Mechanism of Initial Reaction of Phenylhydrazine with Oxyhemoglobin and Effect of Ring Substitutions on the Bimolecular Rate Constant of This Reaction†

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ABSTRACT: Phenylhydrazine in the presence of oxygen causes the oxidative denaturation of hemoglobin. The initial step in this process is a bimolecular reaction, probably a two-electron transfer from phenylhydrazine to oxyhemoglobin. The product of this reaction is neither methemoglobin nor deoxyhemoglobin. Superoxide dismutase and catalase eliminate side reactions that increase the apparent rate of this reaction as measured spectrophotometrically at 577 nm; scavengers for the hydroxyl radical and singlet oxygen do not affect this rate either in the presence or in the absence of these enzymes. Halogen atoms and alkyl groups decrease the rate when ortho and increase the rate when meta or para to the hydrazino group. Chlorine atoms at both ortho positions or the carboxylate group at the ortho or the para position block the reaction. In the presence of phenylhydrazine under air, methemoglobin is converted to the same complex as that produced when phenyldiazene is added to methemoglobin anaerobically. Under N_2 or CO, phenylhydrazine reduces methemoglobin to deoxyhemoglobin or carbonmonoxyhemoglobin.

Addition of phenylhydrazine to a solution of oxyhemoglobin results in precipitation of a form of denatured hemoglobin in which the heme moiety has undergone oxidative changes. When oxidative denaturation takes place in an intact erythrocyte, the resulting intracellular precipitates are known as Heinz bodies, formation of which leads to severe hemolytic anemia and reticulocytosis. Although induction of anemia with phenylhydrazine is the most common method for obtaining reticulocytes for biochemical studies, the chemical mechanism of phenylhydrazine-promoted oxidative denaturation is not known. The severity of arylhydrazine-induced Heinz-body hemolytic anemia is influenced by substituents on the benzene ring of phenylhydrazine (Itano et al., 1976, 1977). In this work, we examine the reaction of phenylhydrazine with oxyhemoglobin and the effect of ring substitutions on this reaction.

Beaven & White (1954) found that phenylhydrazine is oxidized to benzene and nitrogen while oxyhemoglobin is degraded and precipitated as a "green hemoglobin", and Rostorfer & Cormier (1957) reported that the oxygen-combining capacity of hemoglobin is lost when phenylhydrazine reacts with oxyhemoglobin. Others showed that reactions of phenylhydrazine with molecular oxygen and with oxyhemoglobin produce hydrogen peroxide and superoxide radical (Cohen & Hochstein, 1964; Misra & Fridovich, 1976; Goldberg et al., 1976). Phenyldiazene, the product of a two-electron oxidation of phenylhydrazine (Cauquis & Geniès, 1968; Itano, 1970; Mannen & Itano, 1973), reduces oxygen to superoxide radical (Goldberg et al., 1976) and also combines with methemoglobin to form a stable complex (Itano & Mannen, 1976). Castro et al. (1978) concluded that deoxyhemoglobin is the ultimate product of oxyhemoglobin in its reaction with phenylhydrazine; however, this finding conflicts with earlier reports (Beaven & White, 1954; Rostorfer & Cormier, 1957) of products that do not bind oxygen. Itano et al. (1977) proposed that a bimolecular reaction between phenylhydrazine and oxyhemoglobin is the initial step in oxidative denaturation. We have found that the initial change in the electronic absorption spectrum of oxyhemoglobin is due to a simple bimolecular reaction; this reaction is independent of the oxidant species derived from molecular oxygen, and the product of this reaction is neither methemoglobin nor deoxyhemoglobin. We have also recorded spectrophotometrically the reactions that result from the addition of phenylhydrazine to methemoglobin both aerobically and anaerobically.

Experimental Procedures

Whole human blood was obtained by venipuncture from the same subject throughout the study. Reagent grade buffer salts (KH_2PO_4 and K_2HPO_4) were used without further purification. Lyophilized superoxide dismutase from bovine blood and catalase from bovine liver, obtained from Sigma Chemical Co. (St. Louis, MO), were stored below 0 °C. Phenylhydrazine hydrochloride and its ring-substituted homologues were obtained or synthesized as reported (Itano et al., 1977). The arylhydrazines were recrystallized before use in either 2 N HCl or 95% ethanol. Carboxymethylcellulose (CM-52) for column chromatography was obtained from Whatman Chemical Co. (Clifton, NJ). Washed, packed erythrocytes were lysed with 2.5 volumes of water. The hemolysate was centrifuged to remove insoluble material, chromatographed to remove superoxide dismutase and catalase (Lynch et al., 1977), and dialyzed twice against 4 L of 0.1 M potassium phosphate buffer of pH 7.4. The resulting solution of oxyhemoglobin was diluted with this buffer to a heme concentration of 5.0×10^{-5} M. Oxyhemoglobin solutions were stored at 4–5 °C and were used within 5 days of preparation. Solutions of methemoglobin were prepared by the addition of an excess of potassium ferricyanide to oxyhemoglobin, followed by removal of ferrocyanide and excess ferricyanide by gel filtration through Sephadex G-25. Stock solutions of superoxide dismutase and catalase, each 2.5×10^{-5} M in 0.1 M potassium phosphate (pH 7.4), were kept at 5 °C and were used within 4 h of preparation. Stock solutions of phenylhydrazine hydrochloride in the same buffer were bubbled continuously with O_2-free N_2 and were used within 4 h of preparation.

An equimolar reaction mixture was obtained by the addition of 20 μL of 0.01 M arylhydrazine hydrochloride to 4 mL of

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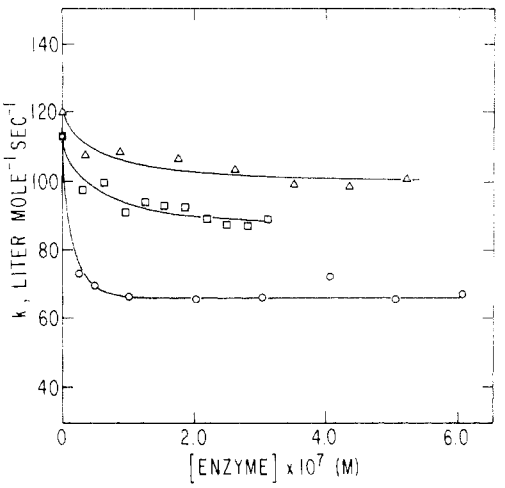

FIGURE 1: Effect of superoxide dismutase and catalase on the apparent rate constant of the bimolecular reaction between phenylhydrazine and oxyhemoglobin. Initial concentrations: [HbO_2] = 5.0×10^{-5} M; [$PhNHNH_2$] = 5.0×10^{-5} M. Buffer: 0.1 M potassium phosphate, pH 7.4. Temperature: 25 °C. (△) Catalase; (□) SOD; (○) catalase and SOD.

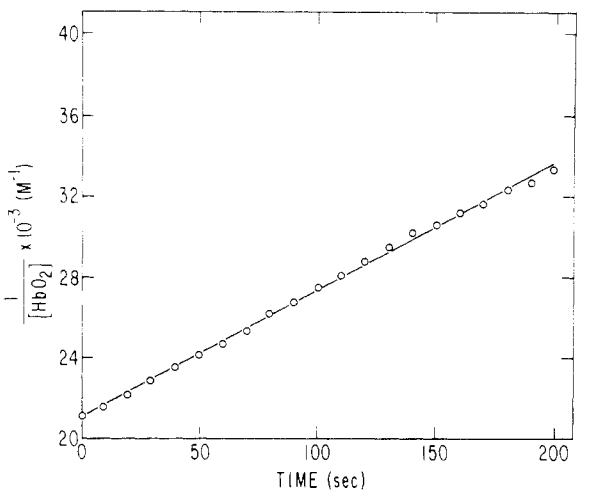

FIGURE 2: Second-order kinetics of the reaction of equimolar concentrations of phenylhydrazine and oxyhemoglobin in the presence of superoxide dismutase and catalase. Reaction conditions are as in Figure 1. Enzyme concentrations: [SOD] = [catalase] = 3.0×10^{-7} M.

5×10^{-5} M (in heme) oxyhemoglobin. The reaction was carried out at 25 °C, and the change in absorbance with time of the reaction mixture at 577 nm was recorded continuously with a Cary Model 17 spectrophotometer. The maximum change in absorbance at 577 nm was determined by adding a 10–12-fold excess of arylhydrazine to oxyhemoglobin and recording the rapid change of absorbance to a constant value. For calculations of reaction rate, absorbances of a reaction mixture were read from the tracing at intervals of 10 or 20 s, and the concentration of unreacted oxyhemoglobin was calculated for each point. Superoxide dismutase, catalase, or both were added in increasing amounts to determine enzyme concentrations required to eliminate side reactions. Thereafter 48 μL each of 2.5×10^{-5} M superoxide dismutase and 2.5×10^{-5} M catalase was added to the solution of oxyhemoglobin before arylhydrazine was added. The effect of ethanol, *tert*-butyl alcohol, triethylamine, or Dabco[1] on the reaction rate in the absence or presence of superoxide dismutase and catalase was determined. Reaction rates with phenylhydrazine at half and twice the concentration of oxyhemoglobin were also measured. Rate constants for unopposed bimolecular reactions with equal and unequal initial concentrations of reactants were computed in the usual manner (Moelwyn-Hughes, 1971). Phenylhydrazine was added to methemoglobin solutions under air, N_2, and CO, and the absorption spectra of these solutions were recorded periodically.

Results

The plot of the reciprocal of concentration vs. time of an equimolar reaction mixture of phenylhydrazine and oxyhemoglobin was nonlinear in the absence of superoxide dismutase and catalase. So that the effect of these enzymes on the change in absorbance of the reaction mixture could be evaluated, apparent second-order rate constants were computed from the approximately linear early segments of data obtained in the presence of increasing concentrations of these enzymes, singly or together. Each enzyme decreased the apparent rate, but not to a constant level. Together the enzymes decreased the rate to a constant level lower than that attained with either alone (Figure 1). A linear second-order rate plot (Figure 2) and a rate constant of 64 M^{-1} s^{-1} were obtained with equimolar phenylhydrazine and oxyhemoglobin when both enzymes were present at concentrations of at least 1.0×10^{-7} M in each. Second-order rate constants obtained in experiments with phenylhydrazine at half (2.5×10^{-5} M) and twice (1.0×10^{-4} M) the concentration of oxyhemoglobin (5.0×10^{-5} M) were 64 and 65 M^{-1} s^{-1}, respectively.

As shown in Table I, the second-order rate constant of the reaction between phenylhydrazine and oxyhemoglobin was not significantly altered by the addition of scavengers for hydroxyl

Table I: Effect of Scavengers for Hydroxyl Radical and Singlet Oxygen on the Bimolecular Rate Constant of the Reaction between Phenylhydrazine and Oxyhemoglobin[a]

scavenger[b]	enzymes[c] absent	enzymes[c] present
none	108	65
ethanol	110	65
tert-butyl alcohol	112	69
triethylamine	109	68
Dabco[d]	103	63

[a] k is in L mol^{-1} s^{-1} with each reactant at an initial concentration of 5.0×10^{-5} M in 0.1 M potassium phosphate, pH 7.4, at 25 °C. [b] 0.05 M. [c] 3.0×10^{-7} M each of superoxide dismutase and catalase. [d] 1,4-Diazabicyclo[2.2.2]octane.

Table II: Second-Order Rate Constants for the Equimolar Reaction of Oxyhemoglobin with Halogen-Substituted Phenylhydrazines[a]

derivative	k	derivative	k
2-fluoro	21 ± 0	2-chloro	25 ± 1
3-fluoro	79 ± 8	3-chloro	292 ± 14
4-fluoro	168 ± 7	4-chloro	553 ± 50
2-bromo	22 ± 1	2-iodo	22 ± 3
3-bromo	346 ± 13	3-iodo	294 ± 4
4-bromo	674 ± 21	4-iodo	381 ± 51
2,3-dichloro	53 ± 2	2,6-dichloro	no reaction
2,4-dichloro	61 ± 3	3,4-dichloro	1819 ± 31
2,5-dichloro	24 ± 1	3,5-dichloro	245 ± 8

[a] In 0.1 M potassium phosphate, pH 7.4, at 25 °C. [SOD] = [catalase] = 3.0×10^{-7} M. The rate constant k is in L mol^{-1} s^{-1}. The value of k for control determinations with unsubstituted phenylhydrazine in this series was 64 ± 4.

[1] Abbreviations: Dabco, 1,4-diazabicyclo[2.2.2]octane; Hb^{II}, deoxyhemoglobin; HbO_2, oxyhemoglobin; $Hb^{IV}O$, ferrylhemoglobin; $PhNHNH_2$, phenylhydrazine; PhN=NH, phenyldiazene; SOD, superoxide dismutase.

Table III: Second-Order Rate Constants for the Equimolar Reaction of Oxyhemoglobin with Alkyl-Substituted Phenylhydrazines[a]

derivatives	k	derivatives	k
2-methyl	41 ± 1	2-ethyl	57 ± 2
3-methyl	184 ± 1	3-ethyl	276 ± 6
4-methyl	147 ± 2	4-ethyl	187 ± 13
2-isopropyl	53 ± 1	2-*tert*-butyl	15 ± 1
4-isopropyl	106 ± 9	4-*tert*-butyl	40 ± 4

[a] In 0.1 M potassium phosphate, pH 7.4, at 25 °C. [SOD] = [catalase] = 3.0×10^{-7} M. The rate constant k is in L mol^{-1} s^{-1}. The value of k for control determinations with unsubstituted phenylhydrazine in this series was 64 ± 1.

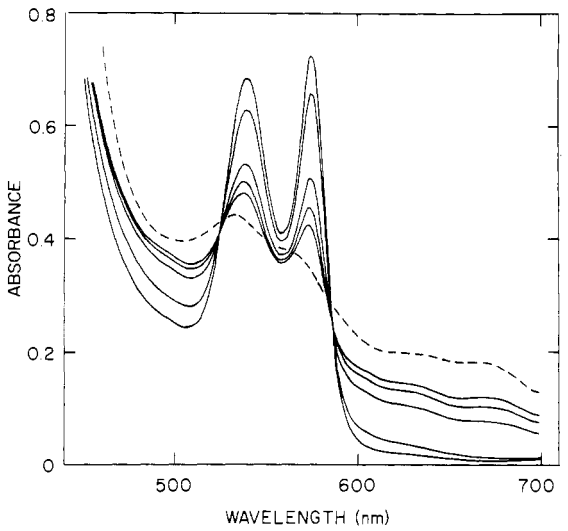

FIGURE 3: Spectral changes during the reaction of oxyhemoglobin with phenylhydrazine. Conditions are as in Figure 2. (—) Spectrum of oxyhemoglobin and spectra of a reaction mixture of 4.9×10^{-5} M HbO_2 and 9.8×10^{-5} M phenylhydrazine 10, 90, 170, and 265 s after the addition of phenylhydrazine to oxyhemoglobin. (--) Spectrum of reaction mixture of 4.9×10^{-5} M oxyhemoglobin and 4.9×10^{-4} M phenylhydrazine (10-fold excess) 6 min after the addition of phenylhydrazine to oxyhemoglobin.

radical and singlet oxygen either in the presence or in the absence of catalase and superoxide dismutase. Second-order rate constants of reactions of halogen-substituted and alkyl-substituted phenylhydrazines with oxyhemoglobin are shown in Tables II and III, respectively. 2-Carboxy- and 4-carboxyphenylhydrazines did not react with oxyhemoglobin. Second-order rate constants of 2 ± 1 and 72 ± 2 M^{-1} s^{-1}, respectively, were obtained with the 2-$COOC_2H_5$ and 4-$COOC_2H_5$ compounds.

The change in the visible absorption spectrum of a reaction mixture of phenylhydrazine and oxyhemoglobin with time was recorded by periodic scans over the wavelength range of 450–700 nm. Spectra taken as the reaction progressed had isosbestic points at 525.5 and 587 nm (Figure 3). When the same reaction was carried out in the presence of a 10-fold excess of KCN, the small increase in absorption at around 630 nm did not appear. The same two isosbestic points were present, and the high absorption between 587 and 700 nm remained. Methemoglobin cyanide has very low absorption above 620 nm and has five isosbestic points with oxyhemoglobin in the visibile region (Castro et al., 1978). Thus, the small amount of methemoglobin evident in the reaction of phenylhydrazine with oxyhemoglobin (Figure 3) must be the product of a minor side reaction.

Possible products of oxyhemoglobin in its reaction with phenylhydrazine were prepared, and isosbestic points in the spectra of the mixtures of these compounds with oxyhemoglobin and methemoglobin were determined. The positions and molar extinctions of these points are given in Table IV. The presence of only two isosbestic points showed that the product of the reaction between phenylhydrazine and oxyhemoglobin is neither methemoglobin, deoxyhemoglobin, nor the product of the reaction between phenyldiazene and methemoglobin, which have three, five, and four isosbestic points, respectively, with oxyhemoglobin between 450 and 700 nm (Table IV and Figure 4). The aerobic reaction of methemoglobin with phenylhydrazine resulted in a compound with substantially the same spectrum as the compound produced when phenyldiazene is added to methemoglobin (Table IV and Figures 4 and 5). The anaerobic reaction produced deoxyhemoglobin under N_2 (Figure 6) and carbonmonoxy-

Table IV: Isosbestic Points in Spectra of Two-Species Mixtures of Oxyhemoglobin or Methemoglobin with Possible Products of Their Reactions with Phenylhydrazine[a]

species[b]	nm (ϵ_{mM})
HbO_2, Hb^{II}	585 (7.6), 568 (11.1), 547.5 (12.3), 521 (6.6), 505 (4.9)
HbO_2, Hb^{III}	589 (3.8), 523 (7.3), 469 (8.4)
HbO_2, HbO_2 + $PhNHNH_2$[c]	587 (5.3), 525.5 (8.5)
HbO_2, Hb^{III} + PhN=NH[d]	586 (6.3), 565.5 (9.3), 554 (9.4), 528 (9.3)
Hb^{III}, Hb^{II}	658 (0.9), 601 (3.3), 524 (7.1), 453.5 (8.8)
Hb^{III}, Hb^{III} + PhN=NH[e]	649 (1.6), 602.5 (3.2), 514 (7.8), 480.5 (7.9)
Hb^{III}, Hb^{III} + $PhNHNH_2$[f]	646 (2.2), 606 (3.2), 514 (7.8), 480 (8.0)

[a] In 0.1 M potassium phosphate, pH 7.4. [b] HbO_2, oxyhemoglobin; Hb^{II}, deoxyhemoglobin; Hb^{III}, methemoglobin; $PhNHNH_2$, phenylhydrazine; PhN=NH, phenyldiazene. [c] Aerobic reaction mixture of oxyhemoglobin and phenylhydrazine. [d] Oxyhemoglobin and the product of the anaerobic reaction of methemoglobin and phenyldiazene. [e] Methemoglobin and the product of the anaerobic reaction of methemoglobin and phenyldiazene. [f] Aerobic reaction mixture of methemoglobin and phenylhydrazine.

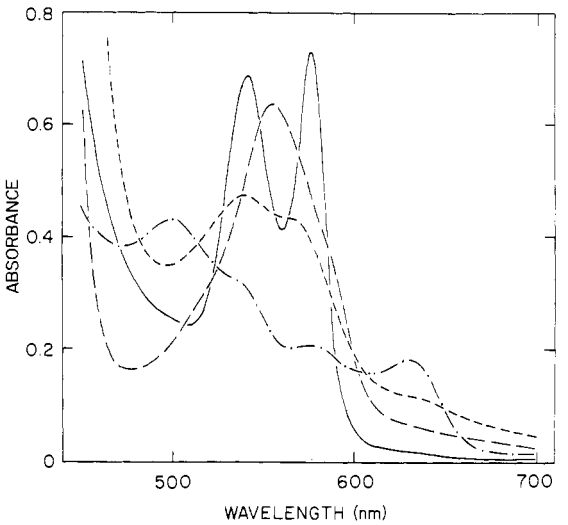

FIGURE 4: Spectra of various hemoglobin derivatives. Conditions are as in Figure 3. (—) 4.8×10^{-5} M oxyhemoglobin; (--) 4.8×10^{-5} M deoxyhemoglobin; (-·-) 4.8×10^{-5} M methemoglobin; (---) reaction mixture of 4.8×10^{-5} M oxyhemoglobin, 2.9×10^{-4} M $K_3Fe(CN)_6$, and 2.4×10^{-4} M phenylhydrazine 20 min after the addition of $K_3Fe(CN)_6$ and phenylhydrazine to oxyhemoglobin. Oxyhemoglobin and phenylhydrazine are converted to methemoglobin and phenyldiazene, respectively, by $K_3Fe(CN)_6$. The spectrum (---) of the product of the reaction of phenyldiazene with methemoglobin is different from that of phenylhydrazine with oxyhemoglobin (Figure 3).

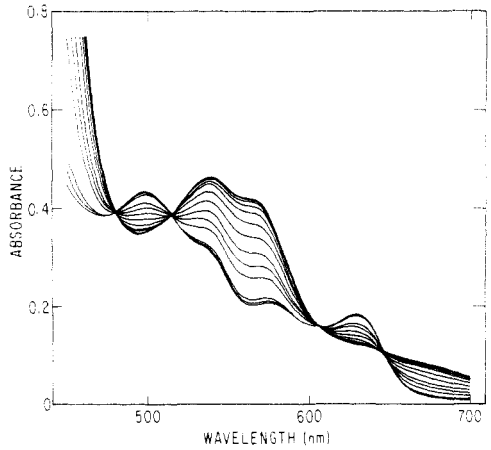

FIGURE 5: Spectral changes under air after addition of 2.5×10^{-4} M phenylhydrazine to 5.0×10^{-5} M methemoglobin. Conditions are as in Figure 2. Spectra were taken at 0, 0.2, 4, and 16 min after addition of phenylhydrazine and then at 5-min intervals until 61 min had elapsed. The final spectrum is the same as that of the product of the reaction between phenyldiazene and methemoglobin (Figure 4).

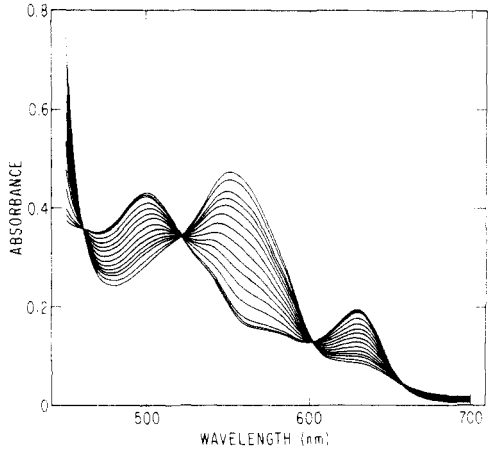

FIGURE 6: Spectral changes under N_2 after addition of 5.0×10^{-4} M phenylhydrazine to 5.0×10^{-5} M methemoglobin. Buffer: 0.1 potassium phosphate, pH 6.2. Temperature: 25 °C. Spectra were taken at 0, 0.25, and 5 min after addition of phenylhydrazine, then at 17.5-min intervals until 110 min was reached, and then every 22–23 min until 222 min had elapsed. A final scan was taken at 270 min. Conversion to deoxyhemoglobin is 66% completed.

hemoglobin under CO (Figure 7).

Discussion

The apparent rate as determined spectrophotometrically of the reaction between oxyhemoglobin and phenylhydrazine was highest in the absence of superoxide dismutase and catalase. Either enzyme decreased the apparent rate, and the two together, each at a concentration of 1×10^{-7} M or greater, minimized the bimolecular rate constant at 64 M^{-1} s^{-1}. This rate was unaffected by scavengers for hydroxyl radical and singlet oxygen in the presence or absence of superoxide dismutase and catalase. The change in spectrum that follows the addition of phenylhydrazine to oxyhemoglobin in the presence of sufficient superoxide dismutase and catalase is therefore due solely to a direct reaction of phenylhydrazine with oxyhemoglobin and not to any reactions with oxidants derived from oxygen.

The inhibitory effects of catalase and superoxide dismutase on the apparent rate of the oxyhemoglobin–phenylhydrazine reaction indicate that hydrogen peroxide and superoxide take part in reactions that increase the rate of conversion of oxyhemoglobin to a product or products of lower absorbance at

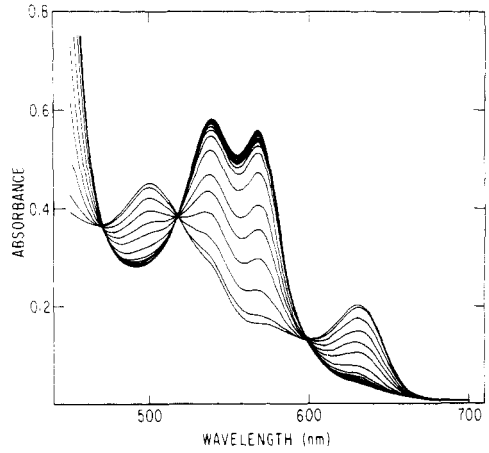

FIGURE 7: Spectral changes under CO after addition of 5.0×10^{-4} M phenylhydrazine to 5.0×10^{-5} M methemoglobin. Buffer: 0.1 M potassium phosphate, pH 6.2. Temperature: 25 °C. Spectra were taken at 0, 0.3, 10, 22.5, 35, 47.5, 70, and 92.5 min after addition of phenylhydrazine. Conversion to carbonmonoxyhemoglobin is 65% completed.

577 nm. Phenyldiazene from the oxidation of phenylhydrazine by oxyhemoglobin reacts rapidly with O_2 to produce superoxide (Goldberg et al., 1976; Vedvick & Itano, 1981). The spontaneous dismutation of superoxide (Bielski & Allen, 1977) and the reaction of superoxide with oxyhemoglobin (Sutton et al., 1976) produce hydrogen peroxide. Superoxide reacts with oxyhemoglobin to produce methemoglobin with a second-order rate constant of 4×10^3 M^{-1} s^{-1} at pH 7 (Sutton et al., 1976), and hydrogen peroxide reacts with deoxyhemoglobin and oxyhemoglobin to produce methemoglobin with second-order rate constants of 125 M^{-1} s^{-1} and about 5 M^{-1} s^{-1}, respectively, at pH 7.4 (Eyer et al., 1975). Since the molar extinction of methemoglobin at 577 nm is lower than that of either oxyhemoglobin or the phenylhydrazine–oxyhemoglobin product, these methemoglobin-producing reactions can make significant contributions to the rate at which absorbance at 577 nm decreases. Superoxide dismutase prevents the first of these reactions but at the same time accelerates the formation of hydrogen peroxide; catalase eliminates hydrogen peroxide but is without effect on superoxide. Thus, in agreement with our results, neither enzyme by itself can abolish reactions that increase the rate of conversion of oxyhemoglobin to a side product, methemoglobin, with a lower absorbance at 577 nm.

Misra & Fridovich (1976) and Castro et al. (1978) postulated that methemoglobin is the initial product in the reaction of phenylhydrazine with oxyhemoglobin, and Castro et al. concluded that the methemoglobin is then reduced by phenylhydrazine to deoxyhemoglobin. However, whereas spectra of oxyhemoglobin and methemoglobin at pH 7.4 have three isosbestic points and of oxyhemoglobin and deoxyhemoglobin five, between 450 and 700 nm, spectra of oxyhemoglobin and the product of the oxyhemoglobin–phenylhydrazine reaction have only two. Moreover, addition of sodium dithionite to the product of this reaction did not produce deoxyhemoglobin, and addition of potassium ferricyanide did not produce methemoglobin. The product of the bimolecular reaction is therefore neither methemoglobin, deoxyhemoglobin, nor a mixture of the two. With insufficient superoxide dismutase and catalase, methemoglobin may be produced in side reactions of oxyhemoglobin with superoxide radical and hydrogen peroxide.

Possible reactions that may result in a change in the electronic absorption spectrum of a compound of hemoglobin are

complex formation, one-electron transfer, or two-electron transfer. The methemoglobin–phenyldiazene complex is the only known complex of hemoglobin or its derivatives with phenylhydrazine or its oxidation products. The product of the phenylhydrazine–oxyhemoglobin reaction is not this complex because spectra of the product and oxyhemoglobin have only two isosbestic points in the wavelength region in which the complex and oxyhemoglobin have four. Addition of phenylhydrazine to methemoglobin in the presence of O_2 produced this complex, not the product of the phenylhydrazine–oxyhemoglobin reaction; methemoglobin accordingly is not an intermediate in the latter reaction. Only in the absence of O_2 did phenylhydrazine reduce methemoglobin, to deoxyhemoglobin under N_2 and to carbonmonoxyhemoglobin under CO, the latter result being spectrophotometric confirmation of the gasometric results of Rostorfer & Totter (1956). A one-electron transfer from a reductant to oxyhemoglobin produces methemoglobin (Wallace & Caughey, 1975; Kawanishi & Caughey, 1979); however, methemoglobin was neither the intermediate nor the final product of the phenylhydrazine–oxyhemoglobin reaction. The remaining possible reaction is a two-electron transfer, which has been postulated in the coupled oxidation of ascorbic acid and hemoglobin in the presence of oxygen (Lemberg et al., 1938) and in the reaction of phenylhydrazine with oxyhemoglobin (Goldberg et al., 1976; Itano et al., 1977). The product of the two-electron transfer has been formulated as $Hb^{II}H_2O_2$ (Lemberg et al., 1938; Itano et al., 1977) or $Hb^{IV}O$ (Goldberg et al., 1976).

$$HbO_2 + 2H \rightarrow Hb^{II}H_2O_2 \rightarrow Hb^{IV}O + H_2O$$

Dithionite would reduce H_2O_2 to water and reduce the ferryl state of iron to the ferrous state. In either case, the product would be deoxyhemoglobin, which, however, was not produced when the reaction product was treated with dithionite. The change in the absorption spectrum shown in Figure 3 must therefore be due to a product other than $Hb^{II}H_2O_2$ or $Hb^{IV}O$. Lemberg et al. (1938) suggested that the ferrous heme of pyridine ferrohemochrome is converted by hydrogen peroxide to a product in which a meso carbon of its porphyrin ring is hydroxylated. Using pyridine octaethylferrohemochrome as a model compound, Bonnett & Dimsdale (1972) confirmed this reaction and, moreover, postulated that the precursor of *meso*-hydroxyoctaethylheme in the reaction is a complex of hydrogen peroxide with the hemochrome.

Bonnett & Dimsdale (1972) postulated that a complex of pyridine ferrohemochrome and H_2O_2 undergoes a reaction that forms ferric heme and hydroxyl radical. Itano et al. (1977) suggested that methemoglobin from the homologous reaction of deoxyhemoglobin with H_2O_2 might bind phenyldiazene from the oxidation of phenylhydrazine by oxyhemoglobin; however, this study has shown that this complex is not a product of the reaction of phenylhydrazine with oxyhemoglobin. Any phenyldiazene produced in the presence of O_2 would rapidly reduce O_2 to superoxide (Vedvick & Itano, 1981) in a one-electron reaction that also must produce phenyldiazenyl radical. The spontaneous decomposition of phenyldiazenyl radical would produce phenyl radical, which can react with heme to form phenyl adducts of biliverdin and protoporphyrin (Saito & Itano, 1981). The initial bimolecular reaction of phenylhydrazine and oxyhemoglobin is therefore the event that activates other reactions that take part in the oxidative degradation of hemoglobin.

Halogen atoms and alkyl groups ortho to the hydrazino group decreased the rate of reaction of an arylhydrazine with oxyhemoglobin. These substitutions also decrease the severity of arylhydrazine-induced Heinz-body hemolytic anemia (Itano et al., 1977). 2,6-Dichloro and both ortho and para carboxylate substitutions, which prevented the bimolecular reaction, prevent the induction of hemolytic anemia (Itano et al., 1976). These relationships show that the initial bimolecular reaction between arylhydrazine and oxyhemoglobin is essential for the occurrence of oxidative denaturation; if this reaction is hindered or blocked, so is the induction of Heinz-body hemolytic anemia. On the other hand, failure of meta and para halogen and alkyl substitutions, which increased the rate of the bimolecular reaction, to increase the severity of induced anemia and reticulocytosis (Itano et al., 1977) suggests that these substitutions inhibit a subsequent reaction essential for the completion of oxidative denaturation. Adducts of the phenyl group of phenylhydrazine with the porphyrin ring of heme have been found in reaction mixtures of phenylhydrazine with oxymyoglobin and oxyhemoglobin (Saito & Itano, 1981). Replacement of heme with these structurally abnormal products would destabilize the hemoglobin molecule and cause it to unfold and precipitate. If adduct formation is slow, this reaction and not the initial arylhydrazine–oxyhemoglobin reaction would be rate limiting in oxidative denaturation.

Acknowledgments

We gratefully acknowledge the contributions of Dr. Thomas S. Vedvick and Cathy Crosby to this study. We thank Robert C. Pegg, Jr., for his help in the design of the computer program for kinetics calculations.

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Acid-Base Properties of Ionophore A23187 in Methanol-Water Solutions and Bound to Unilamellar Vesicles of Dimyristoylphosphatidylcholine†

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ABSTRACT: The acid-base properties of ionophore A23187 in methanol-water solutions (0-95% w/w) and bound to unilamellar vesicles of dimyristoylphosphatidylcholine were examined by ultraviolet and fluorescence spectroscopy, and the spectral properties for the acidic and basic forms were defined under these conditions. Standard mixed-solvent buffers were employed to calibrate pH* measurement in the methanol-water solvents. In 65% methanol-water, two protonation equilibria were observed, the most basic of which displayed a value for the logarithm of the protonation constant ($\log K^*_H$) of 7.19 ± 0.05 at 25 °C and 0.05 M ionic strength. Instability of A23187 was encountered below pH* ~4; however, decomposition was slow enough to allow $\log K^*_H$ for the more acidic equilibrium to be estimated as 1.28. Comparison of these results to those obtained with the methyl ester of A23187 ($\log K^*_H$ = 1.32) and literature values for other model compounds allowed assignment of the more basic equilibrium to the carboxylic acid moiety and the more acidic one to the *N*-methylamino substituent of the benzoxazole ring. $\log K^*_H$ of the carboxylic acid increased from 5.69 ± 0.05 to 9.37 ± 0.05 over the range of solvent polarity encompassed by water to 95% methanol-water. Values for the ground state (absorption) and first excited state (fluorescence) were equal within experimental error. The logarithm of the protonation constant for the membrane-bound ionophore, measured under conditions where the surface potential generated by ionization did not significantly alter the equilibrium, was found to be 7.85 ± 0.05 at 25 °C and at ionic strength of 0.05 M in the aqueous phase. The value agrees with that observed in 80% methanol-water, as does the wavelength of maximum fluorescence emission for the membrane-bound free acid. An interfacial location for the monoprotonated form of the benzoxazolate moiety is proposed, both above and below the membrane phase transition temperature. The location of other regions of the A23187 molecule could not be assessed from these data.

Since the discovery that the polyether antibiotic A23187 is a calcium ionophore with high specificity for divalent cations (Reed & Lardy, 1972), thousands of reports have appeared describing the effects of this compound upon biological systems ranging from subcellular fractions to whole animals. These studies have provided evidence for the involvement of Ca^{2+} in a wide spectrum of biological control mechanisms including muscle contraction, stimulus-secretion coupling, mitosis, fertilization, gluconeogenesis, glycogenolysis, and many others [for reviews, see Rasmussen & Goodman (1977) and Kretsinger (1979)]. In addition to being diverse, the responses of tissues or cells to treatment with A23187 are often complex, depending upon such factors as concentrations of the ionophore, time of exposure, and ionic composition of the medium.

The physicochemical properties underlying the biological responses produced by A23187 are not sufficiently known to explain the biological effects in detail. The structure (see Figure 1) and some chemical properties of the free acid were reported by Chaney et al. (1974). Structures of complexes with a wide range of divalent cations are known in solution (Deber & Pfeiffer, 1976; Anteunis, 1977; Pfeiffer & Deber, 1979) and in the solid state (Chaney et al., 1976; Smith & Duax, 1976). The complex stoichiometries and binding affinity sequence have been studied in solution and by bulk phase extraction techniques (Reed & Lardy, 1972; Pfeiffer et al., 1974; Puskin & Gunter, 1975; Pfeiffer & Lardy, 1976; Young & Gomperts, 1977), and the transport sequence has been investigated in liposomes (Weissman et al., 1980; Pohl et al., 1980). It is generally concluded from these studies that complexes of stoichiometry A_2M[1] are responsible for divalent cation transport, with the overall electroneutral transport reaction arising from M^{2+} for $2H^+$ exchange. The binding affinity sequence for divalent cations arises from the isosteric nature of the complexes combined with the limited confor-

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[1] Abbreviations: Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; *D*, dielectric constant; DMPC, L-α-dimyristoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; HA, the free acid of A23187; A^-, the carboxylate anion of A23187; H_2A^+, the doubly protonated form of the carboxylate anion; A_2M, the 2:1 A23187/divalent cation complex; λ_{ex}, excitation wavelength of maximum fluorescence; λ_{em}, emission wavelength of maximum fluorescence; λ_{max}, wavelength of maximum absorbance.

0006-2960/82/0421-2426$01.25/0