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Oxidation of Heme Proteins by Alkyl Halides: A Probe for Axial Inner Sphere Redox Capacity in Solution and in Whole Cells†

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ABSTRACT: Iron(II) porphyrins in homogeneous solution, in heme proteins, and in intact human erythrocytes and lysed cells are oxidized by certain alkyl halides to the corresponding iron(III) complexes at room temperature. The mechanism established for the oxidation of hemes in homogeneous solution operates at all levels of biological integrity. It is an axial inner sphere process. Deoxyhemoglobin has about the same reactivity within and without cells. The speed of the reaction with the proteins is primarily governed by the steric accessibility to iron. The reactivity of an array of iron(II) proteins accords well with theoretical prediction. In contrast the reactivity of cytochrome b_5 does not. An examination of the oxidation and reduction of this protein with additional mechanistically defined reagents (trinitrobenzene and hydroquinone) shows it to be in the G rather than C conformation. The unusual redox characteristics of this protein can be rationalized on this basis.

The redox capacity of a heme protein may be altered by the microenvironment associated with its location in its native biological matrix. Indeed a sorting out of the reactivity parameters in whole cells at a defined molecular level for any transformation is difficult. To probe this problem, we have attempted to develop a series of mechanistically defined organic reagents with good permeation characteristics which will allow a direct comparison of the homogeneous redox chemistry of an iron porphyrin with that of this same highly ordered array in its native protein matrix and in whole cells. Our general

† From the Department of Nematology, University of California, Riverside, California 92521. *Received July 29, 1977; revised manuscript received April 18, 1978.* This work was supported in part by Research Grant No. AM17936 from the National Institute of Arthritis, Metabolism, and Digestive Diseases and in part by Research Grant No. ES-01588 from the National Institute of Environmental Health Sciences.

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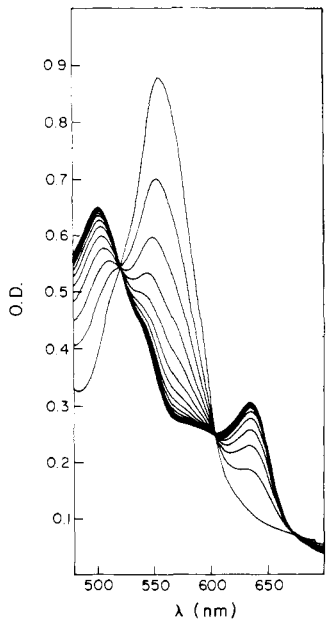


FIGURE 1: The oxidation of deoxymyoglobin by α-bromoisobutyronitrile. Repeat scans at 1-h intervals.

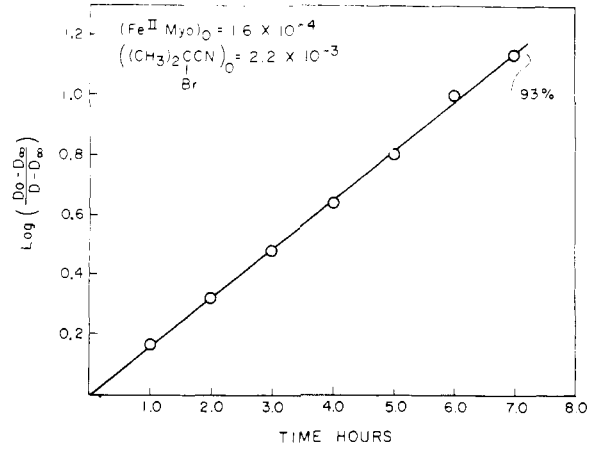


FIGURE 2: Pseudo-first-order plot for the oxidation of deoxymyoglobin by α-bromoisobutyronitrile (Figure 1, λ_{556nm}).

approach has been briefly summarized (Castro et al., 1975). While a simple theory (Castro, 1971, 1978) will accommodate the reactivity we have encountered with heme proteins, until now the validity of its application to whole cells or unusually reactive proteins was untested.

We report here our studies of the oxidation of a range of heme proteins by alkyl halides as well as a direct comparison of the oxidation of human hemoglobin in solution, lysed cells, and intact erythrocytes under identical conditions.

Experimental Section

Materials. Heme Proteins. The proteins were obtained and handled as previously described (Wade & Castro, 1973b; Castro & Bartnicki, 1975). Deoxyhemoglobin was obtained from suspensions of the oxy complex in erythrocytes, lysed cells, or homogeneous solutions by repeated evacuation and purging under argon. Similar procedures were used for obtaining deoxymyoglobin solutions. The reactor and methodology have been described (Castro et al., 1978). Catalase and peroxidase were commercial samples (Sigma Chemical Co.). The peroxidase was from horseradish type II and catalase was the purified powder from bovine liver. The iron(II) complexes for these proteins were generated in situ by reduction with a stoichiometric amount of sodium dithionite. Both the iron(II) and iron(III) species had spectra that matched the literature. It is moot whether the the iron(II) porphyrin complex of catalase λ_{max} 405, 558, 591 nm possesses a native conformation.

Cytochrome b_5 was isolated from rabbit liver microsomes. The microsomes were precipitated with $CaCl_2$ (Schenkman & Cinti, 1972). The solubilized protein was separated from the microsomes by trypsin digestion and ammonium sulfate fractionation (Strittmatter & Ozols, 1966; Kajihara & Hagihara, 1968). Final purification was effected by CM-cellulose and DEAE-Sephadex chromatography (Spatz & Strittmatter, 1971). Reactivity of the solubilized protein in the system NADH, b_5 reductase, cyt b_5, and cyt c was 60 μmol of cyt C/(min mg of P). The reductase was separated from the cytochrome on the DEAE column. The spectrum of the iron(II) and iron(III) cytochrome matched the literature in our standard buffer. Stock iron(II) cytochrome b_5 was prepared from the iron(III) protein in buffer by reduction with hydrogen gas and a catalyst of 5% palladium on carbon. The reduction was conducted in a three-necked flask equipped with stirring bar, argon, and hydrogen inlets and a medium porosity "flat end" (filter disc) filter tube that was fitted with a stopcock and serum cap. Reduction of a 5×10^{-4} M solution of the iron(III) complex was usually complete in 0.5 h. After allowing the catalyst (~10 mg/30 mL solution) to settle, portions of the stock solution were transferred through the filter under argon via stainless steel hypodermic tubing into an argon purged reaction cuvette. Reaction was commenced by addition of 1–10 μL of the substrate in freshly distilled and argon-purged acetone to the reaction cuvette under argon.

Halides. Organics were freshly distilled before use and stored under argon.

Reactions. In general, substances were added neat or in acetone solution. Controls with α-bromoisobutyronitrile showed no difference in products or kinetics in the presence or absence of acetone (<0.1%). Organic products were detected by flame ionization gas chromatography. A standard of the same substance was generally employed for quantitation. Much difficulty was encountered with quantitation of the bromotrichloromethane reaction. High concentrations (>5 $\times 10^{-3}$ M) denatured the protein and, in this case, the first product spectrum did not correspond exactly to metmyoglobin. It did slowly change to metmyoglobin on standing (>3 h).[1] All reactions with isolated heme proteins were conducted under argon at identical conditions of pH and ionic strength. These were pH 7.4, 0.01 M phosphate buffer and 0.1 M potassium chloride.

Kinetics. Rates were monitored spectrally at the α band of the iron(II) proteins in the manner previously described (Wade & Castro, 1973b; Castro & Bartnicki, 1975; Castro et al., 1978). For myoglobin, catalase, peroxidase, and cytochrome b_5, the wavelengths employed were 556, 558, 558, and 556 nm, respectively. A typical trace from the Cary 118C for the oxidation of myoglobin by α-bromoisobutyronitrile is shown in Figure 1, and the corresponding first-order plot is given in Figure 2. The influence of ionic strength and pH upon this reaction is shown in Figure 3.

[1] With this substrate, the process was:

$$Fe^{II}Mb \rightarrow Fe^{III}Mb' \xrightarrow{slow} Fe^{III}Mb$$

However, the $Fe^{III}Mb'$ first formed was not the iron-alkyl intermediate (eq 9). Thus, the yield of chloroform after the initial phase of the reaction did not change upon addition of strong acid. The latter should hydrolyze any iron-alkyl.

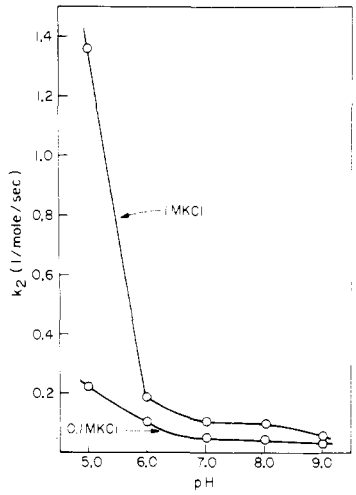


FIGURE 3: The influence of pH and ionic strength on the oxidation of deoxymyoglobin by α-bromoisobutyronitrile.

TABLE I: Products of the Reduction of Alkyl Halides by Iron Porphyrins and Heme Proteins.

substrate	iron porphyrin	product	yield
bromomalononitrile	Fe^{II} deutero-IX	malononitrile	98[a]
	Hb	malononitrile	85[b]
	Mb	malononitrile	87[b]
	cyt *c*	malononitrile	101[c]
	cyt *a*	malononitrile	
	lysed cells	malononitrile	
	intact erythrocytes	malononitrile	90
bromotrichloro-methane	Mb	chloroform	30
bromodiethylmalo-nate	Hb	diethylmalo-nate	81[b]
	Mb	diethylmalo-nate	
α-bromoisobutyro-nitrile	Fe^{II} deutero-IX	isobutyronitrile	101[a]
	Hb	isobutyronitrile	
	Mb	isobutyronitrile	104

[a] Cf. Wade & Castro, 1973a. [b] Cf. Wade & Castro, 1973b. [c] Cf. Castro & Bartnicki, 1975.

Results

Stoichiometry. The general overall stoichiometry (eq 1) had

$$2Fe^{II}\ \text{protein} + BrCH(CN)_2 + H_2O \rightarrow 2Fe^{III}\ \text{protein} + CH_2(CN)_2 + HO^- + Br \quad (1)$$

been established for reduction of the halide bromomalononitrile by iron porphyrins in solution (Wade & Castro, 1973a), with homogeneous solutions of purified hemoglobin and myoglobin (Wade & Castro, 1973b), and with cytochrome *c* and cytochrome oxidase (Castro & Bartnicki, 1975). We now show the same reaction does occur with a suspension of lysed cells and intact human erythrocytes. In all cases, the reaction is quantitative. Actual yields of organic product based upon the general hydrogenolysis reaction (eq 2)

$$2Fe^{II}\ \text{protein} + RX + H_2O \rightarrow 2Fe^{III}\ \text{protein} + RH + HO^- \quad (2)$$

are summarized in Table I for a series of halides for all of the reactions examined. Clearly, the same stoichiometry is obtained at all levels of biological integrity.

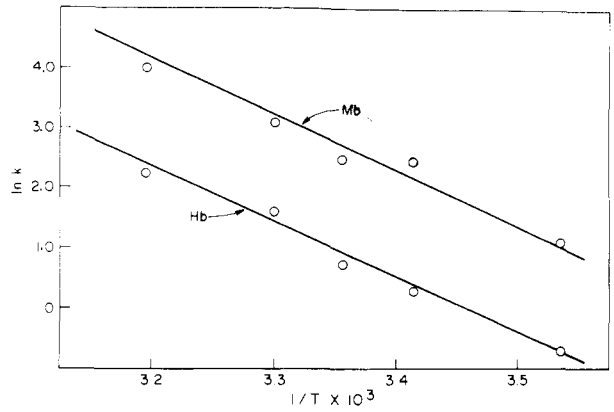


FIGURE 4: Arrhenius plots for the oxidation of deoxymyoglobin and deoxyhemoglobin by bromotrichloromethane.

Kinetics. A summary of second-order rate constants for the oxidation of heme proteins by alkyl halides is presented in Table II. The halides are listed in a decreasing order of reactivity with myoglobin. For all cases, including human erythrocytes

$$\text{rate} = k_2\,(Fe^{II}\ \text{protein})(RX) \quad (3)$$

It will be noted that the relative reactivity of the globins to a given halide is about the same. Thus, with any substrate, myoglobin reacts about twice as fast as hemoglobin, and the reaction proceeds at the same rate in intact or lysed cells.

Activation parameters for the oxidation of hemoglobin and myoglobin by the halide bromotrichloromethane (Figure 4) are similar. For hemoglobin $\Delta H^{\ddagger}$ is 17.8 kcal/mol, $\Delta F^{\ddagger}$, 17 kcal/mol, and $\Delta S^{\ddagger}$, 2.7 eu. For myoglobin these values are 17.8 and 16.4 kcal/mol and 4.6 eu, respectively. The influence of pH and ionic strength upon the oxidation of myoglobin is similar to that observed with cytochrome *c* (Castro & Bartnicki, 1975). As with cytochrome *c*, the more rapid reaction at higher ionic strength and acidity results from easier access to iron (more open conformation).

The general overall reactivity of the iron(II) heme proteins to oxidation by the halides stands in the order: catalase ≫ peroxidase > cytochrome b_5 ≳ myoglobin > hemoglobin ≫ cytochrome a_3 > cytochrome *c* > cytochrome *a*, oxyglobins, carbonylglobins. Their reactivity, however, is much less than that of the corresponding iron(II) porphyrin in homogeneous solution (Wade & Castro, 1973a).

Oxygen and Carbon Monoxide Inhibition. Reactions with the globins are inhibited by oxygen. Thus, oxymyoglobin solutions are inert to α-bromoisobutyronitrile. A slow reaction does ensue with bromomalonitrile ($k_{app} \cong 5$ L/(mol s) at $(O_2) = 7.8$ ppm), but it is the deoxy species that reacts; that is, this apparent rate corresponds well with the necessary dissociation of oxygen before reaction can ensue.

$$FeO_2 \rightleftarrows Fe^{II} + O_2 \quad (4)$$

$$Fe^{II} + RX \rightarrow \text{products} \quad (5)$$

Thus

$$\text{rate} = k_{app}(FeO_2)(RX) = k(Fe^{II})(RX) = kK_{eq}\frac{(FeO_2)}{(O_2)}(RX) \quad (6)$$

Employing K_{eq} as $\sim 1.3 \times 10^{-6}$ (Antonini & Brunori, 1971), the rates expected for eq 5 are within experimental error, the same as that obtained by direct measurement. The corresponding carbonyl derivatives of hemoglobin and myoglobin are also inert.

TABLE II: Rates of Oxidation of Fe^{II} Proteins by Alkyl Halides at 25 °C, 0.01 M Phosphate Buffer, pH 7.4, 0.1 M KCl (k_2 in L mol^{-1} s^{-1}).[a]

alkyl halide	Mb	Hb	Hb (RBC)	Hb (lysed cells)	cat.	perox.	cyt *c*	cyt *a*	cyt a_3	cyt b_5
bromomalononitrile	~600[b]	~300	200[c]	200[c]	*f*	*f*	5[d]	NR[d]	29[d]	~600
bromotrichloromethane	8.0	2.5	2.3	2.3			0.007			0.6
bromodiethylmalonate	0.08	0.03	0.03				NR			1.2, 0.04[g]
carbon tetrachloride	0.06	0.008	0.007				NR			0.06
α-bromoisobutyronitrile	0.04	0.004	0.003		30	0.35	NR			0.15
allyl iodide	0.007	NR	NR				NR			0.007
halothane	NR[e]	NR	NR				NR			

[a] All constants an average of 3–5 determinations, reproducibility, ±15%. [b] Cf. Wade & Castro, 1973a. [c] 0.14 M NaCl was employed. [d] Cf. Castro & Bartnicki, 1975. [e] NR, no reaction in 24 h, 10^{-5} M protein, 10^{-3} M substrate. [f] $k_2 > 600$ L mol^{-1} s^{-1}. [g] This reaction is biphasic.

Discussion

The mechanism for the oxidation of iron porphyrins by alkyl halides has been delineated. It is an axial inner sphere (eq 7–9) (bond cleavage) process (Wade & Castro, 1973a).

$$\mathrm{Fe^{II}} + \mathrm{RX} \rightleftharpoons \mathrm{Fe(XR)} \tag{7}$$

$$\mathrm{Fe^{II}(XR)} \longrightarrow \left[\mathrm{Fe\cdots X\cdots R}\right]^{\ddagger} \longrightarrow \mathrm{Fe^{III}\,X} + \mathrm{R}\cdot \tag{8}$$

$$\mathrm{Fe^{II}} + \mathrm{R}\cdot \xrightarrow[\text{fast}]{\mathrm{H_2O}} \left(\mathrm{(Fe(R))}\right) \longrightarrow \mathrm{Fe^{III}} + \mathrm{RH} + \mathrm{HO^-} \tag{9}$$

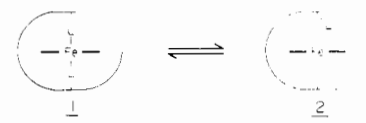
With iron porphyrins, the bond cleavage step (eq 8) is rate limiting. However, with heme proteins, substitution into the inner coordination sphere of iron (eq 7) can be the slow step (cf. Castro & Bartnicki, 1975). The reversibility of eq 8 and the reaction eq 9 is characteristic of the rapid reactions of high-spin iron porphyrins with free radicals in homogeneous solution (Castro et al., 1974).

In all cases at physiological pH, the relative reactivity of the proteins reflects the steric accessibility to iron. Thus, the oxidation of a high-spin heme in homogeneous solution by α-bromoisobutyronitrile or allyl iodide proceeds at least 10^4 more rapidly than the corresponding reactions with myoglobin. The rate of the axial inner sphere oxidation is attenuated by the protein conformation, and substitution into the inner coordination sphere (eq 7) becomes rate limiting. The small differences in enthalpies of activation for the bromotrichloromethane oxidation of hemoglobin and myoglobin are in keeping with a slightly greater steric barrier in the former protein. For hemoglobin, at least, the results demonstrate the same effective redox geometry is present in whole cells and in homogeneous solution.

The relative reactivity of the proteins toward oxidation accords exactly with the predicted reactivity of simple theory (Castro, 1971). However, the high reactivity of cytochrome b_5 toward alkyl halides is an outstanding exception of these views. We elaborate here upon our studies of the general reactivity of this cytochrome and rectify an erroneous conformational assignment.

Cytochrome b_5. Based solely upon its inability to be inhibited by carbon monoxide and cyanide and yet be oxidized by oxygen, a "C" conformation was assigned this protein and an "outer sphere" mechanism for the oxygen oxidation of the heme in b_5 and hemes generally was postulated. We have recently demonstrated this mechanism for the O_2 oxidation of hemes, including an active site model for cyt b_5, does occur in homogeneous solution (Chu et al., 1978). These findings, along with the observations noted below, can explain the seemingly anomalous behavior of this cytochrome. The relatively rapid oxidation of iron(II) b_5 by alkyl halides signifies that an axial position on iron can be substituted. Moreover, it is now known that nitroaromatics also oxidize hemes by a related mechanism (Ong & Castro, 1977) in which the nitro compound must enter the inner coordination sphere. In accord with this, and in complete analogy with the halides, the "G" conformation of myoglobin allows oxidation by the reagent *sym*-trinitrobenzene, while the "C" conformation of cytochrome *c* precludes it. We find that iron(II) cytochrome b_5 *is oxidized* smoothly by *sym*-trinitrobenzene and the rate of the process ($k_2 = 0.64$ L/(mol s)) is somewhat faster than the reaction with myoglobin. Thus, b_5 cannot be in the C conformation.

In contrast to the oxidations by alkyl halides or nitroaromatics, reduction by hydroquinone is a "peripheral outer sphere" process (Castro et al., 1977). With this reagent both a bis(imidazole) heme (an active site model for cyt b_5) and cytochrome *c* react at about the same fast rate (45 L/(mol s)). On the other hand, iron(III) myoglobin is inert as are all high-spin iron(III) porphyrins. In sharp contrast to the high reactivity of its low-spin active site and the low-spin iron(III) complex in cytochrome *c*, cytochrome b_5 is inert to hydroquinone. Therefore, the periphery of the porphyrin in this protein must be sterically encumbered. Thus, b_5 is in the G conformation. That is, the large propionic acid side chains of the porphyrin in cooperation with the bulk of the protein preclude attack at the porphyrin periphery, but an axial position is available for reaction. This assignment is not inconsistent with the X-ray structure of b_5 in the solid state (Mathews et al., 1972). Visual examination of the α-carbon skeleton of the protein suggests a channel to at least one of the imidazoles. This access to imidazole can also explain the oxygen oxidation of this cytochrome. An outer sphere "through axial ligand" process (Castro, 1978) can occur with imidazole as an iron ligand. This mechanism would be consistent with the autoxidation of b_5 (Berman et al., 1976) and its active site (Chu et al., 1978). The known lack of inhibition by carbon monoxide (or cyanide) exhibited by this protein is consonant with a low-spin bis(imidazole) adduct in the G conformation. It has been demonstrated that imidazole is a strong ligand for iron (Broualt & Rouge, 1975). The order of ligand binding to iron(II) porphyrins in benzene is: imidazole > pyridine > CO. Presuming a dissociative mechanism for substitution (eq 10)



(10)

and given the high effective concentration of the imidazoles held in place by the protein, the equilibrium 10 would lie far to the left. A small amount of coordination with CO ($\sim 10^{-3}$ M saturated) would not be expected to be discernible. On the other hand, entrance of an oxidant (alkyl halide, nitro aromatic) into the inner coordination sphere of even a small population of the pentacoordinate cytochrome **2** can result in complete oxidation.[2] The only constraint our data would place on this mechanism is that the imidazole off rate is faster than the rates of oxidation observed by alkyl halides or nitro aromatics and this is not unreasonable.[3]

No stable O_2 adduct is observed with b_5 because like CO any small concentration of a 1:1 adduct would not be discernible. Moreover, the hexacoordinate bis-imidazole adduct **1** is capable of direct oxidation with O_2 by the outer sphere "through axial ligand" route.

The results here with b_5 point to a reactivity pattern that very closely matches that of myoglobin or hemoglobin. Indeed only a very minor difference in structure is consistent with the different spin states of iron in these proteins and their difference in reactivity to oxygen. In contrast to b_5, the distal imidazole in the globins is not held close enough to the iron by the proteins to bind it; hence, the pentacoordinate high-spin iron can be ligated by O_2.

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[2] It is also possible that alkyl halides and nitro aromatics will react with the imidazole and assist in its dissociation.

[3] Using the fastest reacting substrate, BMN, with $k_2 \cong 600$ L/(mol s), an assessment of the off rates would be $\sim 600/(\text{BMN})_0 \cong 0.06\ s^{-1}$. The corresponding rate for the seriously encumbered axial ligand of iron(III) cytochrome *c* is 60 s^{-1} (Sutin & Yandell, 1972).