately 70% of the full time course for Hb^+ production, dependent on the initial $[ArN_2^+]/[Hb]$ ratio, secondary reactions of

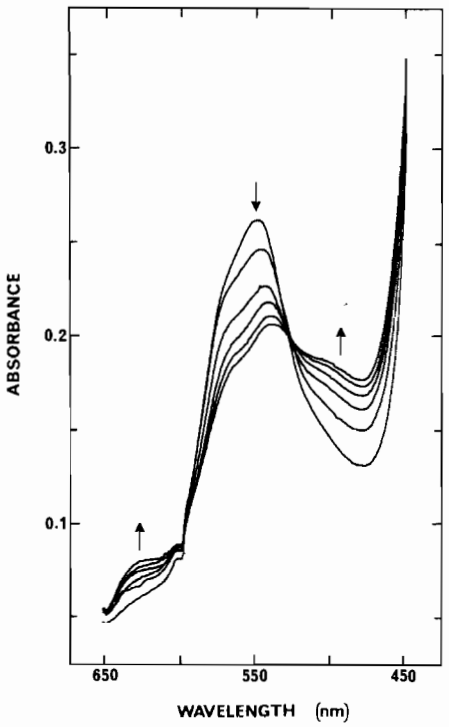

Fig. 1. Spectral time course for the reaction of deoxyhemoglobin with *p*-chlorobenzenediazonium tetrafluoroborate. Reaction was performed on the deoxygenated solution in 0.05 *M* phosphate buffer, pH 7.0, 20.0 °C: 1.95×10^{-5} *M* (heme), 2.9×10^{-5} *M* p-$ClC_6H_4N_2^+BF_4^-$. The initial scan was started 10 sec after addition, scan time for each spectrum was 40 sec, and subsequent scans were repeated at 50-sec intervals. Isosbestic points are observed at 598 and 528 nm.

Hb^+ that result in precipitation of the protein are observed. During this phase, absorbance changes reflect the inhomogeneity of the solution and are not recorded in Fig. 1. Consequently, product determinations were performed following the termination of all observable change but kinetic evaluation was obtained only through the homogeneous phase. In reactions with a stoichiometric amount of *p*-nitrobenzenediazonium tetrafluoroborate, nitrobenzene is the only observed product (49% yield), and the use of 2- to 5-fold excesses of this diazonium salt relative to heme (100% yield of nitrobenzene) suggests the reaction stoichiometry of eqn. 1. The corresponding

$$Hb + ArN_2^+ \rightarrow Hb^+ + ArH + N_2 \quad (1)$$

azo compound and biphenyl product were not observed (<0.5%) even when a 5-fold excess of diazonium salt was employed.

The rates for reactions of deoxyhemoglobin with diazonium salts have been investigated at 25 °C and pH 7.0. Typical time courses for reactions with *p*-chlorobenzenediazonium tetrafluoroborate at different molar ratios of $[ArN_2^+]/[Hb]$ are presented in Fig. 2. Pseudo-first order kinetics was observed for these reactions through greater than 90% of their homogeneous time course, which establishes the direct first order relationship of the rate of reaction

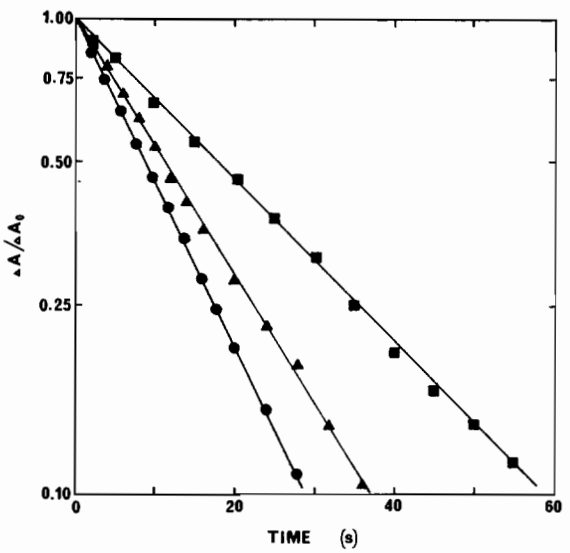

Fig. 2. Typical time courses for reactions of deoxyhemoglobin with *p*-chlorobenzenediazonium tetrafluoroborate. Reactions were observed at 552 nm on deoxygenated solutions in 0.05 *M* phosphate buffer, pH 7.0, 21.0 °C: 5.00 × 10^{-5} *M* (heme); (●) 4.83 × 10^{-4} *M* $ArN_2^+BF_4^-$, (▲) 3.50 × 10^{-4} *M* $ArN_2^+BF_4^-$, (■) 2.42 × 10^{-4} *M* $ArN_2^+BF_4^-$. When plotted against diazonium salt concentration, the reaction rate constants from such time courses describe a linear first order dependence on the concentration of diazonium salt.

with the concentration of hemoglobin. Furthermore, the pseudo-first order rate constants obtained for reactions performed as a function of $[ArN_2^+]$, k_{obs}, describe a first order dependence of the rate on the diazonium ion concentration. Therefore, the reactions of deoxyhemoglobin with arenediazonium salts follow a second order rate law with second order rate constants, k_2^{Hb}, reported in Table I.

TABLE I. Second-order Rate Constants for Oxidation of Hemoglobin at pH 7.0 and 25.0 °C.

Diazonium salt	$10^{-2}\,k_2^{Hb}$ $M^{-1}\,s^{-1}$	$k_2^{HbO_2}$ $M^{-1}\,s^{-1}$
p-$ClC_6H_4N_2^+BF_4^-$	2.62	8.17
p-$NO_2C_6H_4N_2^+BF_4^-$	99.3	166

Oxidation of Oxyhemoglobin

Like deoxyhemoglobin, oxyhemoglobin (HbO_2) reacts with diazonium tetrafluoroborates to produce methemoglobin with similar spectral constraints. Here, also, secondary reactions of Hb^+ with diazonium salts that result in precipitation of the protein are observed at approximately 70% of the full time course for the production of the aquo complex of methemoglobin. Uniform increases in absorption throughout the visible spectrum are characteristic of this complex second phase. The spectral changes observed during the kinetically homogeneous course of the reaction of *p*-chlorobenzenediazonium tetrafluoroborate with oxyhemoglobin in oxygen saturated media are described in Fig. 3. Surprisingly, the phenol is the dominant product, but complete stoichiometric accounting of the fate of the diazonium salt by the combination of substituted phenol and benzene could not be obtained at low molar ratios of diazonium salt to oxyhemoglobin. However, as demonstrated in Fig. 4, the yield of phenol is dependent on the $[ArN_2^+]/[HbO_2]$ ratio.

As indicated by the time courses for reactions of *p*-chlorobenzenediazonium tetrafluoroborate with oxyhemoglobin in oxygen saturated media at 25 °C (Fig. 5), pseudo-first order kinetics was observed through their homogeneous time courses. Pseudo-first order rate constants obtained for reactions performed as a function of $[ArN_2^+]$ describe a first order dependence of the rate on the diazonium ion concentration. Consequently, the rates for these oxidative reactions, like those for deoxyhemoglobin, are dependent on both hemoglobin and diazonium salt concentrations. Second order rate constants for the oxidation of oxyhemoglobin, $k_2^{HbO_2}$ are reported in Table I.

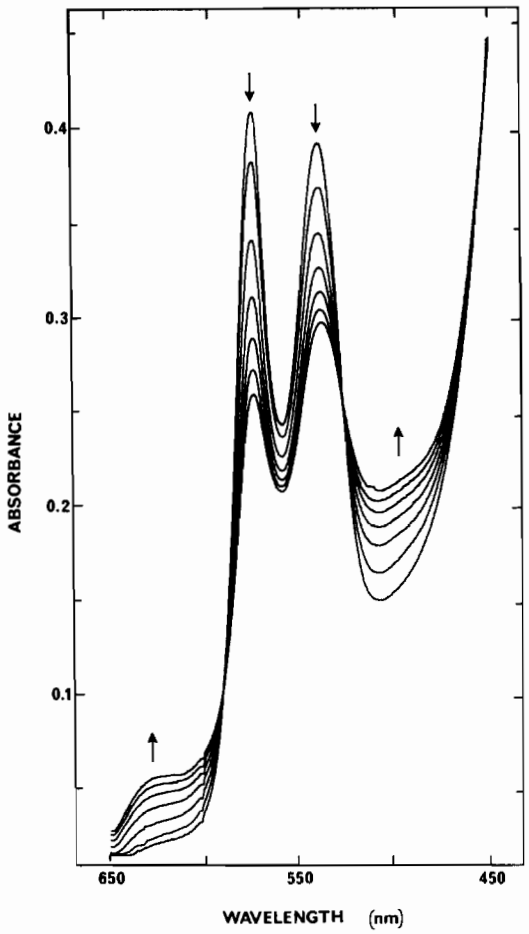

Fig. 3. Spectral time course for the reaction of oxyhemoglobin with *p*-chlorobenzenediazonium tetrafluoroborate. Reaction was performed at oxygen saturation in 0.05 *M* phosphate buffer, pH 7.0, 25.0 °C: 2.50×10^{-5} *M* (heme), 2.50×10^{-4} *M* $p\text{-ClC}_6\text{H}_4\text{N}_2^+\text{BF}_4^-$. The initial scan was started 10 sec after addition, scan time for each spectrum was 100 sec, and subsequent scans were repeated at 120-sec intervals. Isosbestic points are observed at 586, 528, and 456 nm.

Kinetic Dependence on Dioxygen Concentration

That the observed rates for oxidations of oxyhemoglobin by arenediazonium salts are significantly slower than rates for the corresponding oxidation of deoxyhemoglobin suggests that molecular oxygen plays an inhibitory role in the electron transfer process [25]. In order to evaluate the effect of molecular oxygen in diazonium salt oxidations of oxyhemoglobin, the dependence of the second order rate constant, $k_2^{HbO_2}$, on oxygen concentration was determined. The results of this study, in which $k_2^{HbO_2}$ is determined as a function of total oxygen concentration, are described in Fig. 6. A linear inverse dependence of the rate constant is observed throughout a nearly 30-fold change in the molecular oxygen concentration corresponding to oxygen concentrations from those that are nearly equivalent to the initial concentration of hemoglobin heme units to those that are approximately 30 times in excess of heme concentration. This inverse dependence is consistent with oxidation by the diazonium salt of oxyhemoglobin from which molecular oxygen has dissociated. Since the intercept of the line in Fig. 6 is $k_2^{HbO_2} = 0$, electron transfer from HbO_2 is not indicated.

Structure of the Oxidant

Diazonium salts form diazohydroxides and diazoates in alkaline media [27]. To determine the actual reactive oxidant in oxidative transformations of diazonium salts at pH 7.0, the reactions of *p*-chlorobenzenediazonium tetrafluoroborate with oxyhemoglobin were subjected to kinetic evaluation as a function of pH. Kinetic dependence on pH would indicate a role for the diazohydroxide or diazoate. However, the second order rate constants for these reactions at 20 °C in the pH range of 6.6 to 7.7 varied from 1.75 M^{-1} s^{-1} to 2.35 M^{-1} s^{-1} with a maximum near pH 7.0 of 2.36 M^{-1} s^{-1}. This pH dependence is inconsistent with the involvement of either a diazohydroxide or diazoate, and the minor variation in $k_2^{HbO_2}$ with pH is characteristic of oxyhemoglobin oxidations that are inhibited by molecular oxygen [25, 28].

Further evidence that the reactive oxidant is the diazonium salt was obtained by measurement of the rate of loss of diazonium salt at 260 nm in the absence of hemoglobin at pH 7.0. Under these conditions the rate of diazonium ion loss as a result of diazohydroxide formation is two orders of magnitude slower than the dioxygen-inhibited oxidation of HbO_2 by the same diazonium salt. Diazohydroxide and diazoate have no evident role in the oxidation of hemoglobin by diazonium salts.

Influence of Hydrogen Donors

Hemoglobin oxidation by arenediazonium salts might be considered to arise through involvement of the accessible β-93 cysteine residues of tetrameric hemoglobin. However, treatment of hemoglobin with iodoacetamide to form the corresponding β-93 sulfide did not alter the kinetics of hemoglobin oxidation and, with HbO_2, did not influence the relative production of nitrobenzene and *p*-nitrophenol from that reported in Fig. 4.

Discussion

The first order dependence of the rate on the concentrations of both hemoglobin and diazonium salt and the inverse first order dependence on the oxygen concentration are consistent with a mechanism for hemoglobin oxidation that requires molecular oxygen

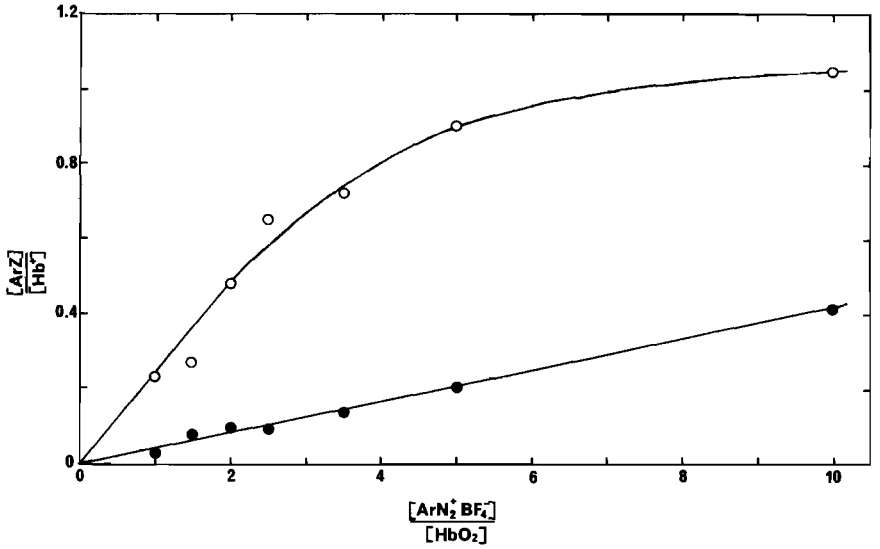

Fig. 4. Dependence of the product yield on the molar ratio of *p*-nitrobenzenediazonium tetrafluoroborate to oxyhemoglobin. All reactions were performed at oxygen saturation in 0.05 *M* phosphate buffer, pH 7.0, 25 °C, 1.0×10^{-4} *M* (heme): (○) *p*-nitrophenol, (●) nitrobenzene.

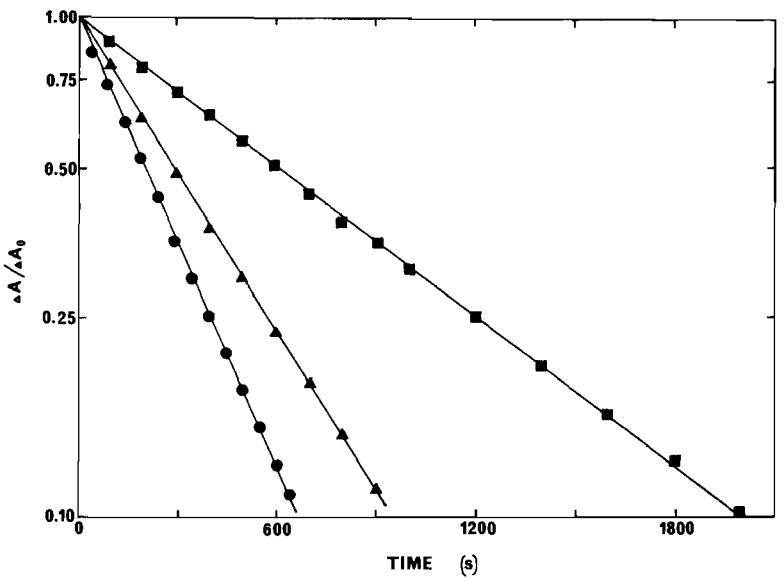

Fig. 5. Typical time courses for reactions of oxyhemoglobin with *p*-chlorobenzenediazonium tetrafluoroborate. Reactions were observed at 576 nm on oxygen saturated solutions in 0.05 *M* phosphate buffer, pH 7.0, 25.0 °C: 2.80×10^{-5} *M* (heme); (●) 4.12×10^{-4} *M* $ArN_2^+BF_4^-$, (▲) 2.91×10^{-4} *M* $ArN_2^+BF_4^-$, (■) 1.41×10^{-4} *M* $ArN_2^+BF_4^-$. When plotted against diazonium salt concentration, the reaction rate constants from such time courses describe a linear first order dependence on the concentration of diazonium salt.

dissociation from HbO_2 prior to oxidation by the diazonium salt (eqn. 2–3). Since dissociation and association of molecular oxygen are fast processes relative to oxidation, the observed rate for electron transfer

$$HbO_2 \overset{K^{O_2}}{\rightleftharpoons} Hb + O_2 \qquad (2)$$

$$Hb + ArN_2^+ \xrightarrow{k_0} Hb^+ + ArN_2\cdot \qquad (3)$$

to the diazonium salt is limited by the equilibrium concentration of hemoglobin from which a molecule of dioxygen has been dissociated. According to this scheme, and in the absence of subsequent reactions of $ArN_2\cdot$ or its derivative free radical products with hemoglobin, the observed rate constant for oxidation of deoxyhemoglobin, k_2^{Hb}, is equal to $k_o{}^T$ whereas that for oxidation of oxyhemoglobin, $k_2^{HbO_2}$, is equal to $k_o{}^R K^{O_2}/(K^{O_2} + [O_2])$. T and R in these

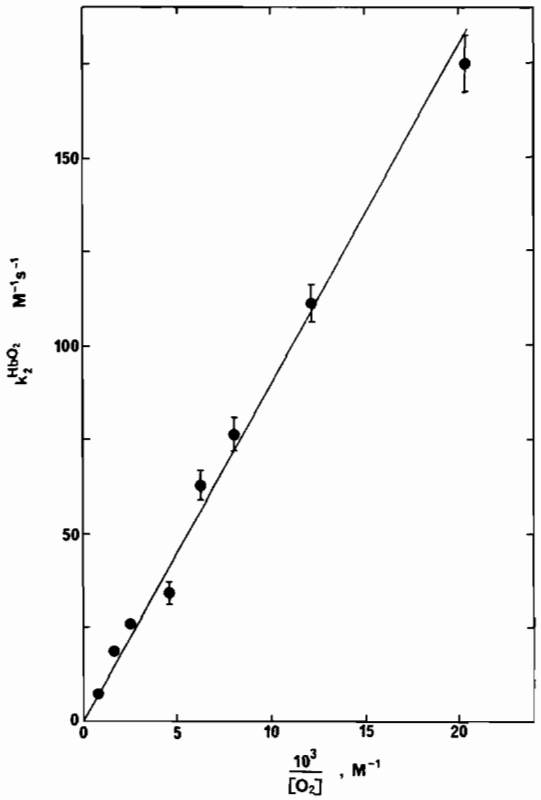

Fig. 6. Dependence of the rate constant for *p*-chlorobenzenediazonium tetrafluoroborate oxidation of hemoglobin on the concentration of dioxygen. All reactions were observed at 576 nm in 0.05 *M* phosphate buffer, pH 7.0, 25.0 °C, and total oxygen concentration is reported.

equations refer to the tense (T) and relaxed (R) states of hemoglobin [29], and values for k_o^T and k_o^R (Table II) indicate the rate enhancement caused by the $T \rightarrow R$ change in hemoglobin conformation. The reactivity ratios k_o^R/k_o^T for electron transfer to the two diazonium salts examined in this study are the largest yet observed for hemoglobin oxidation. Table II lists these values along with those for hemoglobin oxidation by ethyl nitrite [25] and $Fe(CN)_6^{3-}$ [30]. Although the implications of k_o^R/k_o^T values for the detailed mechanism of the electron transfer step are unclear, there is reason to believe that these values may reflect differential distances of

TABLE II. Rate Constants for Oxidation of Hemoglobin in its R- and T-states at pH 7.0.

Oxidant	$10^{-4}\,k_o^R$ $M^{-1}\,s^{-1}$	$10^{-4}\,k_o^T$ $M^{-1}\,s^{-1}$	k_o^R/k_o^T
p-$ClC_6H_4N_2^+BF_4^-$	4.99	0.0262	190
p-$NO_2C_6H_4N_2^+BF_4^-$	101	0.993	102
$Fe(CN)_6^{3-}$	70	0.7	101
EtONO	2.53	0.056	45

electron transfer to hemoglobin in its two conformational states [31].

The formation of nitrobenzene from the reaction of *p*-nitrobenzenediazonium tetrafluoroborate with deoxyhemoglobin is consistent with the intervention of the aryldiazo radical. Loss of dinitrogen and hydrogen atom transfer from a hydrogen donor SH (eqn. 4, 5) account for the production of nitro-

$$ArN_2\cdot \longrightarrow Ar\cdot + N_2 \quad (4)$$

$$Ar\cdot + SH \longrightarrow ArH + S\cdot \quad (5)$$

benzene. That only one-half of the diazonium salt is converted to ArH when a 1:1 molar ratio of ArN_2^+ to Hb is employed suggests that 2 Fe(II) are transformed per ArN_2^+. However, at this ratio of reactants secondary reactions of the diazonium salt with the protein, which are evident in our investigations but have not yet been identified, complicate product analysis. Since reactant ratios of ArN_2^+ to Hb equal to or greater than 2.0 consistently result in a 1:1 stoichiometry between nitrobenzene produced and Fe(II) oxidized, the reaction stoichiometry described in eqn. 1 provides the more reasonable accounting of this transformation.

In oxygenated media phenol production from the *p*-nitrobenzenediazonium salt is dominant, but product accountability is not as straightforward as is found in deoxygenated media. That *p*-nitrophenol is the dominant product demonstrates that dioxygen capture of the generated *p*-nitrophenyl radical (eqn. 6) is favored over hydrogen abstraction (eqn. 5).

$$Ar\cdot + O_2 \longrightarrow ArOO\cdot \quad (6)$$

The observed dependence of product yield on increasing $[ArN_2^+]/[HbO_2]$ (Fig. 4) indicates that the rate for product formation is directly dependent on the concentration of the diazonium salt. These results are consistent with the complex mechanism for autocatalytic decomposition of arenediazonium salts in the presence of dioxygen that has recently been advanced by Besse and Zollinger [32]. Aryl radicals have been reported to react with dioxygen, eventually forming phenols and dihydroxybenzenes [33]. In this study and in the mechanistic investigation of autocatalytic decomposition of ArN_2^+ [32], dihydroxybenzene products were not detected and are, consequently, presumed to be incorporated into hemoglobin and/or polymeric materials.

The reaction characteristics for the oxidation of hemoglobin by diazonium salts have several significant implications for hydrazine oxidations. As a result of the product accountability for ArN_2^+ reactions with deoxyhemoglobin, there is no reason to believe that aryldiazo or aryl radicals are generated inside the heme cavity where a σ-phenyl complex with the heme iron could have been produced. Furthermore, that *p*-nitrophenol is the dominant

reaction product from hemoglobin oxidation by *p*-nitrobenzenediazonium tetrafluoroborate in the presence of O_2, in which the *p*-nitrophenyl radical is a certain reaction intermediate, conflicts with the reported production of benzene, rather than phenol, from reactions of phenylhydrazine with hemoglobin in the presence of dioxygen [12]. If benzene is indeed produced, its origin is not the phenyl radical. Finally, precipitation of hemoglobin in the form of Heinz bodies does not appear to be a unique characteristic of reactions of arylhydrazines with hemoglobin since analogous events are observed for reactions of hemoglobin with diazonium salts both in the presence and absence of oxygen. Our continuing efforts are directed towards resolution of the unique mechanisms for reactions of dinitrogen compounds with hemoproteins.

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