# Chemical Mechanisms of Catalysis by Cytochromes P-450: A Unified View

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Cytochromes P-450 are ubiquitous in nature. The levels of these hemoproteins can be influenced by many chemicals and the enzymes in turn are capable of metabolizing many compounds.[1] Because of the diversity of substrates and the variety of transformations that these enzymes execute, this family of cytochromes has attracted the attention of workers in many fields, including organic and inorganic chemistry, biochemistry, endocrinology, nutrition, pharmacology, bacteriology, toxicology, and oncology. Interest in the latter two fields has been particularly great because of the ability of these enzymes to metabolize rather inert chemicals to electrophilic products that can bind irreversibly to cellular macromolecules and initiate the development of toxic or tumorigenic lesions.[2]

Cytochromes P-450 carry out oxidative reactions with typical mixed-function oxidase stoichiometry:

$$R + NAD(P)H + H^+ + O_2 \rightarrow$$
$$RO + H_2O + NAD(P)^+$$
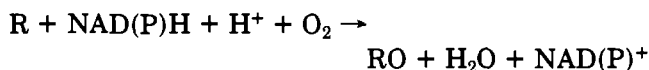
We classify the oxidative reactions into six categories: (1) *Carbon hydroxylation* is the formation of an alcohol at a methyl, methylene, or methine position. (2) *Heteroatom release* is the oxidative cleavage of the heteroatomic portion of a molecule, which results from hydroxylation adjacent to the heteroatom generating a geminal hydroxy heteroatom substituted intermediate (e.g., carbinolamine, halohydrin, hemiacetal, hemiketal, or hemithioketal) that collapses with release of the heteroatom to form a carbonyl compound. (3) *Heteroatom oxygenation* is the conversion of a heteroatom-containing substrate to its corresponding heteroatom oxide, such as the transformation of a tertiary amine to an *N*-oxide, an organohalide to a haloso derivative, a sulfide to a sulfoxide, or a phosphine to a phosphine oxide. (4) *Epoxidation* is the formation of oxirane derivatives of olefins and aromatic compounds. (5) *Oxidative group transfer* involves a 1,2 carbon shift of a group with concomitant incorporation of oxygen

as a carbonyl at the C-1 position. (6) *Olefinic suicide destruction* involves inactivation of the heme, in our considerations here, of cytochrome P-450 by an enzyme product or an enzyme intermediate. Other reactions catalyzed by cytochromes P-450, which will not be considered here, include the reduction of azo dyes, epoxides, *N*-oxides, and halomethanes, the reduction and scission of hydroperoxides (and propagation of lipid peroxidation), and relatively nonspecific oxidations involving forms of partially reduced oxygen that can be released from cytochrome P-450.[1,3]

Elucidation of the chemical mechanisms by which the cytochrome P-450 enzymes catalyze their chemical transformations has been a challenge due to the difficulty in characterization of many of the critical intermediates involved in the catalytic mechanism and the variety of chemical reactions catalyzed by these enzymes.[1,3,4] Two central questions of the catalytic cycle have had to be addressed: the mechanism of oxygen activation to the active intermediate and the mechanism of oxygen transfer from the enzymatic active oxygen species to the substrate.

The early steps in the cytochrome P-450 catalytic cycle are shown in Figure 1.[3–6] The binding of the substrate to the ferric enzyme is rapid and stoichiometric.[7] In some cases the iron is changed from the low- to high-spin state and the oxidation–reduction potential is raised upon binding.[8] Our own studies with the liver microsomal cytochromes P-450 indicate that these changes are not always observed and are not related to rates of catalysis.[6,9] The substrate-bound ferric enzyme accepts one electron from the flavoprotein NADPH-cytochrome P-450 reductase,[9,10] which contains one FMN and one FAD,[11] and which donates the electron from the FMNH$_2$/FADH· complex.[6,12] The

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(1) (1) (a) P. G. Wislocki, G. T. Miwa, and A. Y. H. Lu, *Enzym. Basis Detoxication*, 1, 135 (1980); (b) F. P. Guengerich, *Pharmacol. Ther., Part A*, 6, 99 (1979); (c) A. Y. H. Lu and S. B. West, *Pharmacol. Rev.*, 31, 277 (1980).
(2) (2) (a) E. C. Miller and J. A. Miller, *Cancer (Philadelphia)*, 47, 2327 (1981); (b) S. D. Nelson, *J. Med. Chem.*, 25, 753 (1982).
(3) R. E. White and M. J. Coon, *Annu. Rev. Biochem.*, 49, 315 (1980).
(4) V. Ullrich, *Top. Current Chem.*, 83, 67 (1979).
(5) R. Sato and T. Omura, "Cytochrome P-450", Academic Press, New York, 1978.
(6) F. P. Guengerich, *Biochemistry*, 22, 2811 (1983).
(7) (a) M. J. Coon, D. A. Haugen, F. P. Guengerich, J. L. Vermilion, and W. L. Dean in "The Structural Basis of Membrane Function", Y. Hatefi and L. Djavadi-Ohaniance, Eds., Academic Press, New York, 1976, p 409; (b) F. P. Guengerich, *J. Biol. Chem.*, 253, 7931 (1978).
(8) S. G. Sligar, D. C. Cinti, G. Gibson, and J. B. Schenkman, *Biochem. Biophys. Res. Commun.*, 90, 925 (1979).
(9) F. P. Guengerich, D. P. Ballou, and M. J. Coon, *J. Biol. Chem.*, 250, 7405 (1975).
(10) J. A. Peterson, R. E. White, Y. Yasukochi, M. L. Coomes, D. H. O'Keefe, R. E. Ebel, B. S. S. Masters, D. P. Ballou, and M. J. Coon, *J. Biol. Chem.*, 252, 4431 (1977).
(11) T. Iyanagi and H. S. Mason, *Biochemistry*, 12, 2297 (1973).
(12) (a) T. Iyanagi, N. Makino, and H. S. Mason, *Biochemistry*, 13, 1701 (1974); (b) J. L. Vermilion, D. P. Ballou, V. Massey, and M. J. Coon, *J. Biol. Chem.*, 256, 266 (1981).
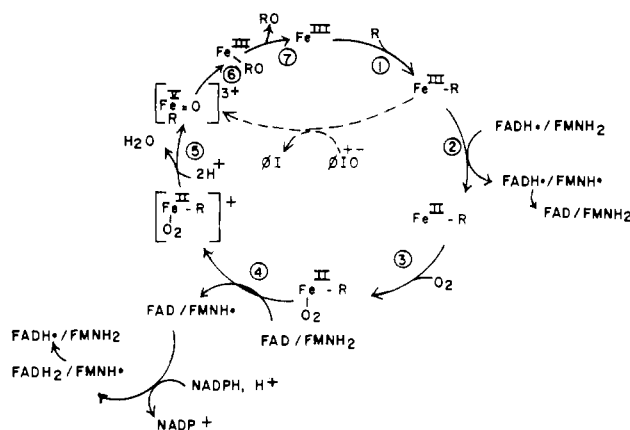
**Figure 1.** Proposed catalytic cycle for reduction and oxidation of cytochrome P-450.

substrate-bound ferrous enzyme binds molecular oxygen rapidly to form an oxygenated complex.[13] Without the addition of another electron, the complex can decay to release superoxide anion (or hydrogen peroxide, after dismutation)[14] or dismute to yield oxidized enzyme products.[9,14c] Alternatively, this oxygenated complex can accept a second electron from the flavoprotein, or possibly cytochrome $b_5$,[6,15] generating an activated iron–oxygen complex. Several lines of evidence suggest that this complex is a perferryl oxygen, formally (Fe=O)$^{3+}$.[16] Oxygen is then added to the substrate. After oxygen addition, the oxygenated product dissociates to leave the iron in the original ferric state. Steps 2, 4, and 6 appear to be rate-limiting under different conditions.[3]

The Account focuses on our efforts directed at understanding the detailed mechanism of enzymatic oxygen transfer. We have purified eight different rat liver microsomal cytochromes P-450 and examined the abilities of the various forms to catalyze certain reaction.[17,18] Common features must exist in all cytochrome P-450 oxidative reactions, since a single form of cytochrome P-450 can carry out many types of reactions. For instance, cytochrome P-450$_{PB-B}$[17] can catalyze all six types of oxidative reactions with certain substrates, i.e., carbon hydroxylation (e.g., cyclohexane[19]), heteroatom oxygenation (e.g., azoprocarbazine[20]), heteroatom release (e.g., benzphetamine[17]), epoxidation (e.g., arachidonate[21]), oxidative group transfer (e.g., trichloroethylene[22]), and olefinic suicide destruction (e.g., vinyl

chloride[23]). Even the relatively specific bacterial cytochrome P-450$_{cam}$ has been shown to catalyze carbon hydroxylation (e.g., camphor[24]), heteroatom release (e.g., 5-bromocamphor[25]), and epoxidation (e.g., dehydrocamphor[26]). As a basis for our initial studies on the mechanism of cytochrome P-450, we reevaluated earlier observations of Ullrich[27] about the electrophilic nature of the active oxygen species and focused our attention on the most electron-rich moieties of cytochrome P-450 substrates, particularly nonbonded and $\pi$ electrons. Our studies were initiated as a part of our work on the metabolism of halogenated compounds[19,22,23,28,29] and have been extended toward development of a unified view of the mechanism of oxidative catalysis.

## Carbon Hydroxylation

The mechanism of hydroxylation of unactivated carbon–hydrogen bonds by cytochrome P-450 has received considerable investigation, due to the high efficiency and specificity of this chemical process and to the critical role played by this transformation in the metabolism of numerous essential physiological compounds such as steroids, as well as in the metabolism of xenobiotic compounds. The mechanism of hydroxylation was believed to be a concerted oxenoid process until the late 1970s. In 1978, Groves presented a series of experiments with deuterated norbornane derivatives which suggested that alkane hydroxylation proceeded by a stepwise process involving the formation of a carbon-centered free radical via hydrogen atom abstraction by the perferryl oxygen intermediate and collapse of the carbon radical–perferric hydroxide radical pair.[30] Data to support this mechanism was derived from the large isotope effect observed in the hydroxylation of tetradeuterated norbornane ($k_H/k_D$ = 11.5) and the loss of stereochemistry (postulated to be a result of radical epimerization) in the hydroxylation. Data from several laboratories, involving studies of both the enzyme and model porphyrin systems, now support the essential features of this mechanism.[16,30,31] The slow step in the oxygen-transfer sequence (not necessarily in the enzyme mechanism) is thought to be abstraction of the hydrogen atom from the alkane carbon by the perferryl oxygen. Collapse of the intermediate carbon radical–perferric hydroxide radical pair is presumably a cage reaction with a low-energy barrier. The

(13) (a) F. P. Guengerich, D. P. Ballou, and M. J. Coon, *Biochem. Biophys. Res. Commun.*, **70**, 951 (1976); (b) Y. Ishimura, V. Ullrich, and J. A. Peterson, *Biochem. Biophys. Res. Commun.*, **42**, 140 (1971); (c) R. W. Estabrook, A. G. Hildebrandt, J. Baron, K. J. Netter, and K. Leibman, *Biochem. Biophys. Res. Commun.*, **42**, 132 (1971).

(14) (a) D. D. Oprian and M. J. Coon in "Microsomes, Drug Oxidations, and Drug Toxicity", R. Sato and R. Kato, Eds., Wiley-Interscience, New York, 1982, p 139; (b) S. G. Sligar, J. D. Lipscomb, P. B. Debrunner, and I. C. Gunsalus, *Biochem. Biophys. Res. Commun.*, **61**, 290 (1974); (c) R. C. Tuckey and H. Kamin, *J. Biol. Chem.*, **257**, 9039 (1982).

(15) (a) A Hildebrandt and R. W. Estabrook, *Arch. Biochem. Biophys.*, **143**, 66 (1971); (b) C. Bonfils, C. Balny, and P. Maurel, *J. Biol. Chem.*, **256**, 9457 (1981).

(16) J. T. Groves, R. C. Haushalter, M. Nakamura, T. E. Nemo, and B. J. Evans, *J. Am. Chem. Soc.*, **103**, 2884 (1981).

(17) F. P. Guengerich, G. A. Dannan, S. T. Wright, M. V. Martin, and L. S. Kaminsky, *Biochemistry*, **21**, 6019 (1982).

(18) A. Y. H. Lu and S. B. West, *Pharmacol. Ther., Part A*, **2**, 337 (1978).

(19) T. L. Macdonald, L. T. Burka, S. T. Wright, and F. P. Guengerich, *Biochem. Biophys. Res. Commun.*, **104**, 620 (1982).

(20) S. W. Cummings, F. P. Guengerich, and R. A. Prough, *Drug Metab. Dispos.*, **10**, 459 (1982).

(21) E. H. Oliw, F. P. Guengerich, and J. A. Oates, *J. Biol. Chem.*, **257**, 3771 (1982).

(22) R. E. Miller and F. P. Guengerich, *Biochemistry*, **21**, 1090 (1982).

(23) F. P. Guengerich and T. W. Strickland, *Mol. Pharmacol.*, **13**, 993 (1977).

(24) M. Katagiri, B. N. Ganguli, and I. C. Gunsalus, *J. Biol. Chem.*, **243**, 3543 (1968).

(25) P. V. Gould, M. H. Gelb, and S. G. Sligar, *J. Biol. Chem.*, **256**, 6686 (1981).

(26) M. H. Gelb, P. Malkonen, and S. G. Sligar, *Biochem. Biophys. Res. Commun.*, **104**, 853 (1982).

(27) V. Ullrich, *Angew. Chem., Int. Ed. Engl.*, **11**, 701 (1972), and references therein.

(28) (a) F. P. Guengerich and P. G. Watanabe, *Biochem. Pharmacol.*, **28**, 589 (1979); (b) F. P. Guengerich, W. M. Crawford, Jr., and P. G. Watanabe, *Biochemistry*, **18**, 5177 (1979); (c) F. P. Guengerich, W. M. Crawford, Jr., J. Y. Domoradzki, T. L. Macdonald, and P. G. Watanabe, *Toxicol. Appl. Pharmacol.*, **55**, 303 (1980); (d) F. P. Guengerich, P. S. Mason, W. Stott, T. R. Fox, and P. G. Watanabe, *Cancer Res.*, **41**, 4391 (1981); (e) L. S. Kaminsky, M. W. Kennedy, S. M. Adams, and F. P. Guengerich, *Biochemistry*, **20**, 7379 (1981); (f) T. L. Macdonald, H. Tachizawa, and R. A. Neal, *Mol. Pharmacol.*, **22**, 745 (1982).

(29) L. T. Burka, A. Thorsen, and F. P. Guengerich, *J. Am. Chem. Soc.*, **102**, 7615 (1980).

(30) J. T. Groves, G. A. McClusky, R. E. White, and M. J. Coon, *Biochem. Biophys. Res. Commun.*, **81**, 154 (1978).

(31) R. E. White, J. T. Groves, and G. A. McClusky, *Acta Biol. Med. Ger.*, **38**, 475 (1979).

stereospecificity often observed in cytochrome P-450 catalyzed hydroxylations is contrary to the expectation for such a radical process and has been attributed to physical factors associated with substrate binding in the enzyme active site.

$$[FeO]^{3+} \xrightleftharpoons[\searrow CH]{} [FeOH]^{3+} + \searrow C\cdot] \longrightarrow Fe^{3+} + \searrow COH$$

## Heteroatom Release

Much of our research has been directed at elucidating the mechanisms of heteroatom release and heteroatom oxygenation. These processes are related conceptually by examination of the site in a heteroatom-containing substrate to which oxygen is transferred. In heteroatom oxygenation, oxidation occurs at the heteroatom, whereas in heteroatom release, oxygen is transferred to the carbon adjacent ($\alpha$) to the heteroatom. Hydroxylation of heteroatom-containing substrates normally demonstrates a marked regioselectivity for the $\alpha$ position as compared with $\beta$, $\gamma$, or more distant positions[32] and thus preference underlies several critical processes mediated by cytochrome P-450 (e.g., N- and O-dealkylation). The relationship between these two biotransformations is further underscored by the observation that the predominant metabolic process of an isoelectronically substituted series of compounds tends to favor heteroatom oxygenation over heteroatom release as the nonmetallic elements progress down a group in the periodic table. Thus, alkylamines produce predominantly intermediate $\alpha$-hydroxy amines, whereas the isoelectronic alkylphosphines generate phosphine oxides; alkyl ethers produce putative $\alpha$-hydroxy ethers, whereas the isoelectronic alkyl sulfides generate sulfoxides. Thus, two major questions have evolved concerning the metabolism of heteroatom-containing compounds by cytochrome P-450: what are the atomic or molecular features of a heteroatom-containing compound that promote heteroatom oxygenation or heteroatom release, and what is the source of the regioselectivity observed for hydroxylation of heteroatom-containing compounds?

$$CH_3CH_2CH_2CH_2X \xrightarrow[NADPH, O_2]{\text{cytochrome P-450}} \begin{array}{l} [CH_3CH_2CH_2CH(OH)X] \\ \downarrow -HX \\ CH_3CH_2CH_2CHO \\ \text{(heteroatom release)} \\ CH_3CH_2CH_2CH_2X\!=\!\!O \\ \text{(heteroatom oxygenation)} \end{array}$$

Several feasible mechanisms for rationalizing regioselective hydroxylation adjacent to heteroatoms by cytochrome P-450 are possible. For example, carbon hydroxylation adjacent to a heteroatom via a stepwise radical hydrogen abstraction process would be facilitated due to the established ability of a heteroatom (except fluorine) to stabilize an adjacent free radical (or an incipient species or a transition state resonance contributor). An alternate pathway for regioselective $\alpha$-hydroxylation was suggested by Griffin, who proposed that cytochrome P-450 abstracts electrons from amines in sequential one-electron processes.[33] Shono has also



**Figure 2.** Possible mechanisms for suicidal inactivation of cytochrome P-450 with cyclopropylamines.

considered the electrochemical oxidation of amines and compared the products to those obtained in enzymatic oxidations.[34] The patterns of regioselectivity are similar, as are the kinetic deuterium isotope effects. These results are important, as the mechanism of electrochemical oxidation of amines is believed to involve sequential one-electron transfers from the nitrogen[35] and enzymatic N-dealkylation (heteroatom release) does not appear to result from simple two-electron oxidation and hydration.[36]

We considered this data along with the knowledge that the nonbonded electrons of heteroatomic molecules should be most easily removed. Cyclopropyl systems appealed to us as mechanistic probes. Cyclopropyl amine and cyclopropoxyl free radicals are known to rearrange rapidly ($k > 10^8$ s$^{-1}$) upon formation, and if formed in the active site of an enzyme, such a species might be anticipated to be highly reactive and destructive.[37] Indeed, cyclopropyl compounds have been used as diagnostic suicide inactivators to study several enzymes, including cytochrome P-450.[38] The mechanism-based inhibition of these enzyme classes by cyclopropylamines was postulated to be a consequence of $\alpha$-hydroxylation to form cyclopropylcarbinolamines, which were proposed to react with enzyme-bound nucleophiles via reactive iminium ions (Figure 2, upper route). However, an alternate pathway for suicide inactivation, postulated to involve initial amine nitrogen oxidation to a radical cation via single-electron transfer, appeared possible (Figure 2, lower route). In order to distinguish between two possible mechanisms of inhibition, we synthesized the cyclopropylamines 1a and 1b.[39] Amine 1b cannot be oxygenated at the amine-bearing ring carbon or form a Schiff's base with the cyclopropyl group due to the unavailability of a cyclopropyl hydrogen adjacent to the nitrogen. Both cyclopropylamines 1a and 1b were equally efficient in suicide inhibition of the enzyme, providing strong support for the single electron transfer–ring opening pathway of suicide inhibition.[39] In addition, the principal mode of cytochrome P-450 inactivation was de-

(32) B. Testa and D. M. Mihailova, *J. Med. Chem.*, **21**, 683 (1978).

(33) B. W. Griffin, C. Marth, Y. Yasukochi, and B. S. S. Masters, *Arch. Biochem. Biophys.* **205**, 543 (1980).

(34) T. Shono, T. Toda, and N. Oshino, *J. Am. Chem. Soc.*, **104**, 2639 (1982).

(35) (a) T. Shono, H. Hamaguchi, and Y. Matsumara, *J. Am. Chem. Soc.*, **97**, 4264 (1975); (b) J. R. Lindsay-Smith and D. J. Masheder, *J. Chem. Soc., Perkin Trans. Trans. 2*, 47 (1976).

(36) (a) R. E. McMahon, H. W. Culp, and J. C. Occolowitz, *J. Am. Chem. Soc.*, **91**, 3389 (1969); (b) J. P. Shea, G. L. Valentine, and S. D. Nelson, *Biochem. Biophys. Res. Commun.*, **109**, 231 (1982).

(37) (a) D. Griller and K. V. Ingold, *Acc. Chem. Res.*, **13**, 317 (1980); (b) A. M. Martinez, G. E. Cushmac, and J. Rocek, *J. Am. Chem. Soc.*, **97**, 6503 (1975).

(38) N. Seiler, M. J. Jung, and J. Koch-Weser, "Enzyme-activated Irreversible Inhibitors", Elsevier–North Holland, New York, 1978; (b) R. P. Hanzlik, V. Kishore, and R. Tullman, *J. Med. Chem.*, **22**, 759 (1979); (c) R. P. Hanzlik and R. H. Tullman, *J. Am. Chem. Soc.*, **104**, 2048 (1982).

(39) T. L. Macdonald, K. Zirvi, L. T. Burka, P. Peyman, and F. P. Guengerich, *J. Am. Chem. Soc.*, **104**, 2050 (1982).
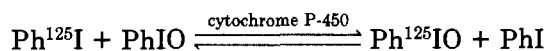
struction (presumably via alkylation) of the heme prosthetic group.

In a similar group, we have examined an analogous series of cyclopropyl- and 1-methylcyclopropyl-substituted ethers, iodides, bromides, amides, and alcohols and cyclopropane hydrate. All of these compounds destroy cytochrome P-450 heme after oxidative metabolism by purified cytochrome P-450. Further, for this series, the log of the inactivation constant (log $k_i$) was inversely related to the single-electron oxidation potential ($e_{1/2}$) (correlation coefficient = -0.89, n = 9),[40] which led us to propose an analogous single electron transfer–cyclopropane ring opening sequence for the mechanism of inactivation. Thus, the suicide inactivation that results with these cyclopropyl compounds and additional evidence provided by Ortiz de Montellano[41] and others demonstrates that one-electron oxidation of heteroatom-containing compounds is the first step in the cytochrome P-450 catalyzed oxidation in many instances. In most cases, such initial electron transfer results in overall $\alpha$-hydroxylation and subsequent heteroatom release; cyclopropyl compounds represent a select subset of substrates for which this process results in ring opening and subsequent enzyme destruction.

## Heteroatom Oxygenation

Many heteroatom oxygenations can be attributed to another mixed-function oxidase, the flavin-containing monooxygenase (EC 1.14.13.8, *N,N*-dimethylaniline N-oxidizing).[42] Several examples of sulfur oxidation by cytochrome P-450 are known,[1] and oxygenation of trivalent phosphorus has also been reported.[43] While the literature attributes many N-oxygenations to cytochrome P-450, the only cases where strong evidence exists for the role of this enzyme involve the substrates 2-(acetylamino)fluorene,[44] 2-aminofluorene,[45] azoprocarbazine,[20] phentermine,[46] and certain tryptophan pyrolysis products.[47]

A number of chemical studies have implicated hypervalent states of halogens in oxidation-dependent solvolysis and elimination reactions.[48,49] The only example of the enzymatic formation of a stable haloso compound comes from work in this laboratory, where we used an exchange reaction to oxidize iodobenzene, with iodosobenzene as both the cytochrome P-450 oxidant and a trap for the product.[29] In this system, the

$$\text{Ph}^{125}\text{I} + \text{PhIO} \underset{}{\overset{\text{cytochrome P-450}}{\rightleftharpoons}} \text{Ph}^{125}\text{IO} + \text{PhI}$$

oxygen of iodosobenzene exchanges rapidly with water in the presence of cytochrome P-450.[19] As in the case of model metalloporphyrins, the perferryl oxygen exchanges with the solvent in the absence of an oxidizable substrate but, if formed in the presence of a substrate, is committed to catalysis.[19,50]

A series of studies on the metabolism of propyl halides also suggest the intermediacy of haloso intermediates.[28f] This study determined the total metabolic profile resulting from the cytochrome P-450 catalyzed metabolism of the propyl halide series (chloride, bromide, and iodide) and examined the influence of the halogen substituent in the halide series on the distribution and types of metabolic products. The distribution of propyl halide metabolites as a function of the halide and the chemical nature of the metabolites was interpreted to be a consequence of the partitioning of the initial metabolic transformation between heteroatom release and heteroatom oxygenation pathways. The relative contribution of heteroatom oxygenation was suggested to increase as the halide progressed down the periodic table.

Why does heteroatom oxygenation occur? We feel that the first step in heteroatom oxygenation is one-electron oxidation of the heteroatom, as in the case of heteroatom release. If $\alpha$-hydrogens are available, they will tend to be abstracted before oxygen rebound can occur to give heteroatom oxygenation, and heteroatom release will eventually result. Heteroatom oxygenation is favored under two conditions: (1) no $\alpha$-hydrogens are available (e.g., several of the aromatic amides,[44] certain primary amines,[45,46] and possibly pyridines[51]), or (2) the radical system has properties that render it relatively stable (e.g., sulfides,[1] phosphines,[43] azoprocarbazine[20]).

$\alpha$-Hydroxylation, the initial event in heteroatom release, can occur via either of two possible pathways. The single electron transfer pathway proceeds identically with the heteroatom oxygenation scheme to generate a transient heteroatom cation intermediate, which rather than oxygen transfer undergoes proton (or hydrogen atom) transfer to produce a transient heteroatom-substituted carbon radical (or carbonium ion) that subsequently collapses to the intermediate alcohol. Alternatively, the postulated transient heteroatom-substituted carbon radical could be produced via hydrogen atom abstraction from carbon by the perferryl oxygen species by analogy with the proposed alkane hydroxylation mechanism. The free energies for reaction of a heteroatomic substrate via the cytochrome P-450 catalyzed hydrogen atom or electron-transfer processes are interrelated by the energies of the intermediates. In order to make accurate a priori predictions about modes of reactivity of a given substrate with the enzyme, several unresolved parameters must be addressed, including the single-electron potential of the activated oxygen complex $(FeO)^{3+}$ and the strength and acidity of the oxygen–hydrogen bond in the preferric hydroxide $(FeOH)^{3+}$ species.

One way in which the ability of heteroatomic molecules to participate in cytochrome P-450 mediated single electron transfer oxidations (both heteroatom

(40) (a) F. P. Guengerich, T. L. Macdonald, L. T. Burka, R. E. Miller, D. C. Liebler, K. Zirvi, C. B. Frederick, F. F. Kadlubar, and R. A. Prough in "Cytochrome P-450: Biochemistry, Biophysics, and Environmental Implications", Elsevier–North Holland, New York, 1982, p 27; (b) F. P. Guengerich, T. L. Macdonald, and R. J. Willard, submitted for publication.

(41) O. Augusto, H. S. Beilan, and P. R. Ortiz de Montellano, *J. Biol. Chem.*, 257, 11288 (1982).

(42) D. M. Ziegler, *Enzym. Basis Detoxication*, 1, 201 (1980).

(43) R. A. Wiley, L. A. Sternson, H. A. Sasame, and J. R. Gillette, *Biochem. Pharmacol.*, 21, 3235 (1972).

(44) E. F. Johnson, D. S. Levitt, U. Muller-Eberhard, and S. S. Thorgeirsson, *Cancer Res.*, 40, 4456 (1980).

(45) C. B. Frederick, J. B. Mays, D. M. Ziegler, F. P. Guengerich, and F. F. Kadlubar, *Cancer Res.*, 42, 2671 (1982).

(46) J. D. Duncan and A. K. Cho, *Mol. Pharmacol.*, 22, 235 (1982).

(47) S. Mita, K. Ishii, Y. Yamazoe, T. Kamataki, R. Kato, and T. Sugimura, *Cancer Res.*, 41, 3610 (1981).

(48) T. L. Macdonald, N. Narasimhan, and L. T. Burka, *J. Am. Chem. Soc.*, 102, 7760 (1980), and references therein.

(49) T. T. Nguyen and J. C. Martin, *J. Am. Chem. Soc.*, 102, 7382 (1980).

(50) J. T. Groves and W. J. Kruper, Jr., *J. Am. Chem. Soc.*, 101, 7613 (1979).

(51) J. W. Gorrod in "Microsomes, Drug Oxidations, and Drug Toxicity", R. Sato and R. Kato, Eds., Wiley-Interscience, New York, 1982, p 621.

Table I
Ionization Potentials of Heteroatoms Found in
Potential Cytochrome P-450 Substrates

| compd type | potential | |
| --- | --- | --- |
| | photoelectron spectroscopy, eV[52] | electro-chemical, V (vs. SCE)[a] |
| Me$_3$P | 8.2 | 0.85 |
| Me$_3$N | 8.5 | 1.0 |
| Me$_2$S | 8.7 | 1.4 |
| MeI | 9.5 | 2.2 |
| Me$_2$O | 9.8 | 2.4 |
| MeBr | 10.6 | 2.6[53d] |
| MeCl | 11.3 | |
| MeF | 13.1 | |

[a] The half-wave potentials for oxidation of the indicated compounds vary slightly depending upon the source even when measured vs. SCE in CH$_3$CN (e.g., MeI, $E_{1/2}$ = 2.12, 2.37 V; Me$_2$S, $E_{1/2}$ = 1.35, 1.60). The values presented here represent averages from three sources[53a-c] unless indicated otherwise.[53d]

oxygenation and release) can be estimated is by consideration of the ionization potentials of the heteroatoms for one-electron oxidation. Table I presents such data. Clearly, this analysis will hold only if substrate oxidation is at least partially rate determining in the enzyme mechanism and if physical factors such as the rates of binding to and orientation within the active site are relatively constant for a given series of heteroatomic compounds. A manifestations of the predominance of chemical differences in determining the differences in the rates of enzyme-catalyzed single electron transfer processes would be that the rates of oxidation of a series of homologous heteroatomic compounds would follow the order indicated in Table I: R$_3$P > R$_3$N > R$_2$S > RI $\simeq$ R$_2$O > RBr > RCl > RF.[52,53] Strong indications that such an order may hold for heteroatom-containing close structural analogues have been presented in the literature. For example, the rates of metabolism of a range of organohalides follows the "halide order": RI > RBr > RCl > RF.[54] We have established that the maximum velocities ($V_{max}$) for hydroxylation of aryl halides follow the halide order.[55] Further, Oae has recently found a correlation between the ionization potentials of a series of sulfides and their rates of oxygenation by cytochrome P-450.[56]

In summary, heteroatom oxygenation preferentially occurs for those heteroatoms, [P, S, (Se, I?)] that form thermodynamically stable heteroatom oxides and that concurrently have relatively low first-electron ionization potentials. The primary reason underlying this metabolic preference is the stability of the heteroatom radical cation (e.g., RCH$_2$X$^+\cdot$) relative to the $\alpha$-heteroatom radical (e.g., RCH·X). Heteroatom release occurs

preferentially for those heteroatoms (except fluorine) in which the $\alpha$-carbon radical is more stable than the corresponding heteroatom radical cation [e.g., N, O, Br, Cl, (I)]. We believe that the single electron transfer pathway for $\alpha$-hydroxylation and subsequent heteroatom release is accessible for all heteroatomic compounds (except alkyl fluorides), although it is probably the dominant or exclusive pathway for heteroatoms with relatively low ionization potentials [e.g., P, N, S, (I)]. The hydrogen atom abstraction pathway may be preferred for heteroatoms with relatively higher ionization potentials [e.g., Cl, Br, (O, I)].

## Epoxidation, Oxidative Group Migration, and Olefinic Suicide Inactivation

These three processes are all considered in the metabolism of olefins and aromatic compounds. The metabolism of unsaturated compounds has traditionally been considered only in terms of epoxidation. The migration of groups from the para to the meta position of aromatic compounds was observed during the para hydroxylation of several aromatic compounds and termed the "NIH Shift".[57] Since similar migrations of para substituents were observed during the rearrangement of 3,4-oxides, the view was taken that epoxides are intermediates in aromatic hydroxylation.

However, studies on suicidal inactivation of cytochrome P-450 by unsaturated compounds recently unraveled the mechanism of alkene oxidation. Synthetic epoxides were shown not to destroy the heme of cytochrome P-450 in cases where destruction was observed during the oxidative metabolism of the parent olefins.[23,58,59] Ortiz de Montellano has deduced the structures of several heme–olefin adducts,[60] which correspond to the structures expected from attack of a porphyrin pyrrolic nitrogen upon an epoxide. However, epoxides have been shown not to be involved and the adducts were explained by attack of a pyrrolic nitrogen upon a transient enzyme intermediate.

We have considered in detail the roles of epoxides in the metabolism of 1,1,2-trichloroethylene, vinylidene chloride (1,1-dichloroethylene), and _trans_-1-phenyl-1-butene. The major product arising from trichloroethylene is chloral (2,2,2-trichloroacetaldehyde).[22] Vinylidene chloride is metabolized to 2-chloroacetic acid and 2,2-dichloroacetaldehyde.[61] In both cases, the distribution of these and other products varies considerably with the isozyme of cytochrome P-450 utilized.[58,61] Although cytochrome P-450 heme is destroyed during the oxidative metabolism of trichloroethylene, the epoxide does not destroy the heme.[58] Moreover, in four different enzyme systems, the level of the epoxide actually formed was 5–28-fold too low to support an obligatory role in chloral formation. Thus, trichloroethylene oxide does not appear to be chemically or catalytically competent as an intermediate.[22] Similar

(52) (a) T. A. Carlson, "Photoelectron and Auger Spectroscopy", Plenum Press, New York, 1975, and references therein; (b) D. W. Turner, C. Baker, A. D. Baker, and C. R. Brundle, "Molecular Photoelectron Spectroscopy", Wiley-Interscience, London, 1970, and references therein; (c) A. D. Baker and D. Betteridge, "Photoelectron Spectroscopy", Pergamon Press., New York, 1972, and references therein.
(53) (a) L. L. Miller, G. D. Nordblom, and E. A. Mayeda, _J. Org. Chem._, **37**, 916 (1972); (b) L. Eberson and K. Nyberg, _Prog. Phys. Org. Chem._, **12**, 1 (1976), and references therein; (c) S. D. Ross, M. Finkelstein, and E. J. Rudd, "Anodic Oxidation", Academic Press, New York, 1975, and references therein; (d) J. Y. Becker and D. Zemach, _J. Chem. Soc., Perkin Trans._ 2, 914 (1979).
(54) T. L. Macdonald, _CRC Crit. Rev. Toxicol._, **11**, 85 (1982).
(55) L. T. Burka, T. Plucinski, and T. L. Macdonald, submitted for publication.
(56) Y. Watanabe, T. Iyanagi, and S. Oae, _Tetrahedron Lett._, **23**, 533 (1982).

(57) J. W. Daly, D. M. Jerina, and B. Witkop, _Experientia_, **28**, 1129 (1972).
(58) R. E. Miller and F. P. Guengerich, _Cancer Res._, **43**, 1145 (1983).
(59) P. R. Ortiz de Montellano, G. S. Yost, B. A. Mico, S. E. Dinizo, M. A. Correia, and H. Kumbara, _Arch. Biochem. Biophys._, **197**, 524 (1979).
(60) (a) P. R. Ortiz de Montellano, H. S. Beilan, K. L. Kunze, and B. A. Mico, _J. Biol. Chem._, **256**, 4395 (1981); (b) P. R. Ortiz de Montellano, K. L. Kunze, H. S. Beilan, and C. Wheeler, _Biochemistry_, **21**, 1331 (1982); (c) P. R. Ortiz de Montellano, B. L. K. Mangold, C. Wheeler, K. L. Kunze, and N. O. Reich, _J. Biol. Chem._, **258** 4208 (1983).
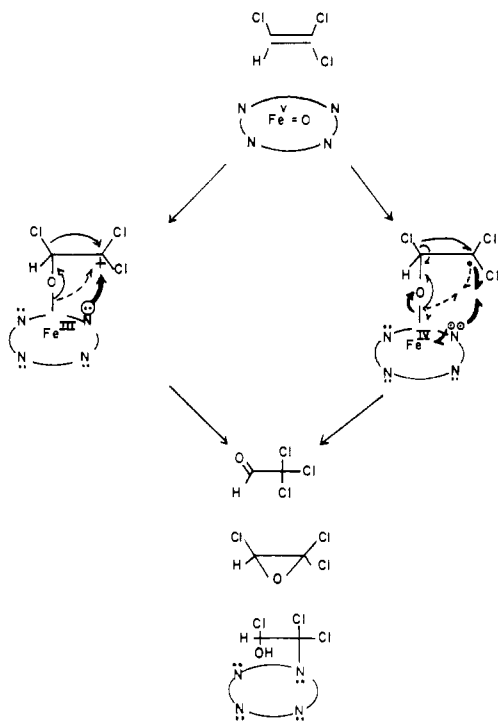(61) D. C. Liebler and F. P. Guengerich, _Biochemistry_, in press.

**Figure 3.** Rationalization of the products of cytochrome P-450 mediated trichloroethylene metabolism in terms of an intermediate that decomposes in heterolytic or homolytic steps. Other resonance forms of the intermediate are possible and have been considered elsewhere.[22,60,61]

results were obtained in a study of vinylidene chloride metabolism.[61] The possibility can be considered that an epoxide is formed in a concerted manner and that the cytochrome P-450 acts as a Lewis acid to catalyze rearrangement to the observed products. However, studies with stable epoxides (which can be shown to be bound in the active site) indicate that neither model iron porphyrins nor cytochrome P-450 heme are good Lewis acids when compared to free ferric iron.[61] Further, *trans*-1-phenyl-1-butene was metabolized to significant quantities of 1-phenyl-1-butanone and 1-phenyl-2-butanone in addition to *trans*-1-phenyl-1-butene oxide, under conditions where the epoxide is stable.[61]

We rationalize the results in the following way. The $Fe^V{=}O$ form of cytochrome P-450 adds to the double bond in a manner analogous to olefin radical addition to form an intermediate, which may have several resonance forms.[22,61] Since hydrogen exchange has been shown to occur between vinylidene chloride and water during the reaction, the intermediate may have some iron–carbon bond character.[22,61] It is possible that formation of the preferryl oxygen–olefin intermediate is preceded by a single electron transfer step that generates a transient $[Fe^{IV}{-}O]^{2+}$–olefin radical cation complex (vide infra). Subsequent collapse of the transient electron-transfer complex would generate the postulated [Fe–O]–olefin addition intermediate. Such an electron transfer–recombination sequence parallels the proposed mechanism for heteroatom oxygnation and heteroatom release. The addition intermediate, shown in Figure 3, has several possible fates: (1) The electrons in the Fe–O bond may attack the carbonium ion (or radical) to form an epoxide. (2) The electrons in the Fe–O bond may be shifted to form a carbonyl and 1,2-migration of a group (chlorine or hydrogen) may occur. (3) A pyrrolic
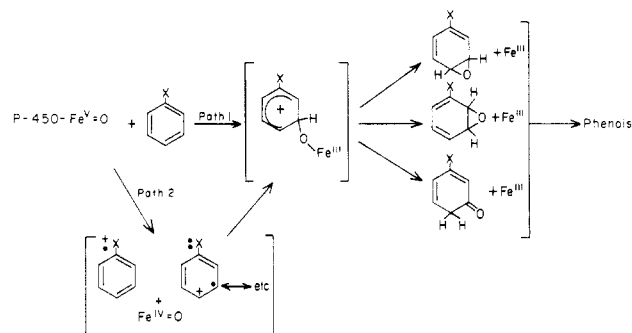


**Figure 4.** Possible mechanism for hydroxylation of monohalobenzenes.

nitrogen of the porphyrin may attack the electropositive center of the intermediate to produce an *N*-alkylporphyrin derivative. In the epoxidation of olefins, stereochemistry is often maintained; such findings are consistent with a nonconcerted mechanism in which orbital overlap[60c] (iron–carbon bond formation) or enzyme steric features prevent rotation. Ortiz de Montellano has characterized alkyne–heme adducts and rationalized the mechanism of alkyne oxidation in a similar way.[62]

We have also investigated the mechanism of hydroxylation of halobenzenes by a cytochrome P-450 system.[55] The halophenol metabolites are postulated to be derived from rearrangement of intermediate arene oxides generated via cytochrome P-450 catalyzed arene epoxidation. We determined the kinetic parameters for hydroxylation of the halobenzene series and found the rate $(V_{max}/K_m)$ to follow the halide order: PhI > PhBr > PhCl > PhF > PhH. In addition, an excellent correlation of $V_{max}$ to the Hammett $\sigma^+$ parameter[63] was obtained with a $\rho$ value of $-1.1$ and a correlation coefficient of 0.99. This correlation implies a positively charged transition state or intermediate in the hydroxylation. In addition, a good correlation ($r = 0.97$) between $K_m$ for total hydroxylation and parameters associated with ease of oxidation and/or polarizability of the halobenzenes was found (e.g., the Hansch $\pi$ parameter;[64] the Allred–Rochow electronegativity of the substituent atoms).

Our data are inconsistent with a direct, intermediateless mechanism for epoxidation of the aromatic ring, since little charge separation would be anticipated to occur in a concerted mechanism. We have considered two mechanisms, depicted in Figure 4, to be compatible with our data. The first mechanism (path 1) is essentially electrophilic aromatic substitution of the perferryl oxygen intermediate at the *m*-halobenzene position to give a tetrahedral intermediate that could close to give either of two epoxides. Alternatively, a 1,2 hydride shift could occur to give what would become the metahydroxylated product. Ortho- and para-hydroxylated products would arise from rearrangement of the 2,3- and 3,4-arene oxides.[57] An alternate mechanism, which we favor, inserts a rate-determining one-electron oxidation of the halobenzene to give a radical cation and the one electron reduced oxygenated heme intermediate

(62) P. R. Ortiz de Montellano and K. L. Kunze, *Biochemistry*, 20, 7266 (1981).

(63) C. D. Ritchie and W. F. Sager, *Prog. Phys. Org. Chem.*, 2, 323 (1964).

(64) C. Hansch, A. Leo, S. H. Unger, K. H. Kim, D. Nikaitani and E. H. Lien, *J. Med. Chem.*, 16, 1207 (1973).
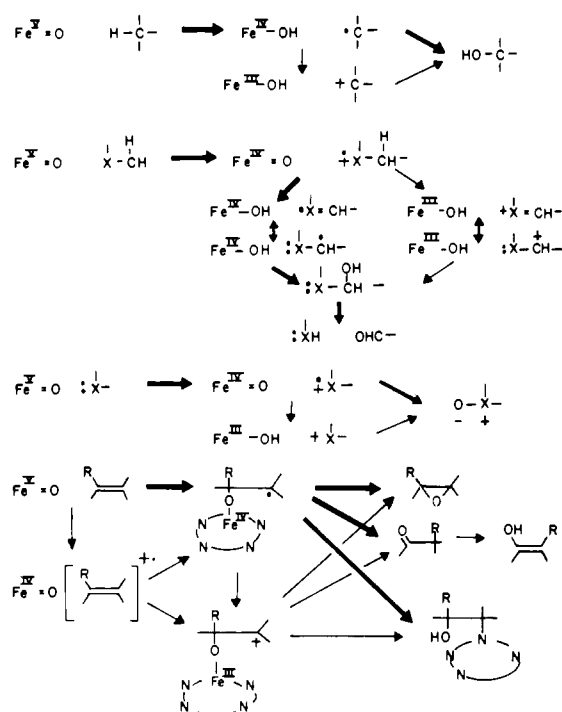
**Figure 5.** Minimal schemes for the rationalization of oxidative reactions of cytochrome P-450 in terms of perferryl oxygen and electron and hydrogen abstraction. The simplest pathways are shown with heavy arrows.

$[Fe^{IV}=O]$, which has radical character (path 2). These radicals would collapse to give essentially the meta-substituted intermediate of the electrophilic aromatic substitution pathway. Whatever the precise mechanism, our studies, and the work of others, demonstrate that enzymatic oxidation by cytochrome P-450 of unsaturated compounds proceeds not by concerted oxygen delivery from the enzyme to the substrate but instead by stepwise processes involving discrete radical and/or cationic substrate intermediates.

## A Unified View

A minimal unified mechanism for oxidative cytochrome P-450 catalyzed reactions is presented with the heavy arrows in Figure 5. In all cases we have utilized the formal perferryl oxygen as the species involved in catalysis, although the perferryl oxygen–cysteine–porphyrin complex may be best viewed as a resonance hybrid.[3,65] In this regard, Groves has presented strong evidence in model systems that the formal $Fe^V$ might better be represented as a $Fe^{IV}$ porphyrin radical cation.[16] In all of the reactions of this complex, the first step is the abstraction of electrons (nonbonded or $\pi$) or hydrogen atoms (i.e., protonated electrons) or radical (odd-electron) addition to an olefin.

In carbon hydroxylation, the perferryl oxygen abstracts a hydrogen atom. Oxygen rebound from the formal $Fe^{IV}$–OH complex occurs with the carbon radical within a caged pair to give the alcohol. The heteroatom release mechanism may proceed via either of two mechanisms that result in net hydroxylation adjacent to the heteroatom. The perferryl oxygen could selectively abstract a hydrogen atom adjacent to the heteroatom due to the stability afforded the radicaloid transition state by the heteroatomic substituent. Al-

ternatively, single-electron transfer from the heteroatom to the perferryl species could occur producing an intermediate heteroatom radical cation. The intermediate radical cation would transfer a labile proton (or hydrogen atom) on the $\alpha$-carbon to generate a carbon-centered radical (or cation). Oxygen rebound from the iron–hydroxide species to the carbon radical (or cation) produced either from the electron- or hydrogen-transfer mechanisms would produce the geminal hydroxy heteroatom species and lead to heteroatom release. Heteroatom oxygenation is initiated by single-electron transfer to generate a heteroatom radical cation and oxygen rebound occurs to the electrophilic heteroatom. Epoxidation, oxidative group migration, and olefin suicide inactivation are all initiated by nonconcerted oxygen addition to an unsaturated substrate site; disproportionation of the intermediate according to substrate and isozyme active site structural factors leads to the observed outcome.

Thus, all of the known oxidative reactions catalyzed by cytochrome P-450 can be described in the context of a general mechanistic scheme that involves stepwise electron abstraction and oxygen transfer (rebound) to electropositive atomic centers. The activated iron-oxygen complex, described here as a perferryl oxygen, can be viewed as an electrode of sorts. The lighter arrows of Figure 5 show an expanded view of the unified mechanism. Briefly, the expansion includes both homolytic and heterolytic mechanisms and also allows for carbonium ions and their heteroatomic analogues. Different substrates may exert some influence on the polarity of the mechanism, and some of the details may still be somewhat variable in this regard.

What, then, really determines the rate and course of cytochrome P-450 mediated reactions? We feel that three factors are involved with each form of cytochrome P-450. (1) The binding of substrate has been shown in certain cases, but not others, to facilitate the kinetics of the first one-electron reduction.[66] Conceivably the binding of certain substrates may also differentially regulate the kinetics of the entry of the second electron. (2) The ease of electron (or hydrogen atom) abstraction by the perferryl oxygen is a key factor. Factors such as the single electron oxidation potential of a heteroatomic substituent and energies of the carbon-centered and heteroatom cation radical intermediates must be considered. In this regard, our preliminary work on cyclic voltammetry of cyclopropyl derivatives and model oxygenated metalloporphyrins suggests that the oxidation–reduction potential of the formal cytochrome P-450 $(Fe=O)^{3+}$ complex is approximately 1.5–2.0 V. (3) The proximity of a given oxidizable site to the perferryl oxygen is determined by structural features specific to each cytochrome P-450, and this proximity effect is combined with the chemical potential at each site of a substrate to determine the regioselectivity and kinetics of catalysis. In the lack of structural information about the active sites of cytochrome P-450, specific features of binding are still unknown. Jerina has used data on the regio- and stereoselective metabolism of a family of polycyclic aromatic hydrocarbons to begin mapping features of the active site of one form of cytochrome P-450.[67] We have determined the rates

(65) V. Ullrich, *J. Mol. Catal.*, **7**, 159 (1980).

(66) T. Iyanagi, F. K. Anan, Y. Imai, and H. S. Mason, *Biochemistry*, **17**, 2224 (1978).

of regioselective metabolism of the $R$ and $S$ isomers of warfarin[17] and testosterone.[17,68] by eight different rat liver cytochromes P-450 and are using these results to initiate model building studies of the active sites.[68]

## Concluding Remarks

What does the future hold? Our own efforts are directed toward elucidation of the general aspects of the

working hypothesis we have elaborated here. Further, if the general scheme is appropriate, many details remain to be filled in, such as the physical description of the iron–oxygen complexes involved and the kinetic and thermodynamic parameters of the individual steps. The modeling of the active sites has only begun. When more information becomes available, the catalytic activity of cytochromes P-450 may ultimately be understood in molecular terms and of course of reactions with new substrates may be predicted.

(67) D. M. Jerina, D. P. Michand, R. J. Feldmann, R. N. Armstrong, K. P. Vyas, D. R. Thakker, H. Yagi, P. E. Thomas, D. E. Ryan, and W. Levin in "Microsomes, Drug Oxidations, and Drug Toxicity", R. Sato and R. Kato, Eds., Wiley-Interscience, New York, 1982, p 195.

(68) L. S. Kaminsky, F. P. Guengerich, D. C. Waxman, and C. Walsh, unpublished results.

# Binuclear Oxygen Carriers: Hemerythrin

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With few exceptions, all living cells take up oxygen.[1] In simple, small animals, in which body surface is large compared to the mass of the organism, the oxygen essential for respiration reaches the cells by diffusion from the external medium. In larger animals, however, the ratio of surface to bulk drops markedly. Furthermore, internal tissues are too far from the exterior to have access to sufficient oxygen if it is supplied only by simple, slow diffusion from the outer environment. Thus in the course of evolution in animals from annelids upwards, nature has developed blood vessels to transport oxygen into proximity to deeper cells and has created several different molecular devices to provide for oxygen accumulation in blood.[1] The oxygen content of sea water, 0.5 mL of $O_2$/100 mL of solvent, has thereby been raised progressively to about 25 mL of $O_2$/100 mL of blood in warm-blooded mammals.[1]

There is surprising diversity in molecular structure, particularly at the functional site, of the different naturally occurring carriers of dioxygen.[2-5] All are constituted of protein frameworks, but the molecular weights, subunit constitution, and symmetry in supramolecular assembly vary enormously (Table I). Within the hemoglobins, which are the most familiar as well as the most ubiquitous $O_2$ carriers, the tetrameric oligomer of 64 000 molecular weight is the best known, but

monomeric myoglobin, of 16 000 molecular weight, and multisubunit extracellular hemoglobins, as high as 4 × 10^6 in molecular weight, have also been studied extensively. Hemoglobins are distributed throughout the animal kingdom (Figure 1) and even among some plants. On the other hand, the non-heme $O_2$ carriers hemerythrin and hemocyanin are found only among invertebrates, particularly annelids, molluscs, and arthropods. Most hemerythrins show molecular weights near 108 000 and are constituted of eight subunits.[2] Nevertheless, smaller oligomers, with two, three, or four subunits, are also known,[6-10] and even monomers have been found in some organisms. In contrast, in the hemocyanins, the natural form of the pigment is multisubunit in constitution, and functional monomers are unknown under physiological conditions.

Despite their many differences, hemerythrin, hemocyanin, and hemoglobin all function effectively as oxygen carriers. To a chemist, relationships between

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(1) Baldwin, E. "An Introduction to Comparative Biochemistry"; Cambridge University Press: Cambridge, 1940.

(2) Klotz, I. M.; Klippenstein, G. L.; Hendrickson, W. A. *Science* 1976, *192*, 335.

(3) Kurtz, D. M.; Shriver, D. F.; Klotz, I. M. *Coord. Chem. Rev.* 1977, *24*, 145.

(4) Loehr, J. S.; Loehr, T. M. *Adv. Inorg. Biochem.* 1979, *1*, 235.

(5) Lamy, J., Lamy, J., Eds. "Invertebrate Oxygen-Binding Proteins"; Marcel Dekker: New York, 1981.

(6) Klippenstein, G. L.; Van Riper, D. A.; Oosterom, E. A. *J. Biol. Chem.* 1972, *247*, 5959.

(7) Liberatore, F. A.; Truby, M. F.; Klippenstein, G. L. *Arch. Biochem. Biophys.* 1974, *160*, 223.

(8) (a) Addison, A. W.; Bruce, R. E. *Arch. Biochem. Biophys.* 1977, *183*, 328. (b) Smith, J. L.; Hendrickson, W. A.; Addison, A. W. *Nature (London)* 1983, *303*, 86.

(9) Manwell, C. *Comp. Biochem. Physiol.* 1977, *B58*, 331.

(10) Sieker, L. C.; Bolles, L.; Stenkamp, R. E.; Jensen, L. H.; Appleby, C. A. *J. Mol. Biol.* 1981, *148*, 493.