# Oxidation of Heme Proteins by Alkyl Halides

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**Abstract:** Bromomalonitrile and bromodiethyl malonate rapidly and quantitatively oxidize dilute solutions of iron(II) human hemoglobin and sperm whale myoglobin at 18° to the iron(III) proteins without protein denaturation. Under identical conditions iron(II) cytochrome *c* (horse heart) is inert. The latter can be induced to react with a high threshold excess of bromomalononitrile (80-fold). The halides are converted in high yield to the corresponding alkane in a second-order process. Rate constants for hemoglobin and myoglobin are $\sim 3 \times 10^2$ l./mol per sec and $\sim 6 \times 10^2$ l./mol per sec, respectively. A variety of halides cause the oxidation of human hemoglobin. The results verify recent theoretical predictions and demonstrate bromomalononitrile to be an efficient reagent to probe axial inner sphere electron transfer capacity in heme proteins.

A recent theory of hemoprotein reactivity[1] assigns specific electron transfer properties to these entities. One set of predictions is that certain hemoproteins in the iron(II) state will be oxidized to the iron(III) complexes by alkyl halides, while others will be inert or slow to react. This prediction provides the basis for a sharp experimental test of the theory. Thus, the iron(II) proteins hemoglobin and myoglobin, both of which reversibly complex oxygen, and hence are not readily oxidized by it, are considered typical of a protein conformation and axial bond type that should be rapidly oxidized by organic halides! On the other hand, cytochrome *c* is predicted to be inert. The X-ray structures for iron(III) complexes of hemoglobin,[2] myoglobin,[3] and cytochrome *c*[4] are known, and work on iron(II) cytochrome *c* has recently been completed.[5] It is essential for an adequate test of the theory that work be performed with defined hemoproteins in order to allow an extrapolation to proteins of unknown structure. Thus, a correct fitting of predicted oxidation–reduction reactivity with proteins of characterized structure (in at least one of the valence states) suggests a substrate design that will probe effective protein conformation for specific oxidation–reduction mechanisms and thereby allow a broad experimental classification of these important respiratory substances.

As a parallel to our work with hemes[6] we present here our initial studies of the oxidation of hemoproteins by alkyl halides. The halide substrates employed, bromomalononitrile and bromodiethyl malonate, were chosen because of their water solubility and extreme rapidity of reaction with hemes.

## Results

**Stoichiometry.** Reactions with the hemoproteins were conducted under argon in an aqueous solution that contained $6.7 \times 10^{-4}$ *M* phosphate buffer at pH 7.4, $3 \times 10^{-3}$ *M* potassium chloride, and 0.1 *M* sodium chloride. The iron(II) spectra obtained by reduction with a slight stoichiometric excess of sodium dithionite were stable under these conditions for hours, and they were obtained reproducibly. There was no observable precipitation or denaturation of the protein. Initial concentrations of heme protein were $\sim 5 \times 10^{-4}$ *M* for hemoglobin and $10^{-3}$ *M* for myoglobin. Initial halide concentrations ranged from $4 \times 10^{-3}$ to $10^{-2}$ *M*. Under these conditions reactions were instantaneous and the production of Fe(III) protein was quantitative. No protein denaturation occurred. The stoichiometry for the oxidation of human hemoglobin and horse heart myoglobin by bromomalononitrile and bromodiethyl malonate is sketched in

$$\mathrm{Hmb(Fe^{II})_4 + 2BrCH(CN)_2 + 2H_2O \longrightarrow Hmb(Fe^{III})_4 + 2CH_2(CN)_2 + Br^- + 2OH^-} \quad (1)$$
(85%)

$$\mathrm{Hmb(Fe^{II})_4 + 2BrCH(CO_2Et)_2 + 2H_2O \longrightarrow Hmb(Fe^{III})_4 + 2CH_2(CO_2Et)_2 + Br^- + 2OH^-} \quad (2)$$
(81%)

and similarly

$$\mathrm{2Myo(Fe^{II}) + BrCH(CN)_2 + H_2O \longrightarrow 2Myo(Fe^{III}) + CH_2(CN)_2 + Br^- + OH^-} \quad (3)$$
(87%)

The determined yields of alkane based upon this stoichiometry are noted. No other products could be detected, and the yields reflect the adequacy of the work-up and analysis. Under these conditions, cytochrome *c* ($Fe^{II}$) was inert. In addition to these halides, several others caused the oxidation of Fe(II) hemoglobin (*cf.* Experimental Section). The general reactive halide bond types are those reactive toward hemes.[6] Although slower reacting halides can be studied, a turbidity in the solutions suggests some protein denaturation has occurred. This in turn reflects a conformation change that renders any mechanistic interpretation ambiguous.

**Kinetics.** The kinetics of the reaction with bromomalononitrile were monitored spectrophotometrically by following the decrease in concentrations of an iron(II) protein Soret band. The wavelengths employed were: hemoglobin, 428 mμ; myoglobin, 434 mμ; and cytochrome *c*, 415 mμ. A comparison of the rates of oxidation of the three proteins is given in Figure 1 under these conditions: $(\mathrm{Fe(II)protein})_0 = 5 \times 10^{-6}$ *M* and $(\mathrm{BMN})_0 = 5 \times 10^{-5}$ *M* for myoglobin and cytochrome *c*, with $Fe^{II}$ hemoglobin, $(\mathrm{BMN})_0 = 20 \times$

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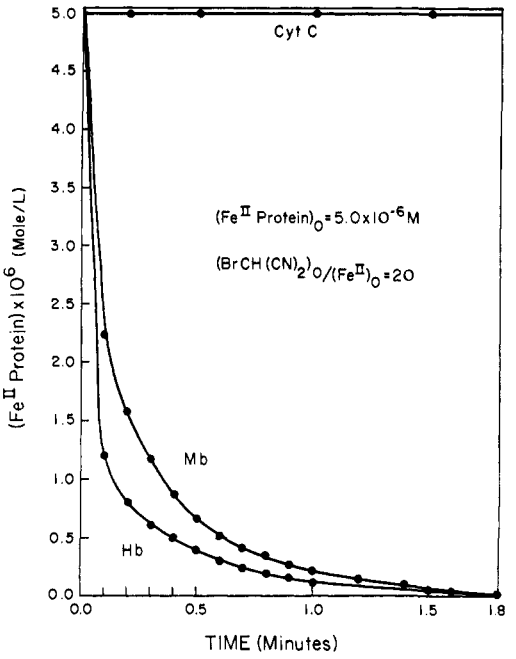



Figure 1. Relative rates of oxidation of the iron(II) complexes of hemoglobin, myoglobin, and cytochrome *c* by bromomalononitrile.

$10^{-5}$ *M*. The spectrum of Fe(II) cytochrome *c* was not altered after 1 day. Indeed this cytochrome was inert to a 40-fold excess of BMN. However, an 80-fold excess of the reagent did cause a slow oxidation indicating a conformation change is a requisite for reaction. The reactions with hemoglobin and myoglobin were determined to be first order in halide and $Fe^{II}$ protein from initial slopes. Approximate second-order rate constants for myoglobin and hemoglobin are $6 \times 10^2$ and $3 \times 10^2$ l./mol per sec, respectively. Estimates from initial slopes (Figure 1) indicate approximate rate constants of $10^3$ l./mol per sec and $3.2 \times 10^2$ l./mol per sec for these proteins. Pseudo-first-order plots of data with excess BMN yield numbers of $6.4 \times 10^2$ and $3.6 \times 10^2$. These approximations are at the limits of our methodology in that the first points at 6–12 sec represent 50% completion. A more precise instrumentation will allow a sharper kinetic analysis of these reactions. At this time, however, it is clear that myoglobin is about twice as reactive as hemoglobin and the rate of these reactions is of the same order of magnitude as the reaction of hemes in solution with related substrates. We presume the initial rates reflect the reactivity of the first heme of hemoglobin.

## Discussion

The rapid oxidation of hemoglobin and myoglobin by bromomalononitrile and bromodiethyl malonate as contrasted with the inertness of cytochrome *c* lends strong support to the recently advanced theory. The results accord exactly with the prediction for these defined proteins. Thus, oxidation of hemes by alkyl halides has been demonstrated[6] to occur by an "axial inner sphere process." The rate-determining step is an initial scission to radicals (eq 4) followed by a fast reduction of the latter (eq 5). The relative reactivity of

$$Fe^{II} + RX \longrightarrow Fe^{II}(XR) \longrightarrow \left[ Fe\text{---}X\text{---}R \right]^{\neq} \longrightarrow Fe^{III}\text{—}X + R\cdot \quad (4)$$

$$Fe^{II} + R\cdot + H_2O \longrightarrow Fe^{III} + RH + HO^- \quad (5)$$

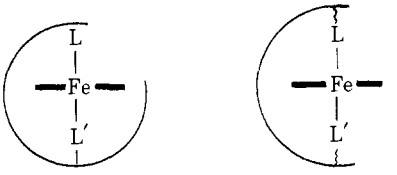
the globins to halides and the kinetics and products of the reactions with bromomalononitrile and bromodiethyl malonate are most reasonably accommodated by this mechanism. From the previous work[6] it will be noted that an alternate path to alkanes from hemes entails the attack of a heme upon a 1:1 heme–alkyl halide complex (eq 6). This latter mechanism is precluded with hemoproteins by the steric bulk of the protein cage. That is, it is impossible for an alkyl halide ligand of a heme in hemoglobin or myoglobin to be approached by another heme imbedded in a second protein molecule. The elimination of this mechanism (eq 6) represents one of the controlling influences of the apoprotein upon heme reactivity.

$$Fe^{II}(XR) + Fe^{II} \longrightarrow 2Fe^{III} + X^- + RH \quad (6)$$

If the mechanism (eq 4 and 5) is correct, then the Fe(II) globins must be capable of reducing radicals (eq 5). Although we have demonstrated this with hemes[6] mechanistic details are obscure. At this point the reasonable intermediacy of iron alkyls (eq 7) remains to be unambiguously demonstrated. With the hemoglobin tetramer it is possible that a heme within the same protein aggregate is the site for reaction (eq 5) following the initial cleavage (eq 4). A greater definition of these events awaits further investigation.

$$Fe^{II} + R\cdot \longrightarrow Fe\text{—}R \xrightarrow{H_2O} Fe^{III} + RH + HO^- \quad (7)$$

The conformation of the protein and its control of the axial ligands on iron have been identified as the major factors that modulate the chemical reactivity of hemes in their native protein matrices.[1] Thus, typical extremes for globin and cytochrome conformations have been formulated in two dimensions as those drawn (**1** and **2**). The salient features of protein structure

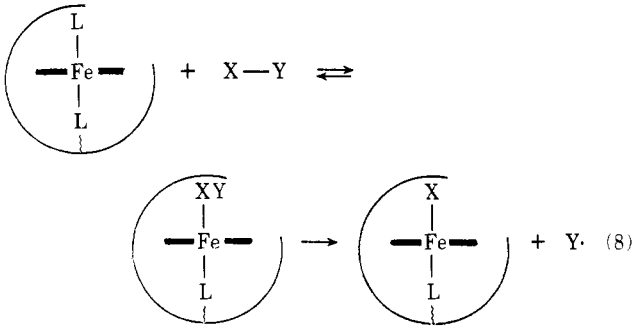



**1** **2**

intended to be conveyed by these simple drawings are briefly noted below.

The globins are characterized by a protein conformation ("G" conformation) that allows easy access to one of the axial positions on iron but the porphyrin periphery is cooperatively blocked in these units by the protein and the protruding propionic acid side chains of protoporphyrin IX. In contrast, the cytochromes ("C" conformation) contain a heme enveloped by the protein in such a way that the axial ligands are blocked, but the porphyrin periphery is exposed. Thus, the Fe(II) globins should be capable of oxidation by an

axial inner sphere process (eq 8). If the oxidant is a "low-field" ligand




If the oxidant contains no easily dissociable bond[7] but possesses appropriate empty orbitals such that iron dxz,dyz electrons can bond to it, a stable low-spin complex ensues. Such is the case for the oxygen complexes of hemoglobin and myoglobin. On the other hand the cytochromes with an exposed periphery should be capable of peripheral $\pi$ or outer sphere electron transfer processes. An amplified account of these views has been given.[1] We consider the oxidation of hemoglobin and myoglobin by alkyl halides to be a good example of an axial inner sphere oxidation by a low-field ligand.

In this context iron(II) cytochrome *c* would be expected to be inert because the oxidant cannot approach iron. The fact that it can begin to be oxidized with an ~80-fold threshold excess of BMN is indicative of other reactions with the protein that alter the conformation to enable an approach to an axial position. Presuming that the thiomethyl of methionine-80 in cytochrome *c* would be more easily substituted as an axial ligand than the imidazole of histidine-18, an alkyl halide complex of the heme in all three proteins studied herein would contain essentially the same[8] inner coordination sphere. Thus, it is most unlikely that the reactivity of the proteins for BMN reflects a thermodynamic control of the bond scission process. Based upon the findings of this and the preceding work, we believe bromomalononitrile is a reagent that can be successfully utilized to probe the electron transfer characteristics of heme proteins at the microgram level. Thus, at $\sim 10^{-4}$ *M* concentrations, if a hemoprotein is oxidized by BMN, it must be capable of an axial inner sphere electron transfer process. Hence, it must possess a G or "short C" conformation.[1] If the iron(II) protein is inert, a C conformation[9] is likely.

The relevance of the present observations to the reductive biodehalogenations noted previously[6] is obvious. Moreover, the rapid destruction of respiratory capacity that can ensue through the breathing of organic halides can be envisaged.

## Experimental Section

**Materials and Methods.** Human hemoglobin, twice recrystallized, and sperm whale myoglobin were purchased from Mann Biochemicals. Horse heart cytochrome *c* was obtained from Sigma Chemicals. Fresh buffered salt solutions of hemoglobin were filtered into the reaction vessel (*cf.* the typical procedure below). The myoglobin was subjected to G-75 Sephadex chromatography just before use. Cytochrome *c* was employed directly. The hemoproteins employed all possessed the correct literature spectrum for the iron(III) and iron(II) complexes. The latter were obtained by reductions with a slight stoichiometric excess of sodium dithionite. Fresh batches of this reagent were standardized by spectrophotometric titration with Myb. Moreover, all of the proteins employed had the requisite physiological behavior under reaction conditions. Thus, hemoglobin and myoglobin solutions rapidly formed oxy complexes (Soret 415 and 424, respectively) upon admission of air. Iron(II) cytochrome *c* was rapidly oxidized by fresh cytochrome oxidase. The latter was isolated from beef heart by essentially the Morrison modification[10] of the Yonetani preparation. Bromomalononitrile was prepared from bromine and malononitrile[11] and recrystallized from chloroform to mp 63–64°. Bromodiethyl malonate was purchased from Eastman Organic Chemicals and freshly distilled before use.

**Reactions.** All reactions were conducted under purified and scrubbed argon. Gas scrubbers containing chromous sulfate were most effective for ensuring traces of oxygen were removed from the argon line.

The following procedure for the quantitative oxidation of hemoglobin by bromomalononitrile is typical.

Human methemoglobin (~0.40 g) from Mann Biochemicals (twice recrystallized) is dissolved in $6.7 \times 10^{-4}$ *M* phosphate buffer (pH 7.4) that contains 0.1 *M* NaCl and $3 \times 10^{-3}$ *M* KCl. The solution is filtered through a small wad of glass wool into a 25-ml round-bottomed flask. The clear solution was diluted with more buffer until 10 ml of $5.0 \times 10^{-4}$ *M* pure hemoglobin was obtained, as assessed by its visible spectrum ($\lambda_{max}$ (sharp) 406 m$\mu$, ($D_{406}/D_{430}$) = 4.7), or by the Drabkin cyanomethemoglobin assay.[12] The reaction flask is fitted with two serum caps for argon flushing and a Teflon-coated magnetic stirrer. Argon is passed over the gently stirred solution through no. 26 hypodermic needles. The exit needle is connected to a mercury trap. After flushing for 15 min, 25 $\mu$l ($1.25 \times 10^{-5}$ mol, 25% mol excess) of a freshly prepared buffer solution of sodium dithionite is injected under an argon sweep. With continued slow stirring the reduction to hemoglobin is complete in 30 min. This is assessed by removing an aliquot with a hypodermic syringe and transferring it under argon to a prepurged serum capped spectrophotometric cell that contains the buffer solution. (A sharp single soret at 430 m$\mu$ in the hemoglobin spectrum indicates complete reduction ($\lambda_{max}$ (sharp) 430 m$\mu$, ($D_{406}/D_{430}$) = 0.46.) A buffer solution of freshly recrystallized 2-bromomalononitrile (1.0 ml, $4.0 \times 10^{-5}$ mol, fourfold excess) is injected into the red solution. The color changes immediately to brown. Work-up was usually begun about 15 min after mixing. Before proceeding with it an aliquot was removed from the reaction mixture under argon to record the methemoglobin spectrum. At an initial methemoglobin concentration of $2 \times 10^{-6}$ *M*, the entire reaction sequence can be followed in a spectrophotometric cell. The isobestic point for the soret is at 420 m$\mu$ in the conversion of the Fe(II) to the Fe(III) protein.

The flask is opened to the atmosphere and 4 g of ammonium sulfate and 1 drop of concentrated hydrochloric acid are added. The reaction mixture is centrifuged in a clinical centrifuge for 10 min. The clear colorless supernatant solution is transferred to a separatory funnel and extracted with 10 ml of ether. The pellet is triturated with 1 ml of ether and recentrifuged. The supernatant solution is extracted three additional times with 10 ml of ether. Combined ether extracts are washed once with 10 ml of water, dried over sodium sulfate, filtered, and evaporated to dryness. Final concentration is performed in a 10-ml flask. The residue is taken up in 0.4 ml of 1:1 methanol–water and analyzed by flame ionization gas chromatography. A peak coemergent with authentic malononitrile at four different temperatures on two different columns (20% DC-710 on Chromosorb W and 3% DEGS on Porapak P) is obtained. The yield determined by comparison with an authentic standard is 85%. No other products are detectable. A portion of the product solution was hydrolyzed with concentrated sulfuric acid at 90° for 5 min. Neutralization with ammonium hydroxide and direct gas chromatographic analysis afforded a peak

(7) A case of outer sphere oxidation of an organic halide that contains a carbon–halogen bond in conjugation with a low lying $\pi$ orbital has been noted: L. G. Marzilli, P. A. Marzilli, and J. Halpern, *J. Amer. Chem. Soc.*, **92**, 5752 (1970).

(8) The porphyrin in cytochrome *c* is different in that $\alpha$-thioethyl ether linkages are present in the 2 and 4 positions.

(9) The conformations referred to here are the minimum necessary to explain current reactivity patterns. Clearly others are possible. For example, a completely inert heme could result from deep cavity shielding.

(10) S. Horie and M. Morrison, *J. Biol. Chem.*, **238**, 1855 (1963).

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coemergent with authentic malonamide. The hydrolysis is complete and no other peaks are visible. A blank without the hemoprotein does not reduce the bromide.

With horse heart myoglobin the identical procedure can be employed except that the purchased material (Mann Biochemicals) is treated first with potassium ferricyanide and passed through a short G-75 Sephadex column in buffer before commencing with its reduction.

**Kinetics.** Two milliliters of $5 \times 10^{-6}$ *M* iron(II) hemoprotein was prepared directly in 1-cm spectrophotometric cells in the fashion described for reaction. The same buffer and salts were employed. The cell was fitted with a tight serum cap and thoroughly flushed with argon *via* hypodermic needles. The requisite amount of a fresh dithionite solution (10 $\mu$l) was injected to generate the iron(II) complex. In all cases reduction was complete in 15 min or less. At time 0, 10–20 $\mu$l of a stock freshly prepared and argon purged $2.5\text{–}6.25 \times 10^{-3}$ *M* bromomalononitrile solution was injected. The solution was tipped once and placed in the spectrophotometer cavity. The decrease in the Fe(II) soret band was plotted by the recorder. A reference cell contained salts and buffer. All operations were conducted at ambient temperature (18°).

**Other Reactive Substrates.** A $5 \times 10^{-6}$ *M* solution of hemoglobin was oxidized by the addition of 1–2 $\mu$l of a saturated buffer solution of the following substrates: iodoacetic acid, 2,3-dibromosuccinic acid, $\alpha$-bromobutyrolactone, allyl bromide, allyl chloride, and 2,3-dibromopropanol. The halides are grouped in a decreasing order of reactivity. However, all of these reactions caused some denaturation of hemoglobin. A turbidity of the solution and an incompleteness of reaction were typical of this phenomenon.

# Diffusion Studies on Phosphatidylcholine Vesicles

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**Abstract:** Diffusion measurements of homogeneous phosphatidylcholine vesicles in 0.1 *M* KCl–0.01 *M* Tris–11.5% $D_2O$, pH 8.0, have been carried out at 20° in the ultracentrifuge. Since the apparent specific volume of phosphatidylcholine vesicles is nearly equal to the reciprocal of the density of this medium, no appreciable sedimentation occurs during the high speed experiment. Consequently, the measurement of diffusion coefficients of the lipid system in the ultracentrifuge at high speed is virtually identical with that of the free diffusion experiments performed in the stationary diffusion apparatus. Procedures are described in detail for calculating the diffusion coefficient from Rayleigh interference data. The averaged diffusion coefficient is $2.03 \pm 0.04 \times 10^{-7}$ cm$^2$ sec$^{-1}$.

Reports on determinations of the translational diffusion coefficients of proteins and viruses in solutions have been well documented in the literature.[1-3] Since the determination of diffusion coefficients from data obtained *via* the optical systems built into the analytical ultracentrifuge is relatively simple and straightforward to perform in comparison with *free diffusion* experiments of stationary diffusion apparatus, the ultracentrifuge has frequently been employed for diffusion studies. A general account on the evaluation of diffusion coefficients of both native and denatured proteins from sedimentation boundary curves obtained at various speeds with a synthetic boundary cell has been extensively discussed by Kawahara.[4] In the case of a pure lipid system, however, no detailed reports on the measurements of diffusion coefficients have yet appeared in the literature.

Based on the theoretical works of Fujita,[5-7] the diffusion coefficient, $D$, of macromolecules can be related to the maximum height–area ratio ($H/A$) of the sedimenting boundary obtained with a synthetic boundary cell and the schlieren optical system according to the following equation[4]

$$(A/H)^2 = 4\pi Dt(1 - z)(1 + s\omega^2 t) \qquad (1)$$

with $z = 2r_0\omega^2 s^0 k C_0 (H/A)t$ and $0 < z < 0.16$. Here $t$ is the time, $\omega$ the angular velocity of the ultracentrifuge rotor, $r_0$ the radial position of the initial synthetic boundary, $C_0$ the initial solute concentration, and $s$ the sedimentation coefficient which varies with concentration, $C$, obeying the linear relation: $s = s^0(1 - kC)$, where $s^0$ is the extrapolated value of $s$ at infinite dilution and $k$ is a constant.

It is obvious from eq 1 that the values of $z$ and $s\omega^2 t$ may become negligibly small compared with unity if the experiment is performed at low speed. At the rotational speed of 12,590 rpm, for example, the values of $z$ are all smaller than 0.02 and the values of $s\omega^2 t$ are less than 0.001 for native proteins such as aldolase, bovine serum albumin, hemoglobin, myoglobin, and ribonuclease.[4] The diffusion coefficient can, therefore, be calculated from the experimental data obtained at low speed by the familiar simple equation for the *free diffusion* method[1] which can be easily derived from eq 1 as follows

$$D = \frac{1}{4\pi t}(A/H)^2 \qquad (2)$$

Alternatively, eq 1 can also reduce to eq 2 if the value of $s$ for a given system is so small that the values of both $z$ and $s\omega^2 t$ are much less than unity. Under the condition $s \simeq 0$, the diffusion experiment carried out in the ultracentrifuge is equivalent to that performed at low speed. Consequently, the measurement of diffusion coefficients is virtually identical with that of the *free diffusion* experiment.

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