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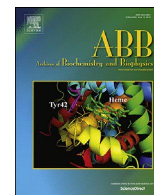

Oxidative hemoglobin reactions: Applications to drug metabolism

Tatyana Spolitak [a, *], Paul F. Hollenberg [b], David P. Ballou [a, **]

[a] Department of Biological Chemistry, Medical School, University of Michigan, Ann Arbor, MI 48109-0600, USA
[b] Department of Pharmacology, University of Michigan School of Medicine, Ann Arbor, MI 48109-5606, USA

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ABSTRACT

Hb is a protein with multiple functions, acting as an O_2 transport protein, and having peroxidase and oxidase activities with xenobiotics that lead to substrate radicals. However, there is a lack of evidence for intermediates involved in these reactions of Hb with redox-active compounds, including those with xenobiotics such as drugs, chemical carcinogens, and sulfides. In particular, questions exist as to what intermediates participate in reactions of either metHb or oxyHb with sulfides.

The studies presented here elaborate kinetics and intermediates involved in the reactions of Hb with oxidants (H_2O_2 and mCPBA), and they demonstrate the formation of high valent intermediates, providing insights into mechanistic issues of sulfur and drug oxidations. Overall, we propose generalized mechanisms that include peroxidatic reactions using H_2O_2 generated from the autooxidation of oxyHb, with involvement of substrate radicals in reactions of Hb with oxidizable drugs such as metyrapone or 2,4-dinitrophenylhydrazine and with sulfides. We identify ferryl intermediates (with a Soret band at 407 nm) in oxidative reactions with all of the above-mentioned reactions. These spectral properties are consistent with a protonated ferryl heme, such as Cpd II or Cpd ES-like species (Spolitak et al., JIB, 2006, 100, 2034–2044). Mechanism(s) of Hb oxidative reactions are discussed.

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1. Introduction

Hemoglobin is a highly studied O_2 transport protein that is also involved in several biologically important redox reactions *in vivo*. The protein consists of two α-subunits and two β-subunits, with each subunit containing an iron protoporphyrin IX prosthetic group [1]. Although Hb is not generally thought of as an enzyme, it was reported as far back as 1950 that in the presence of H_2O_2 the ferric state (metHb) can catalyze peroxidase-like oxidation reactions [2]. A mechanism was postulated that included a Hb-bound form of H_2O_2. Importantly, peroxidatic reactions can lead to tissue damage, but because under normal conditions the physiological concentrations of H_2O_2 in the blood plasma and in tissues are low, such reactions are not usually a problem. However, under some conditions, such as those that promote excessive autooxidation of oxyHb, superoxide radical anion is formed and dismuted by superoxide dismutase into H_2O_2 and O_2 [3,4]. In addition, free radicals can be generated from processes such as the activation of blood platelets [5]. Under these scenarios H_2O_2 levels may increase to deleterious levels. It has been shown that the reaction of H_2O_2 with either metHb or oxyHb forms a ferryl species (oxidoiron(IV), $Fe^{IV}{=}O$) that can persist for many minutes when an excess of H_2O_2 is present [6]. When oxyHb is in excess, which is the normal condition, intermediate ferryl Hb is converted to metHb [6,7]. This latter process can occur both *in vitro* and *in vivo* [4,8,9] and is proposed to occur by a radical mechanism that is facilitated by Tyr-mediated intersubunit electron transfer pathways [10,11].

In general, classical peroxidases function via the Poulos-Kraut mechanism [12] by first reacting with H_2O_2 to form Cpd I, the Fe(IV)-oxo porphyrin π-cation radical species [$Fe^{IV}{=}O$/porphyrin

Abbreviations: Hb, hemoglobin; MetHb, met-hemoglobin; oxyHb, HbO_2, oxyhemoglobin; Mb, myoglobin; MetMb, met-myoglobin; oxyMb, MbO_2, oxymyoglobin; SHb, sulfhemoglobin; P450, cytochrome P450; P450cam, cytochrome P450Cam (CYP101) isolated from *Pseudomonas putida*; CcP, cytochrome *c* peroxidase; Cpd I, state of a heme protein that is 2 equivalents of oxidation greater than the ferric form and contains a ferryl center ($Fe^{IV}{=}O$) and a porphyrin π-cation radical; Cpd II, state of heme protein that is 1 equivalent of oxidation greater than the ferric form and contains a ferryl center; Cpd ES, the 2-electron-oxidized state of CcP containing both a ferryl center and the Trp-191 free radical; EPR, electron paramagnetic resonance spectroscopy; SVD, singular value decomposition; mCPBA, *meta*-chloroperbenzoic acid; TMPD, *N,N,N,N*-tetramethyl-*p*-phenylenediamine; ABTS, 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonate); metyrapone, 2-methyl-1,2-di(pyridin-3-yl)propan-1-one; PHZ, phenylhydrazine.

* Corresponding author.
** Corresponding author.
E-mail addresses: spolitaktat@gmail.com (T. Spolitak), dballou@umich.edu (D.P. Ballou).

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π-cation radical] that is formally oxidized by two electrons relative to the ferric state. Cpd I then is reduced by one electron to form Cpd II, which has the heme remaining in the $Fe^{IV}{=}O$ state while the porphyrin π-cation radical has been reduced. A second electron reduces Cpd II to the ferric state. Both electrons are generally provided by an electron rich substrate such as a phenol. In some peroxidases and cytochrome P450s Cpd I rapidly converts to Cpd ES, which consists of a ferryl heme center ($Fe^{IV}{=}O$ (d^4, S = 1)) plus an amino acid residue radical (Tyr or Trp). This phenomenon was first reported for Cyt *c* peroxidase [13] in which Cpd ES is also oxidized by two electrons compared to the Fe^{III} enzyme, but the oxidizing equivalents appear as a ferryl heme plus a Trp radical. Other heme proteins and enzymes have also been shown to have Tyr or Trp radicals in a Cpd ES-like form of the two-electron oxidized state. These include myoglobin [14], bovine catalase [15], the F172Y mutant of HRP [16], turnip peroxidase [17], and prostaglandin-H synthase [18–20]. These proteins all contain a heme unit with a histidine coordinating to the proximal side of the heme iron, leaving the distal side capable of binding peroxide or O_2. Whereas the extinction coefficient of the Soret band of Cpd I is significantly less than either that of the ferrous or ferric forms of the heme (because the π-cation radical porphyrin is not fully aromatic), the Soret band of Cpd ES has normal heme intensity because the porphyrin macrocycle is fully aromatic. Spectrally, Cpd ES in Cyt *c* peroxidase is characterized by a Soret peak at ~420 nm, very similar to that of Cpd II. In addition, like Cpd II there are alpha and beta bands at ~575 and ~540 nm, respectively. However, Cpd ES in P450s often show a Soret at ~407 nm. Because this form predominates at low pH, it is considered to be protonated, possibly on the ferryl group or else on some nearby protein residue [21].

EPR data and the proximity of the heme group suggest that the protein-based radicals associated with the ferryl oxidation state of HbA [22] resides on the Tyr42 residue on the α-subunit [9,23,24]. Ferryl hemoglobin is highly oxidizing and therefore can oxidize a variety of electron donors [25] and even undergo internal reactions leading to irreversibly oxidized heme derivatives (e.g., choleglobin) or modifications of globin amino acid residues [26,27]. In its oxidative reactions, Hb has been shown to catalyze reactions such as lipid peroxidation [28], decarboxylation of DOPA by lysed erythrocytes [29,30], dealkylation of certain aromatic *N*,*N*-dimethylamine-*N*-oxides, and hydroxylation of aniline [31–33], all reactions typically catalyzed by Cyt P450s, which utilize Cpd I as the principal oxygenating agent [34,35]. In the case of aniline, P450 reductase and NADPH were required to obtain efficient catalysis [31]. Hb has been shown to catalyze the oxidation of redox-active xenobiotics and metabolites to form radicals that initiate radical chain reactions, often resulting in the eventual oxidative denaturation of Hb [6,36]. More recently, it was reported that hemeproteins are also involved in hydrogen sulfide activation [37,38]. For example, Hb ferryl species can react with sulfides to form sulfhemoglobin (SHb) by a mechanism that is not fully understood [37–39].

Thus, Hb can be considered as a protein with multiple functions, primarily acting as an O_2 transport protein but having some peroxidase activity, including the capability of hydroxylating aniline [31] and forming substrate radicals in reactions with xenobiotics. The large concentrations of Hb in blood can make such reactions significant under stressed conditions, and a better understanding of such reactions will be important in meliorating cellular damage from these reactions. However, there is a lack of direct evidence for intermediates involved in the reactions of Hb with oxidants, including oxidative reactions with myriad xenobiotics such as drugs, chemical carcinogens, and sulfides. Particular questions exist as to what intermediates participate in reactions of either metHb or oxyHb with sulfides to yield thiosulfate and polysulfides as products [40,41]. For example, it is known that the reaction of metHb with HS^- requires oxygen, but the mechanism is not understood. Our work presented in this manuscript further elucidates mechanistic aspects of these reactions, including the oxygen dependence and kinetic propensities of metHb sulfide oxidation to yield oxidation products.

In this study we present spectroscopic evidence for hemoglobin peroxidase-type species ($Fe^{IV}{=}O$) that are involved in oxidative reactions with sulfides, oxidizable drugs such as metyrapone, and drug derivatives such as phenylhydrazine. We attribute a Soret band observed at 407 nm in oxidative reactions with all of the above-mentioned compounds to a protonated form of Cpd ES or Cpd II. These studies elaborate kinetic properties of the reactions and provide new insights into mechanistic issues of sulfur oxidation by metHb and oxyHb. Based on the studies presented in this manuscript we suggest that the reactions of sulfides with both metHb and oxyHb involve similar intermediates and utilize peroxidatic cycles involving H_2O_2. Overall, we propose generalized mechanisms that include peroxidatic reactions with H_2O_2 and the involvement of substrate radicals for reactions of Hb with both oxidizable drugs and sulfides. We propose explanations for how metHb reacts with sulfides, and why these reactions are dependent on the presence of oxygen. These reactions, which go far beyond the basic O_2 transport function of hemoglobin, are relevant to many biological systems.

2. Materials and methods

All regents were obtained from commercial sources, human metHb was purchased from Sigma. Spectrophotometric measurements were performed using a Shimadzu UV-2501PC instrument. The peracids were standardized spectrophotometrically using the triiodide assay ($\varepsilon_{353\ nm} = 25.5\ mM^{-1}\ cm^{-1}$) [42].

All kinetic experiments were performed at 25 °C at pH 7.4, 6.2, or 8.0 in 50 mM phosphate buffer containing 75 mM KCl. All stopped-flow kinetic and diode array studies were carried out using freshly prepared heme proteins and peroxidase substrates. Concentrations of metHb were determined from absorbance values at 405, 500, and/or 631 nm ($\varepsilon_{405} = 179\ mM^{-1}\ cm^{-1}$, $\varepsilon_{500} = 10.0\ mM^{-1}\ cm^{-1}$, and $\varepsilon_{631} = 4.4\ mM^{-1}\ cm^{-1}$) [43]. Solutions of oxyHb were prepared by stoichiometrically reducing metHb with sodium dithionite under anaerobic conditions, followed by exposure to air. Concentrations of oxyHb solutions were determined from absorbance values at 415, 541, and/or 577 nm ($\varepsilon_{415} = 125\ mM^{-1}\ cm^{-1}$, $\varepsilon_{541} = 13.8\ mM^{-1}\ cm^{-1}$, $\varepsilon_{577} = 14.6\ mM^{-1}\ cm^{-1}$) [43]. H_2O_2 concentrations were determined from the absorbance at 240 nm ($\varepsilon_{240} = 43.6\ M^{-1}\ cm^{-1}$). In stopped-flow experiments relatively large concentrations of H_2O_2 were used to accelerate the time courses, which helped resolve intermediates. Where indicated, 2 mM sodium sulfide (Na_2S) was used to react with the ferryl heme oxidation state to produce sulfhemoglobin, using established methods [39]. Na_2S has been shown to react with ferryl heme groups in Hb and Mb to produce quantifiable derivatives (SHb) that give an absorbance peak at ~ 620 nm [39].

Kinetic data were acquired using a Hi-Tech Scientific, Ltd Model SF-61 stopped-flow spectrophotometer equipped with a xenon lamp. The dead time was determined to be 1.5 ms. Concentrations given in figure legends are those before mixing. Data collection and analysis were performed using KinetAsyst software from TGK Scientific and the Singular Value Decomposition (SVD) and global analysis methods in the Specfit program (Spectrum Software Associates). Because peracids and hydrogen peroxide were always greatly in excess in these experiments, fitting to models described below treated the first step as a pseudo-first-order reaction. Kinetic

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