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COMPREHENSIVE INVITED REVIEW

The Redox Activity of Hemoglobins: From Physiologic Functions to Pathologic Mechanisms

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Abstract

Pentacoordinate respiratory hemoproteins such as hemoglobin and myoglobin have evolved to supply cells with oxygen. However, these respiratory heme proteins are also known to function as redox enzymes, reacting with compounds such as nitric oxide and peroxides. The recent discoveries of hexacoordinate hemoglobins in vertebrates and nonsymbiotic plants suggest that the redox activity of globins is inherent to the molecule. The uncontrolled formation of radical species resulting from such redox chemistry on respiratory hemoproteins can lead to oxidative damage and cellular toxicity. In this review, we examine the functions of various globins and the mechanisms by which these globins act as redox enzymes under physiologic conditions. Evidence that redox reactions also occur under disease conditions, leading to pathologic complications, also is examined, focusing on recent discoveries showing that the ferryl oxidation state of these hemoproteins is present in these disease states *in vivo*. In addition, we review the latest advances in the understanding of globin redox mechanisms and how they might affect cellular signaling pathways and how they might be controlled therapeutically or, in the case of hemoglobin-based blood substitutes, through rational design. *Antioxid. Redox Signal.* 13, 1087–1123.

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I. Introduction

Myoglobin (Mb) and its "blood" relation hemoglobin (Hb) are some of the most extensively studied proteins and have been the subject of many tens of thousands of articles. These vertebrate respiratory pigments belong to an ancient superfamily of heme-associated globin proteins. The favorability of these proteins to be used in research in the nineteenth and twentieth centuries resulted from their high abundance and easy method of isolation (10). Despite the history of research on respiratory heme proteins, new information on their cellular and extra cellular functions is continually being brought to light. More than just simple oxygen-binding proteins, pentacoordinate globins such Mb and Hb can catalyze a series of reactions under both physiologic and pathophysiologic conditions, such as nitric oxide (NO) metabolism and transport, hydrogen sulfide transport (H_2S), and peroxidase activities, consuming both hydrogen peroxide and lipid hydroperoxides. The redox activities of these hemoproteins can induce free radical production and oxidation of free fatty acids and membrane lipids. These reactions have been observed *in vitro* and predicted to occur *in vivo* for many decades; however, only in the past decade or so has definitive evidence has emerged showing the occurrence of respiratory hemoprotein redox cycling under pathologic conditions. Additionally, these reactions occur, to a much lesser extent, under normal physiologic conditions. Notwithstanding this evidence, controversy still exists over the relative roles that intact hemoproteins play in the pathogenesis of certain posthemolytic and myolytic disease states, compared with other initiators of oxidative and nitrosative stress, such as free iron released from degraded hemoproteins or from other sources.

The recent discovery of a range of low-abundance hexacoordinate globins has shown that globins are ubiquitous throughout nature. The proposed physiologic functions of many of these ancient series of globins have been linked to their redox chemistry. This reinforces the conviction that enzymatic reactions are an inherent function of all globins and may be used for protective functions *in vivo*. However, this redox chemistry may also play a critical role in the pathogenesis of various diseases and in the difficulties in developing safe hemoglobin-based blood substitutes. Therefore, the mechanisms of the redox chemistry of these globins, Mb and Hb in particular, must be fully understood. New information has emerged on the importance of tyrosine residues in the regulation of redox activity of respiratory hemoproteins and the mechanism of the strong pH dependence of their peroxidatic activity. This knowledge suggests new targets for therapeutic intervention of disease states after hemolytic and myolytic events such as delayed vasospasm after subarachnoid hemorrhage or acute renal failure after rhabdomyolysis. Therefore, the redox activity of respiratory hemoproteins, although mainly suppressed under physiologic conditions, must be considered when these globins are removed from their protective environments, such as after a hemolytic or myolytic event, or are used extracellularly as blood substitutes. The physiologic and potential roles in pathologic conditions for various hemoproteins are explored herein and summarized in Table 1.

II. More Than Just Oxygen-Binding Proteins

The primary function of respiratory hemoproteins Mb and Hb is to bind oxygen reversibly and hence to function as oxygen-storage or oxygen-transport proteins. A ferrous iron [Fe(II)] is coordinated to four nitrogens of a tetrapyrrole ring (making the heme moiety) and a nitrogen of an imidazole ring, linking the heme to the protein (via the proximal histidine). This leaves one coordination site free to bind reversibly gaseous molecules such as oxygen, but also carbon monoxide (CO) and NO, amongst others. Thus, these proteins are able to perform their main functional roles of facilitated oxygen diffusion (Mb) and oxygen transport (Hb).

Mammalian globin chains are typically ~150 amino acids in length, consisting of six to eight α helical chains arranged with a three-on-three α helix "sandwich," forming the hydrophobic pocket in which the heme resides (Fig. 1). Other Hbs, such as the truncated Hbs (trHb) found in bacteria, plants, and unicellular eukaryotes, may have a two-on-two α helical sandwich (204).

The central role of the tetrameric Hb is undoubtedly oxygen transport, with allosteric functional regulatory mechanisms for optimal loading and unloading of oxygen in the lungs and tissues, respectively. The key to the allosteric properties of Hb is the transition from a structure that has low affinity for oxygen, known as the T state, and a high-oxygen-affinity form, known as the R state. From crystal structure studies, the transition between these two states is known to involve movement of the iron from "out of the plane" of the heme ring in the deoxy ferrous state to "in the plane" of the heme ring when in the oxygenated form. The movement of the iron causes movement of the proximal histidine (F8), together with Val67 (E11) of the β chain to allow oxygen to bind. The F-helix moves to avoid steric interactions with the porphyrin ring, causing a rotation of the two α/β dimers of the tetramer by 15°, so that β-His97 jumps from contact with α-Thr41 (C6) to the next α-helical turn at α-Thr38 (C3) (17, 201). The T state is stabilized by 2,3,bisphosphoglycerate binding to the central tetramer cavity, which is expelled in the R-state because of a narrowing of the cavity (12).

Table 1. Reported Physiologic and Pathologic Functions of Hemoglobins

Protein	*Deoxyferrous coordination state*	*Physiologic functions*	*Roles in pathologic diseases involving suspected redox chemistry*	*References*
Vertebrate myoglobin	Pentacoordinate	Facilitate O_2 diffusion/storage	Kidney dysfunction or acute renal failure after rhabdomyolysis	(5, 8, 34, 35, 65, 106, 128, 176, 286)
		NO scavenging (protects mitochondrial enzymes)		
Vertebrate hemoglobin	Pentacoordinate	O_2 transport	Delayed vasospasm after subarachnoid hemorrhage	(3, 5, 12, 19, 39, 56, 75, 110, 138, 218, 223, 286)
		NO transport	NO scavenging Hypertension (HBOCs) Hemoglobinopathies	
Vertebrate neuroglobin	Hexacoordinate	Hypoxia, ischemic signaling molecule (binds G proteins) Protects against oxidative stress NO regulation Modulates apoptosis (reduced cytochrome *c*) Modulates sulfide Protects in stroke model	–	(24, 29, 36, 44, 82, 187, 205, 281, 282)
Vertebrate cytoglobin	Hexacoordinate	Hypoxic sensing	Fibrotic organ disorder	(43, 85, 86, 161, 166, 183, 203, 205, 246, 292)
		O_2 or NO regulation	Downregulated in certain cancer cells	
		Tumor-suppressor gene		
Symbiotic plant hemoglobin	Pentacoordinate	O_2 regulation for nitrogen fixation	–	(11, 152)
Nonsymbiotic plant hemoglobin	Hexacoordinate	Regulating NO bioavailability (defense, signaler molecule)	–	(124, 200, 212, 272)
Truncated hemoglobin	Hexacoordinate	NO scavenger (defense)	–	(153, 175, 193, 291)
Erythrocruorins	Pentacoordinate	O_2 transport	–	(23, 135)

The optical properties of Hbs enable us to study its redox and ligand chemistry through optical spectroscopy, among other methods. The intense color of the protein stems from the extended conjugation of the protoporphyrin ring, not the iron, as is commonly misconceived. Thus, the energy required to excite electrons from the π to π^* orbital states places the optical absorption bands in the near UV region (the Soret or γ peak) and the visible region (the visible or α and β bands) of the electromagnetic spectrum. Factors that affect the positioning and intensities of these bands include the heme environment (*e.g.*, changes in the structure of the heme pocket), the oxidation state of the iron (*e.g.*, ferric [Fe(III)] or ferrous), the iron ligand (*e.g.*, oxyferrous or deoxyferrous), and the heme structure (*e.g.*, heme b or heme d). The optical characteristics of isolated human hemoglobin in various oxidations states and ligation states are shown in Fig. 2.

Mb is generally considered an oxygen-storage protein that augments oxygen diffusion in high-oxygen–consuming, mitochondria-rich muscles (*e.g.*, heart) (286). Mb lacks the multisubunit allosteric regulatory mechanisms that make Hb such an effective oxygen carrier. However, the oxygen affinity of Mb from sperm whale and horse heart has been found to be affected by lactate, the product of glycolysis, in essence, making them monomeric pseudoallosteric proteins (99). However, the observation that mice lacking the gene to produce Mb had no obvious change in phenotype, other than "white" muscle, required that the physiologic role of Mb be reassessed (106). The requirement that the protein contain a redox-active metal porphyrin allows the globins to behave as redox enzymes. Oxyferrous Mb can react with nitric oxide (NO), an important signaling molecule, to form ferric Mb and nitrate. Thus, Mb is now considered to be an intracellular scavenger of NO under aerobic conditions, in addition to its

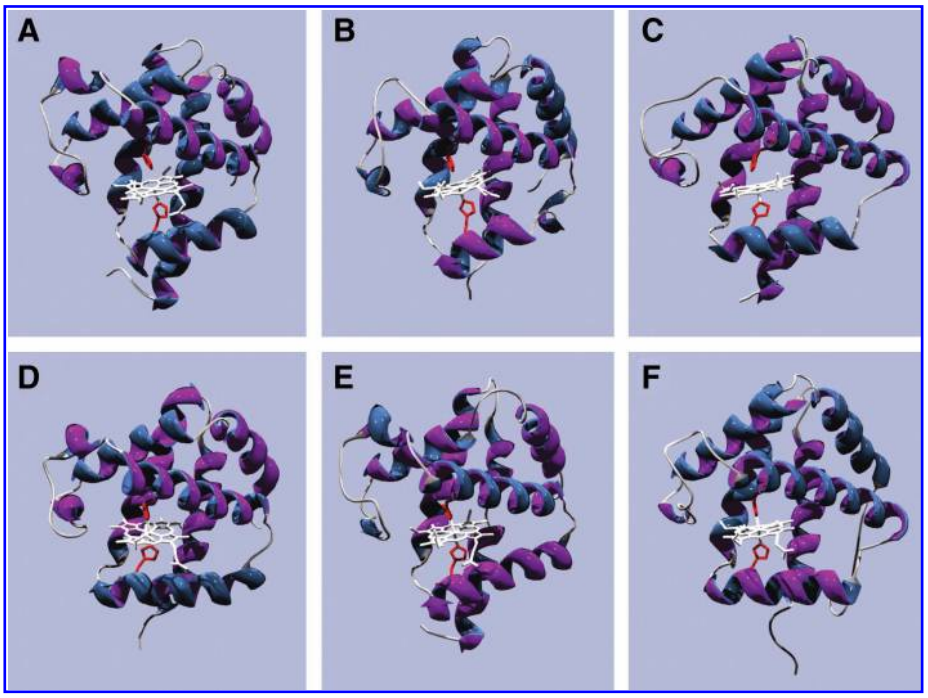

FIG. 1. Comparison of structures of pentacoordinate and hexacooordinate hemoglobins. Top row: pentacoordinate globins, sperm whale myoglobin (**A**), human hemoglobin alpha subunit (**B**), and yellow lupine leghemoglobin (**C**). *Bottom row:* hexacoordinate globins, human neuroglobin (**D**), human cytoglobin (**E**), and Asian rice nonsymbiotic hemoglobin (**F**). Heme moiety and proximal and distal histidines are shown in ball and stick. Protein structure orientations were fitted by Swiss PDB viewer; raytracing was performed by POVRAY for Windows v3.6. Files used were 1VXA, 1A3N, 1LH2, 1OJ6, 1V5H, and 1D8U for **A–F**, respectively. All files were obtained from the RSCB protein databank. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

role as an oxygen carrier, thus protecting NO-sensitive respiratory enzymes such as cytochrome *c* oxidase (34, 35, 80). Under hypoxic conditions, Mb has been reported to generate NO, leading to vasodilation and inhibition of mitochondrial enzymes, hence arresting the production of potentially damaging reactive oxygen species (ROS). Thus, Mb functions as both a dioxygenase and a reductase (65) (see Section VI.A).

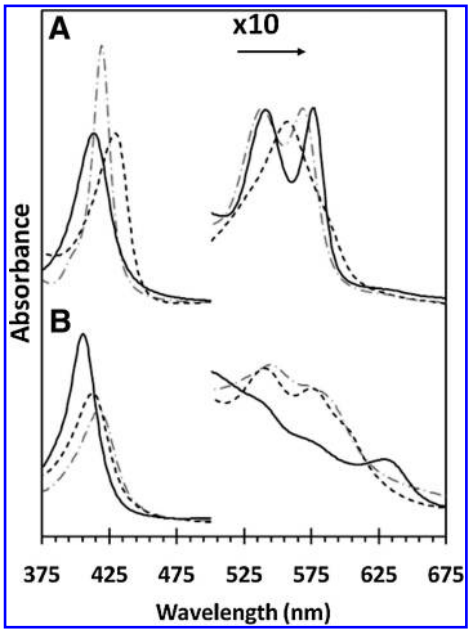

FIG. 2. Optical properties of human hemoglobin. (**A**) Soret and visible regions of the spectrum of hemoglobin in its oxyferrous form [P-Fe(II)-O^2, solid line], deoxyferrous form [P-Fe(II), *dotted line*], and carbonmonoxy ferrous form [P-Fe(II)-CO, *gray dot-dashed line*]. (**B**) Spectrum of hemoglobin in its acid ferric form [P-Fe(III) (H_2O), pH 7, *solid line*], alkali ferric form [P-Fe(III)–OH^-, pH 10, *dotted line*], and unprotonated ferryl form [P-Fe(IV) = O^{2-}, *gray dot-dashed line*].

III. Neuroglobin and Cytoglobin: Redox-Active Hemoglobins?

It was long believed that Hb and Mb were the only heme-containing globins in vertebrates. However, at the turn of the millennium and with the advent of the ability to search genomes, new proteins have been discovered, greatly expanding the family of globins. The recent discovery from genetic sequences of two new vertebrate globins, neuroglobin [Ngb, reported in 2000 (44)] and cytoglobin [Cygb, also previously known as histoglobin and stellate cell activation–associated protein, reported in 2001 (143)], has affected the way in which the function of Hbs in vertebrates is considered. Both Ngb and Cygb are low-abundance proteins and are hexacoordinate (Fig. 1). Ngb is expressed in the central and peripheral nervous system, cerebrospinal fluid, retina, and endocrine tissues (44, 270), whereas Cygb is ubiquitously expressed in vertebrate tissues but found in higher concentrations in the brain, eyes, liver, heart, and skeletal muscle (43, 86). Despite having the sixth coordination site occupied by the distal histidine (His E7), Ngb and Cygb have affinities for oxygen in the low nanomolar range but are rapidly autoxidized on contact with oxygen. Therefore, they are unlikely to function as classic oxygen-carrier proteins, but it appears that their function is instead related to redox chemistry.

Phylogenetic analyses suggest a common ancient evolutionary origin of all vertebrate globins (44, 203). Both Ngb and Cygb share only 21–25% sequence identity with Mb and Hb; however, the key amino acids required for Mb and Hb function are conserved (203). Despite intensive research over the past decade, the functions of both Ngb and Cygb remain unclear, but are currently a subject of considerable interest. Numerous proposed functions exist, most of which involve the heme redox chemistry. They both appear to be related

to hypoxia in cells because of their upregulation under hypoxic conditions (85). They have also been shown to scavenge peroxynitrite, hydrogen sulfide, and peroxides (29, 187, 203).

The oxygenated ferrous form of Ngb rapidly reacts with NO, yielding ferric Ngb and nitrate, reportedly through a heme-bound peroxynitrite intermediate (36). Ngb, but not Cygb, reacts with nitrite to form *S*-nitrosothiol derivatives that may have a function in NO regulation (205). Overexpression of Ngb has been found to protect neurons against hypoxic/ischemic insults and preserve mitochondrial function after ischemic reperfusion, suggesting a role in protection against oxidative stress (164). It has been reported that Ngb binds G proteins in its ferric form; thus, it may be a signaling molecule under ischemia/reperfusion oxidative stress (281, 282). It also was reported that Ngb binds to cytochrome *c*, rapidly reducing ferric cytochrome *c* to the ferrous form, suggesting a role in modulating apoptotic response (24, 82).

Amphibians that lack Mb have a higher expression of Cygb in the nuclei of heart cells and cytoplasm of skeletal muscle (292). Thus, the function of Cygb under these conditions may be related to oxygen diffusion; however, under these circumstances, it also may be case that the globins may function to protect mitochondria from NO toxicity, similar to reported functions of Mb. Neuroblastoma cells, exposed to a variety of stresses, including UV radiation, hydrogen peroxide, kainic acid, high extracellular $CaCl_2$, high osmolarity, and heat stress, showed no upregulation in Cygb. Only hypoxia increased the expression of Cygb. Additionally, Cygb knockdown neuroblastoma cells exacerbated cell death on treatment with hydrogen peroxide, suggesting a role for protection against oxidative stress (161). Cygb has been reported to have no appreciable catalase activity (143), but has considerable peroxidatic activity, consuming both hydrogen peroxide and lipid peroxides (143, 166). Cygb is also induced in fibrosis and may regulate collagen gene expression. The expression of Cygb also was upregulated in fibrotic lesions of the pancreas and kidney, with overexpression of Cygb in NIH 3T3 cells inducing a decrease in migratory activities and increasing the expression of collagen alpha1(I) mRNA (183), indicating that Cygb may play a role in fibrotic organ disorder. Upregulation of Cygb expression has been postulated to reduce oxidant stress and possibly may be used as a therapy to prevent the progress of liver fibrosis, as peroxides can trigger stellate cell activation and hence promote liver fibrosis (143, 246). Recently, Cygb was reported to be a tumor-suppressor gene. Certain cancer cells downregulate Cygb expression, significantly upregulating Cygb expression when these cells become hypoxic (246). Cygb expression is controlled by both hypoxia and promoter methylation, with a correlation between Cygb expression and pathoclinical aggression of the tumor (246). Thus, expression of Cygb may also be a new target for therapy of cancer (166).

IV. Plant Hemoglobins

Hbs are ubiquitously produced not only in animals, but also in plants. Plant Hbs generally fall into one of two categories. Symbiotic plants Hbs have been known for some time and, through extensive studies, the protein structure and functions have been well characterized. However, nonsymbiotic plant Hbs are a more recently discovered class of globins, having structural similarities with Ngb and Cygb. Although the functions of nonsymbiotic Hbs are currently unclear, they are unlikely to function as oxygen-carrier proteins.

A. Symbiotic plant hemoglobins

Symbiotic Hbs are found in root nodules of leguminous plants and hence termed leghemoglobin (Lb). Lb also was reported to be associated with the root nodules of some nonleguminous plants (11). The structure for Lb is very similar to that of Mb (152). Lb is a monomeric heme protein with a five-coordinate heme iron, allowing the sixth coordination site to bind oxygen (Fig. 1). However, the affinity for oxygen is much higher than that in vertebrate Hb and 20-fold higher than in Mb (152). This difference in oxygen affinities is attributed to the presence of a Ser/Thr residue at position F7 in Mb that is absent in Lb (120). The globin works in symbiosis with nitrogen-fixing bacteria, binding oxygen at the surface of the root nodule to facilitate oxygen supply to the bacteria for nitrogen fixation. The globin also keeps oxygen concentrations low enough as not to disrupt nitrogenase enzyme activity, which is highly sensitive to oxygen. The redox chemistry of Lb *in vitro* is similar to that of Mb and Hb, showing oxidation with peroxides and protein radical formation and is functionally inactive in the ferric form (178). Reduction mechanisms are in place to prevent Lb redox chemistry, including enzymatic reduction of Lb by various compounds, such as NADH, thiols, and flavins (20).

B. Nonsymbiotic plant hemoglobins

Unlike symbiotic plant Hbs, the recently discovered nonsymbiotic plant Hbs (nsHbs) do not appear to function as oxygen-carrier or scavenging proteins, but instead, their functions may be linked to redox chemistry. These nsHbs have little sequence homology with their symbiotic counterparts. Like that of the Ngb and Cygb, heme iron in these nonsymbiotic plant globins is also hexacoordinate, with the sixth coordination site occupied by a distal histidine (271) (Fig. 1). Also like those of Ngb and Cygb, both proteins are reported to have high oxygen affinity (p50 of 1.6 n*M* for *Arabidopsis* Hb1 at pH 7, 20°C, 130 n*M* for *Arabidopsis* Hb1), but whereas AHb1 forms a stable oxy-complex, AHb2 rapidly autoxidizes to the ferric form (32). Thus, both proteins do not appear to function as classic oxygen-transport or storage proteins. Significantly, both the animal and plant hexacoordinate globins are upregulated during hypoxia or after ischemic reperfusion (272). Therefore, it was suggested that a common physiologic function might exist for both plant and animal hexacoordinate hemoglobins. However, the details of the physiologic functions of these hexacoordinate proteins are currently unclear. In plants, three classes of nsHbs exist, in addition to the symbiotic bacterial Lb.

Arabidopsis Hb1 (AHb1) is a typical example of the plant nsHbs. AHb1 is a class-1 nonsymbiotic Hb, a homodimeric protein, each chain comprising 160 amino acids, and its expression is strongly induced in both roots and rosette leaves in hypoxic conditions (272). AHb1 may modulate NO bioactivity: treatment of the oxy-complex with an excess of NO oxidizes the iron to the ferric state and formation of a stoichiometric quantity of nitrate. AHb1 can also scavenge NO

through *S*-nitrosothiol formation and reduces NO emission under hypoxic stress (200). This NO activity appears to be related to the pathogen invasion response, upregulating expression of the defense genes PR-1 and PDF1.2 and conferring enhanced disease resistance, possibly by modulating the NO level and the ratio of peroxide to NO in the defense process (212). The ferric AHb1 can be directly but very slowly reduced by NADPH. On the assumption that a relatively efficient reductase system is present *in vivo,* it has been proposed that AHb1 may function as a terminal oxidase, by using NO as the acceptor under hypoxic conditions. The silencing of AHb1 leads to NO accumulation to high levels (272), resulting in the delay of plant bolting (flowering), with earlier bolting seen in Arabidopsis Hb2 (AHb2)-overexpressed plants (124). The interface residues between the homodimer are highly conserved. This may mean that the interface may serve as a binding site to other proteins and that dimerization *in vivo* is an unlikely event (109). This supports the hypothesis that these proteins are molecular sensors, with changes in the coordination or redox chemistry or both to trigger other signaling events.

Arabidopsis Hb2 is a class 2 nonsymbiotic Hb, homodimeric protein, each chain comprising 158 amino acids. It is present in rosette leaves and is overexpressed at low temperatures (272). Seedlings overexpressing AHb2 show enhanced survival to hypoxic stress, whereas AHb2 knockout combined with AHb1 silencing leads to seedling death. Protein dynamic studies of CO binding to the heme shows that AHb2 has significant differences in the internal structure of the protein compared with AHb1, supporting the idea that the two proteins have different physiologic roles (33). However, silencing AHb2 results in a delay in plant bolting, similar to that observed from silencing AHb1, suggesting that AHb2 may also play a role in NO regulation (124).

As yet, no definitive consensus exists as to the physiologic functions of nsHbs. It has been suggested, however, that the presence of multiple genes in some plant species may imply that these proteins serve more than one function (121). A third class of nonsymbiotic hemoglobins is that of the truncated Hbs. They are related to bacterial trHbs; their functions appear to be similar and are examined later.

V. Other Hemoglobins

A. Truncated bacterial and plant hemoglobins

Truncated Hbs (trHbs) are a distinct class of globins found in plants and bacteria but are typically 20–40 amino acids shorter than other globins. They are distinct from bacterial flavohemoglobins, but appear often to coexist in the same bacterium (291). Crystal structures show that trHbs have a classic globin fold with antiparallel α helices in a B/E and G/H conformation forming a heme-binding pocket. Of the 8 α-helical regions of the typical globin, the N-terminal A-helix is almost nonexistent; the whole of the CD/D helix region is clipped to only two or three amino acids; and the F helix comprises only one helical turn. This makes trHbs very compact, with a two-on-two α-helical fold rather than the more common three-on-three α-helical fold. In trHbs, very few amino acids show a high degree of conservation between sequences, the proximal histidine (F8) being the only invariant residue. Strongly conserved residues include a Phe-Tyr pair at the B9-B10 sites involving heme ligand stabilization, a Phe at site CD1, a commonly conserved residue in other nonvertebrate Hbs, and another Phe at site E14, which may be related to shielding the heme from bulk solvent (291). TrHbs are generally hexacoordinate; however, the distal E7 position can be occupied by various different residues in trHbs from different bacilli, including tyrosine glutamine, threonine, serine, and histidine.

Found in many pathogenic bacteria such as *Mycobacterium tuberculosis,* the truncated Hb function is related to counteracting host defenses. As a causative agent of most cases of tuberculosis, *M. tuberculosis* infects inactivated macrophages. More than 1.8 billion people are infected with *M. tuberculosis* worldwide, resulting in 15 million deaths annually (193). The immune system contains the bacillus in the vast majority of otherwise healthy infected hosts, isolating the bacillus and forcing it to lie dormant, maybe for many years, without the host displaying symptoms (260). Key to this macrophage defense is inducible NO synthase, generating large concentrations of NO (54, 169), inactivating key bacillus enzymes, such as the terminal respiratory oxidases (31, 37, 59) and the iron–sulfur protein aconitase (89, 90). Key to the survival of the bacillus is its ability to deal with the NO. Now strong evidence suggests that trHbs function to protect bacillus like *M. tuberculosis* from nitrosative stress through its action as a scavenger of NO. The second-order rate constant for the reaction of trHbs with NO is $\sim 7.5\times10^{8}$ M/s, about 20-fold higher than that reported for Mb (193). Silencing the glbN gene for trHbN expression in *M. bovis* bacillus caused a dramatic decrease in the NO-scavenging activity of stationary-phase cells, and that activity could be restored fully by complementing knockout cells with glbN (193). Deletion of the N-terminus pre–A-helix motif significantly decreases the ability of trHb from *M. tuberculosis* to function as an NO scavenger. Additionally, the motif insertion into a trHbs from *M. smegmatis* (generally considered a nonpathogenic microorganism) enhanced its NO dioxygenase activity (153). Therefore, the pre-A helix motif affects the protein dynamics and allows an open pathway for diatomic ligands to enter the heme pocket, a gateway that is closed in the absence of the motif.

B. Erythrocruorins

Annelid hemoglobins, known as erythrocruorins (ecHbs) are extracellular Hbs that do not require compartmentalization because of their immense size. They comprise standard Mb-like 16- to 17-kDa subunits that form multisubunit hexagonal bilayer structures, typically containing 144 subunits and 36 non-Hb linker proteins, creating a macromolecule with molecular masses in excess of 3 million Da. The Hb chains are arranged in dodecameric substructures with 12 trimeric linker complexes, resulting in a scaffold on which 12 hemoglobin dodecamers assemble (233). At $>$240Å in diameter and 160Å in width, the hexagonal structure of these macromolecules can be readily observed with electron microscopy (91).

The function of ecHbs is undoubtedly related to oxygen transport. The large molecular size of ecHbs allows the transport of oxygen without the need for an erythrocyte and also has a high degree of allostery, with a high Hill coefficient and a large Bohr effect (135). The potential to oxidize ecHbs is increased at alkaline pH, in contrast to vertebrate Hbs (23). These globins are also resistant to autoxidation,

because of uniquely positive redox potentials (122). Thus, no known functions of the redox chemistry of ecHbs have been reported.

VI. The Redox Chemistry of Myoglobin and Hemoglobin in Their Reactions with Ligands and Oxidants

A. Nitric oxide

The reactions of vertebrate Mb and Hb with the gaseous NO have been reported for a considerable time. However, the reaction came into intense focus when it was discovered that NO functions as an important signal transmitter in numerous physiologic pathways (66). The reactions of Hb and NO are a complex subject and could fill a comprehensive review by themselves. Many controversial aspects exist to the reactions and physiological significance of such reactions, which have not yet been resolved. A more-complete picture of the views of NO chemistry with Hb can be found in several recent reviews (102, 103, 110, 254). All hemoproteins have the capacity to react with NO, the chemistry of which is summarized for human Hb in Fig. 3. It has been known for many decades that human Hb reacts with NO in one of two possible reactions: (a) high-affinity binding of NO to deoxyferrous Hb (used in the 1960s to study the EPR of ferrous Hb, normally EPR silent without the NO ligand); and (b) the conversion of NO to nitrate by oxyferrous Hb. The compartmentalization of Hb in the erythrocyte protects endothelial cells from Hb scavenging NO and hence prevents increased blood vessel vasoconstriction. However, such NO-scavenging reactions have been of great interest to the engineering of cell-free blood substitutes, a subject discussed in further detail in Section XII. The reaction of NO with ferryl [Fe(IV)] Hb also has been detailed. Acting as a hemoprotein reductant instead of an oxidant, NO reacts with ferryl Hb to produce a peroxynitrite intermediate, finally yielding the ferric protein and nitrite (125). This reaction, unlike the reaction with ferrous protein, does not require hypoxic conditions and is relatively rapid, $\sim 10^7$ M/s (125). It has been proposed that NO might function as an antioxidant, playing a role in the mechanism to detoxify high oxidation states of Hb or Mb under oxidative conditions.

Nitrite (NO_2^-) is a ubiquitous circulating anion, and accumulating evidence suggests that nitrite is a vasodilator and intrinsic signaling molecule (104). Nitrite reacts with deoxyHb to form ferric Hb and NO, and recent evidence suggests that this reaction provides a major pool of NO for hypoxic vasodilation (64, 105). This nitrite reductase activity was believed responsible for the vasodilation observed when nitrite was infused into patients (64). However, the

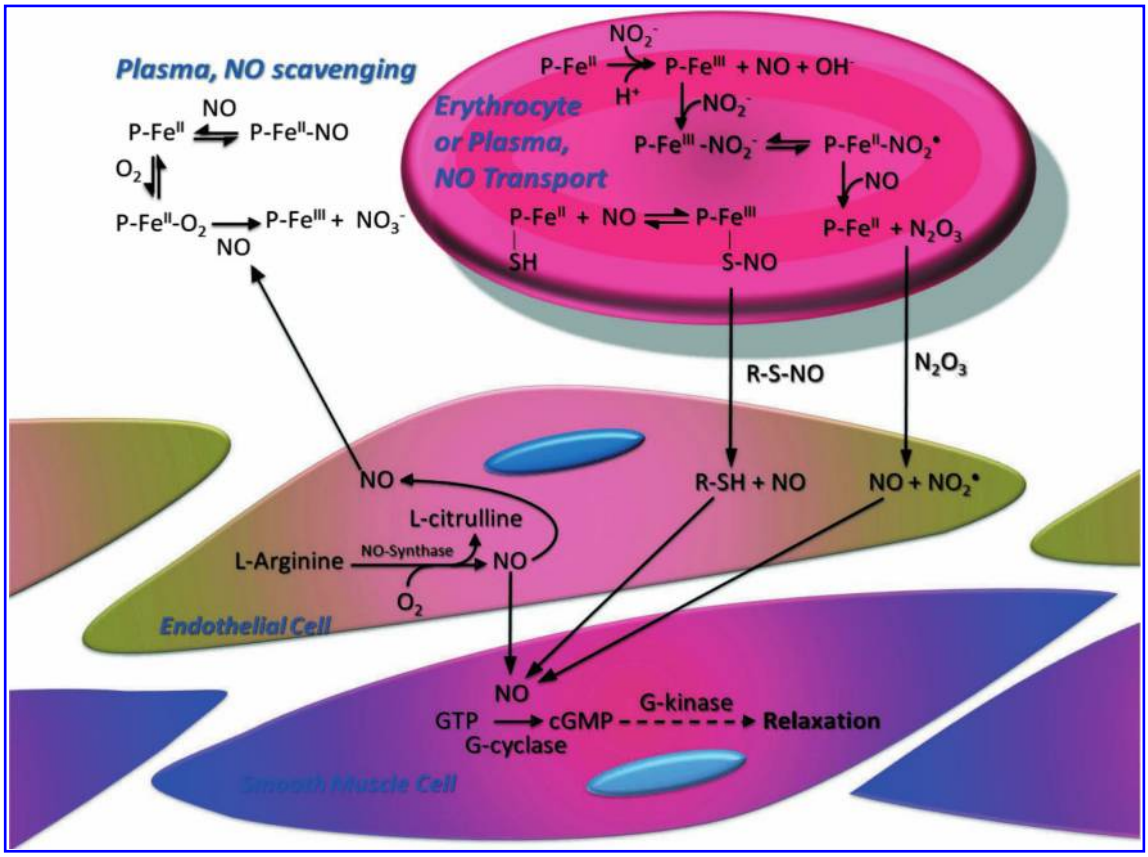

FIG. 3. Summary of the main reactions of hemoglobin with nitric oxide. Hemoglobin is considered both a scavenger and a transporter of NO. The scavenging is most physiologically relevant when the hemoglobin is outside the erythrocyte, such as flowing cell lysis or through addition of cell-free hemoglobin-based oxygen carriers. The physiologic relevance of NO transport as a mediator of vasodilation from the erythrocyte to the endothelium is still under debate. Scavenging or supply to endothelial cells affects the NO signaling for smooth muscle contraction (88). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

physiological relevance of the scavenging of NO before it could leave the confined interior of the erythrocyte is unclear. The mechanism of escape of NO from the erythrocyte remained elusive until it was reported to involve a mechanism in which the NO/NO_2^- was converted into dinitrogen trioxide (N_2O_3) (19),

$$\text{P-Fe}^{(\text{II})} + \text{NO}_2^- + \text{H}^+ \rightarrow \text{P-Fe}^{(\text{III})} + \text{NO} + \text{OH}^- \quad \text{Eq. 1}$$

$$[\text{P-Fe}^{(\text{III})}\text{-NO}_2^- \leftrightarrow \text{P-Fe}^{(\text{II})}\text{-NO}_2^{\bullet}] + \text{NO} \rightarrow \text{P-Fe}^{(\text{II})} + \text{N}_2\text{O}_3 \quad \text{Eq. 2}$$

where P represents the protein. Therefore, under hypoxic conditions, N_2O_3 is generated, and, being an uncharged molecule, N_2O_3 can pass lipid membrane barriers and hence can diffuse out of the erythrocyte. This exportation of the bioactivity of NO to the endothelium has been challenged, with data that support a high affinity of ferric Hb for nitrite ($K_d \approx 1.8$ m*M*) (242), contradicting the data of Basu *et al.* ($K_d \approx 7\ \mu M$) (19). Thus, the physiological relevance for this role of Hb redox activity *in vivo* requires definitive measurement of the affinity of ferric Hb for nitrite.

It is now generally accepted that Hb can function as transporter of NO through the formation of *S*-nitroso-Hb (SNO-Hb) and can deliver this NO to areas of hypoxia or restricted blood flow (110). Currently, however, considerable debate exists as to the mechanism of SNO-Hb formation and its role in mechanisms of vascular tension. First reported in 1996, Hb from erythrocytes was proposed to take up both oxygen and NO in the lung and release both under conditions of low po_2 (138, 258). Therefore, under hypoxic conditions, Hb could regulate blood flow through releasing NO and causing vasodilatation and hence could increase oxygen delivery. The proposed mechanism of this function was the binding of NO to a cysteine residue (Cys93) on the β chain of Hb. This residue is as highly conserved in mammals and birds as the proximal histidine, implying an essential function (202), but previously had no known physiological function (110). When Hb is in the high-oxygen-affinity R state, the Cys93 thiol affinity for NO is also high. In the low-oxygen-affinity T state, the Cys93 affinity for NO is reduced, so that under low po_2, the Hb releases its oxygen and also NO (138).

The formation of nitrosothiol from NO and thiol requires a one-electron oxidation step ($\text{R-S}^- + \text{NO} \rightarrow \text{R-SNO} + \text{e}^-$), for which the electron acceptor has been reported unidentified for SNO-Hb under physiologic conditions (231). The heme iron of ferric Hb has been ruled out by some as an acceptor (making ferrous Hb), as the reaction is very slow (103). However, others have reported that nitrosothiol synthesis requires oxygen to drive the reaction, and that SNO-Hb formation can occur by direct reaction with subsequent electron abstraction by oxygen, making oxygen the electron acceptor (111). EPR studies show evidence of a transient radical during the transition of NO from the heme iron to the thiol, which is oxygen dependent (173). Reports that superoxide dismutase enhances SNO-Hb formation may be explained through the provision of copper as a catalyst and electron acceptor (232), or may simply be explained by scavenging superoxide, which would otherwise consume NO (111). However, it has been noted that Hb has a copper-binding site close to Cys93, the affinity of which is dependent on the Hb conformation (225).

The physiologic importance of this NO-delivery system is currently under considerable debate. Numerous studies using sensitive methods to detect NO liberation from SNO-Hb have reported extremely low ($\sim$200 n*M*) or undetectable concentrations of SNO-Hb *in vivo*, too low for any significant effect on vasoregulation. Additionally, other nitrosative reaction species, such as nitrous anhydride, are also capable of generating SNO-Hb directly (231) and could be generated after the addition of a bolus of NO and may explain the *in vitro* experimental observations (103). Transgenic mice that express human Hb, either with the β-chain Cys93 or a substituted Cys93 $\rightarrow$ Ala mutation, showed that the loss of the cysteine exhibited no measurable changes in systemic or pulmonary hemodynamics. Additionally, the Cys93 $\rightarrow$ Ala mutation did not affect isolated erythrocyte-dependent hypoxic vasodilation (136). This showed that, even though a measured decrease in SNO-Hb concentration occurred, the SNO-Hb was not essential for vasoregulation. Erythrocytes from the βCys93-mutant mice stimulated hypoxic vasodilation *ex vivo* as efficiently as do erythrocytes from mice with wild-type human Hb, thus demonstrating that βCys-93 is not essential for stimulating NO signaling from erythrocytes (199). Fetal Hb was also reported as undetectable and unlikely to have SNOHb at compensatory levels.

The interpretation of the results from the transgenic humanized mice has been challenged with regard to the significance of SNOHb as a physiologic transporter of NO (110). Blood pressure and time to fatigue measured in the transgenic humanized mice are believed to be unrelated to hypoxic vasodilation, but aggravates hypoxemia-mediated pulmonary hypertension, a parameter that was not measured in the transgenic humanized mice (259). Additionally, the physiologic importance of increased low-mass nitrosothiols that were observed in the β-chain Cys-93 $\rightarrow$ Ala mice is in dispute, in addition to other parameters, such as measurements of vasodilation across an oxygen gradient, membrane SNO content, and levels of fetal and embryonic hemoglobins (which may contain compensatory βCys-93-SNO) (259).

The presence of small but detectable quantities of SNO modification to human Hb *in vivo* is one detail that may be agreed on. However, the ability of Hb to self-regulate oxygen delivery to hypoxic tissue through release of NO from Hb is a question that currently remains unresolved. It may be that neither SNO-Hb nor nitrite may fully explain the self-regulation of oxygen delivery by Hb. Release of ATP from erythrocytes as a result of reduced oxygen tensions has been proposed to function as a vascular control mechanism, for which a considerable literature base exists. An in-depth appraisal of these mechanisms may be found in a recent review by Ellsworth *et al*. (81). In brief, an increase in oxygen demand induces adenylyl cyclase activity to generate cAMP, leading to ATP release. This ATP in turn activates endothelial P2y receptors to stimulate $InsP_3$ synthesis, promoting the release of calcium from the endoplasmic reticulum (48). Increased levels of calcium generate NO through activation of eNOS and the release of membrane lipids by phospholipase $cPLA_2$. This in turn induces production of cGMP and cAMP from endothelium-released NO and modified lipids (*e.g.*, prostaglandin I_2), respectively (45, 88). Both cGMP and cAMP promote vascular smooth muscle cell relaxation and dilation (81).

B. Sulfide

The Hbs from animals in symbiosis with chemoautotrophic bacteria have functions related to H_2S transport. These sulfur-oxidizing bacteria are commonly found in the sunlight-deprived deep-sea environments where the only source of nutrients is chemical deposits from hydrothermal vents. Such vents were first discovered along the Galapagos Rift but now are known to be common close to volcanoes and tectonic plate boundaries. Chemoautotrophic bacteria are internalized as intracellular symbionts in molluscs and annelids, such as the giant tube worm, giant clam, and a snail (51). These species often lack a mouth and guts and depend almost entirely on the symbionts for their supply of organic carbon (50).

The key to the symbiosis between the chemoautotrophic bacteria and their host organisms is the host Hb. The Hb transports H_2S to cells harboring their symbiotic bacteria (30). Three specific Hbs have been identified in the mollusc *Lucina pectinata* (149). Two (HbII and HbIII) are tetrameric proteins and readily bind oxygen, but do not appear to have oxygen-binding cooperativity. These proteins also remain oxygenated in the presence of H_2S and are believed to function as oxygen transporters to the autotrophic bacteria in a way that is analogous to the way in which leghemoglobin supplies oxygen to nitrogen-fixing bacteria in the root nodules of legumes (149). A third Hb, HbI, is a monomeric protein found in the cytoplasm and reacts rapidly with H_2S to form a ferric-sulfide derivative (149). This reaction is highly pH dependent, with kinetics 3,000-fold faster at pH 5 than at pH 10.5. With a pK of 7, the attacking ligand appears to be undissociated H_2S (149). The reaction of ferric HbI with H_2S is 40-fold faster than HbII or Mb, with a 4,000-fold difference in affinity, implying that HbI does not have a distal histidine to rough crystal structure analysis to be a glutamine residue. This glutamine (Gln 64, E7), together with two other residues (Phe 29, B10 and Phe 68, E11), are not found in vertebrate globins and are thought to be important for high affinity for H_2S in HbI (226). The ferrous oxidation state of HbI is destabilized by sulfide anions, displacing superoxide in a nucleophilic process to form the ferric protein. (149). The ferric oxidation state is further stabilized by the electron-withdrawing character of the out-of-plane heme vinyl group, facilitating H_2S binding (253).

The mitochondria of vestimentiferan tubeworms have been found to be sensitive to H_2S, with mitochondrial cytochrome *c* oxidase inhibited by concentrations of sulfide as low as 10 μM (210). To combat this toxicity, vestimentiferan tubeworms have several large extracellular Hbs that can simultaneously and reversibly bind sulfide and oxygen, thus giving the extra function of protecting mitochondria aerobic respiration as well as supplying chemoautotrophic bacteria with H_2S and O_2 (83). Tubeworm *Riftia pachyptila* has three distinct Hbs, termed V1, V2, and C2. Globin V1 is a typical annelid ecHb with mass of 3.6 million Da, made up of Hb and non-Hb components (277). Globins V2 and C2 are much smaller, with masses of $\sim$400 kDa, and consist of only Hb chains, six for V1 and five for C1 (298). The primary sequence of Hb chain b, common to all three proteins, revealed three cysteines, one of which (Cys-75) was reported to be the site for binding sulfide (298), but was later reported from crystal structures to involve zinc ions ligated to the protein through several histidines and a glutamate (83). These zinc sites for sulfide ligation have also been reported in *Calyptogena elongate* (58).

C. Peroxide

It has long been known that the rate of the oxidation of ferrous to ferric Mb increases with increasing partial pressure of oxygen (96–98). Therefore, oxygen has an inherent ability to oxidize the ferrous heme iron, oxygen being a natural oxidizing agent (hence its name). Outside of the heme pocket, the heme iron becomes oxidized by oxygen, forming ferric heme and superoxide, with half time in the range of a few seconds. Inside the globin, the heme is partially protected from oxidation, with half times measured from hours to days.

$$\text{P-Fe}^{2+} - \text{O}_2 \leftrightarrow \text{P-Fe}^{(\text{III})} + \text{O}_2^- \left(\text{O}_2^- + 2\text{H}^+ \rightarrow\rightarrow \text{H}_2\text{O}_2\right) \quad \text{Eq. 3}$$

This reaction is highly pH dependent, with the pH profile of the rate of oxidation crudely parabolic in shape. The kinetics for the autoxidation of bovine Mb ferrous heme iron is most stable around pH 9 ($k_{obs} \approx 0.03$ per h), the autoxidation rate increasing $\sim$1,000 times at pH 5 (249). The acid-base pH dependence of ferrous Mb autoxidation involves at least two protonation events, giving three possible prototropic states (AH-BH, AH-B^-, and A^--B^-). The pK values for these two groups are reported as 6.75 and 10.4, giving probable identities of histidine and tyrosine residues, respectively (249). Substitution of the distal histidine (E7) significantly enhanced autoxidation at pH 7.0 (257), with the pH profile losing its acid-catalyzed process (262). These observations have led to the proposal that the distal histidine plays a dual role in the autoxidation mechanism. One is in a proton-relay mechanism, facilitating the movement of a proton from the solvent to the bound dioxygen through its imidazole ring, enhancing autoxidation at acid pH. The other is in the protection of the iron-dioxygen center against a water molecule or an hydroxyl ion that can enter the heme pocket from the surrounding solvent, thus reducing the autoxidation rates at neutral and alkaline pH values (262). Therefore, a tradeoff exists between a higher autoxidation at acidic pH values and a lower rate of autoxidation at neutral pH. Thus, under conditions of acidosis, which often follow hemolytic/myolytic events such as rhabdomyolysis, the globin undergoes rapid autoxidation. Under these oxidative conditions, superoxide is formed that, in the presence of superoxide dismutase, generates hydrogen peroxide and oxygen in the triplet state. In the absence of superoxide dismutase, peroxide is formed at a slower rate; however, the oxygen formed is in the singlet state, a highly reactive oxygen species (148). Under conditions of acidosis, therefore, hemoproteins such as Mb and Hb undergo rapid oxidation and generate peroxides, which can react with globins in a function analogous to peroxidases, but also inducing damaging radical-mediated reactions.

The ability of the respiratory globins Mb and Hb to function as pseudo-peroxidatic enzymes is an important biochemical property, with the reaction with peroxides generating ferryl heme- and protein-based radicals. Since the first report in 1958 that sperm whale Mb could form protein-based radicals (100), radicals in hemoproteins have been extensively studied, yet much is still not understood about their properties. Many radical reactions are vital to the biochemistry of life and are used in photosynthesis catalytic reactions and signaling. Many of these processes use heme-containing enzymes; examples include cytochrome P450, catalase, prostaglandin H

synthase, and photosystem II. The radicals generated in these systems are part of their catalytic cycle and, as such, are closely controlled. Unlike classic peroxidases, however, respiratory hemoproteins such as Mb and Hb cannot safely use radicals generated, and radical leakage is unavoidable, leading to oxidative reactions that are invariably detrimental to the cell.

The reaction between hydrogen peroxide and ferrous or ferric Mb/Hb is considered a classic one, due to the slow kinetics compared with true peroxidases, which allowed the reaction to be followed with early spectroscopic equipment (93–95). Hydrogen peroxide is a two-electron acceptor, forming ferryl heme iron from either the ferrous or ferric oxidation states. If reacted with the ferric oxidation state, a cation radical species is formed, in addition to the ferryl:

$$\text{P-Fe}^{(\text{II})} + \text{H}_2\text{O}_2 \rightarrow \text{P-Fe}^{(\text{IV})}{=}\text{O}^{2-} + \text{H}_2\text{O} \qquad \text{Eq. 4}$$

$$\text{P-Fe}^{(\text{III})} + \text{H}_2\text{O}_2 \rightarrow \text{P}^{\bullet+}\text{-Fe}^{(\text{IV})}{=}\text{O}^{2-} + \text{H}_2\text{O} \qquad \text{Eq. 5}$$

The radical is believed to be initially located on the porphyrin, equivalent to peroxidase compound I. However, the characteristics of compound I are not observed, even through the use of stopped-flow spectroscopy or by rapid-freeze EPR techniques; the optical properties of the protein observed are equivalent to peroxidase compound II (ferryl alone). The radicals observed with EPR spectrometry are assigned to various protein-based radical species because of its rapid migration away from the porphyrin (264). Various neutral radicals are observed by EPR spectrometry after the formation of ferryl heme, often assigned to tryptophan or tyrosine residues. These radicals are neutral because of the rapid deprotonation of the cation radical and partial stabilization of neutral radical on these residues (263, 266). This lack of control of the protein radical for Mb and Hb is in contrast to more-classic peroxidatic enzymes, which exhibit a tight control of the radical formed for use in its catalytic cycle, preventing intermolecular leakage of radicals and unwanted oxidative reactions. For many classic peroxidases, the loss of the radical to form ferryl (compound II) is a catalytic dead end. Mb from *Aplysia fasciata* (Sea hare, sometimes referred to under the junior pseudonym of *Aplysia limacine*) is a protein of interest structurally, as it has no distal histidine group. This protein reacts with hydrogen peroxide to form a short-lived Mb-peroxide adduct known as compound 0, which can also be observed in sperm whale Mb mutants with no distal histidine (28, 62, 267). The absence of the distal histidine in sperm whale Mb does not significantly affect the formation of protein radicals, only their kinetics of formation (114). In *Aplysia* Mb, the formation of ferryl heme and protein radicals can be observed after compound 0 (62). The radicals observed on *Aplysia* Mb are, as yet, unidentified. Despite the presence of two tryptophan residues, *Aplysia* Mb does not show any signals associated with classic tryptophan peroxyl radicals, and *Aplysia* Mb has no tyrosine residues.

The formation of ferryl heme without generating the protein-based radical (Eq. 4) is difficult to achieve, due in part to a comproportionation reaction (sometimes referred to as synproportionation), which rapidly yields two ferric proteins from a ferrous and ferryl protein (171).

$$\text{P-Fe}^{(\text{II})} + \text{P-Fe}^{(\text{IV})}\text{-OH}^- \rightarrow 2\times\text{P-Fe}^{(\text{III})} + \text{OH}^- \qquad \text{Eq. 6}$$

Thus, the reaction between both ferrous and ferric hemoproteins tends to yield ferryl heme plus protein-based radical species because of the more-rapid reaction of peroxide with ferric heme in comparison with oxyferrous heme. Comproportionation has been proposed to be facilitated by tyrosine residues, permitting intermolecular electron transfer between the ferrous and ferryl globins (101). Vertebrate Mb and Hb contain such tyrosine residues and have been shown to participate in comproportionation reactions (101). Additionally, globins that lack a tyrosine like that of the plant globin leghemoglobin do not exhibit comproportionation, thus altering the extent of radicals generated from the reaction of ferrous protein and peroxide (171).

Specific modifications to Hb have been observed under oxidative conditions. When challenged with hydrogen peroxide, the heme may become oxidatively modified, with some heme becoming covalently linked to the protein, possibly through a histidine residue under conditions of acidosis and a serine residue under more-neutral pH conditions. The mechanism and implications of the generation of heme-to-protein cross-linking *in vivo* are discussed in more detail in Section VII. In addition to heme damage, specific irreversible modifications to the protein have been observed in human Hb. These included oxidation of sulfur-containing amino acids, including βMet-55, βCys-93, and βCys-112, all being oxidized to the methionine sulfoxide and cysteic acid forms, respectively; βTrp-15 was found to oxidize irreversibly to oxyindolyl and kynureninyl products (139). Surprisingly, however, no modifications to amino acids in the α subunit were observed, suggesting that the β chain collapses when challenged with peroxide. This suggests an explanation for the observation that, under oxidative conditions, Hb with cross-linked β chains is more resistant to peroxide-mediated damage than α-chain cross-linking, namely that the β cross-linked protein is more stable, and hence protein denaturation and heme loss are prevented (5, 190).

The peroxidatic activity of Hb and Mb can function as a protective mechanism, consuming peroxides and safely removing the radicals formed through internalization of the radical burden, preventing the depletion of important intracellular antioxidant molecules such as glutathione and ATP (285). Therefore, a balance exists between the beneficial effects of globins reacting (and hence removing) peroxides, and the damaging effects of oxidizing other molecules, such as lipids.

D. Hemoglobin redox activity and lipid-oxidation biochemistry

Because of their high redox potentials, both the protein-based radical and ferryl heme that result from the peroxide-induced oxidation of Hb and Mb can induce a wide range of oxidation reactions. Such uncontrolled oxidation reactions *in vivo* induce a wide array of physiologic and pathophysiologic responses. Recently, it has become apparent that many of these responses involve the formation of signal molecules, often derived from the free radical–based oxidation of lipids (208). Foremost among these radicals are the lipid hydroperoxide radicals formed by the reaction of oxygen with carbon-centered lipid radicals. It is from these peroxide radicals that a large class of stable signal molecules is formed, through

peroxide radical self-termination reactions or interaction with other molecules. Lipid oxidation is of immense physiological importance because of their role in cell-signaling pathways. The central concept underlying this field of "redox cell signaling" is that changes in the reductive or oxidative capacity of the cell lead to posttranslational modification of proteins by ROS/RNS species (63, 160, 250). These posttranslational modifications, induced by Hb redox chemistry, are emerging as an important field of research in pathologies such as atherosclerosis, ischemia/reperfusion injuries, posthemolytic/myolytic vasoactivity, and blood substitutes.

Both the ferryl heme iron and protein-based radicals are capable of initiating the oxidation of a wide variety of substrates, including DNA, other proteins, or, of course, lipids. Initiation of lipid oxidation arises from abstraction of hydrogen from the lipid (LH) to create a lipid alkyl radical ($L^{\bullet}$):

$$P^{\bullet}\text{-}Fe^{(IV)}=O^{2-}+LH \rightarrow PH\text{-}Fe^{(IV)}=O^{2-}+L^{\bullet} \qquad \text{Eq. 7}$$

$$P\text{-}Fe^{(IV)}=O^{2-}+LH \rightarrow P\text{-}Fe^{(III)}\text{-}OH+L^{\bullet} \qquad \text{Eq. 8}$$

In the presence of oxygen, the alkyl radical will rapidly react to form a peroxyl radical ($LOO^{\bullet}$):

$$L^{\bullet}+O_2 \rightarrow LOO^{\bullet} \qquad \text{Eq. 9}$$

This peroxyl radical can undergo a number of reactions. Certain unsaturated lipid peroxyl radicals can rearrange to form epoxides (220). Some peroxyl radicals will terminate to form a lipid alcohol, lipid ketone, and singlet oxygen ($^1O_2$, Eq. 10). Most significant of these reactions is the peroxyl radical reacting with another lipid molecule to form a lipid peroxide molecule and a further lipid alkyl radical (Eq. 11).

$$LOO^{\bullet}+LOO^{\bullet} \rightarrow L{=}O+LOH+{}^1O_2 \qquad \text{Eq. 10}$$

$$LOO^{\bullet}+LH \rightarrow LOOH+L^{\bullet} \qquad \text{Eq. 11}$$

This latter propagation reaction is an autocatalytic cycle that, in the absence of sufficient concentrations of antioxidants, can lead to a cascade of lipid oxidation. In the presence of Mb or Hb, the lipid peroxides can react with ferrous, ferric, and ferryl oxidation states of the protein generate further lipid radicals and a ferric-ferryl redox cycling of the hemoprotein (Fig. 4). Lipids containing unsaturated fatty acid chains are vulnerable to lipid oxidation by hemoproteins. High-abundance membrane lipid chains, such as eicosatetraenoic acid (arachidonic acid) or docosahexaenioc acid (cervonic acid), are particularly vulnerable because of their extensive diene structure.

Figure 5 shows the time course of the oxidation of liposomes by Mb. Ferrous and ferric Mb/Hb does not react with

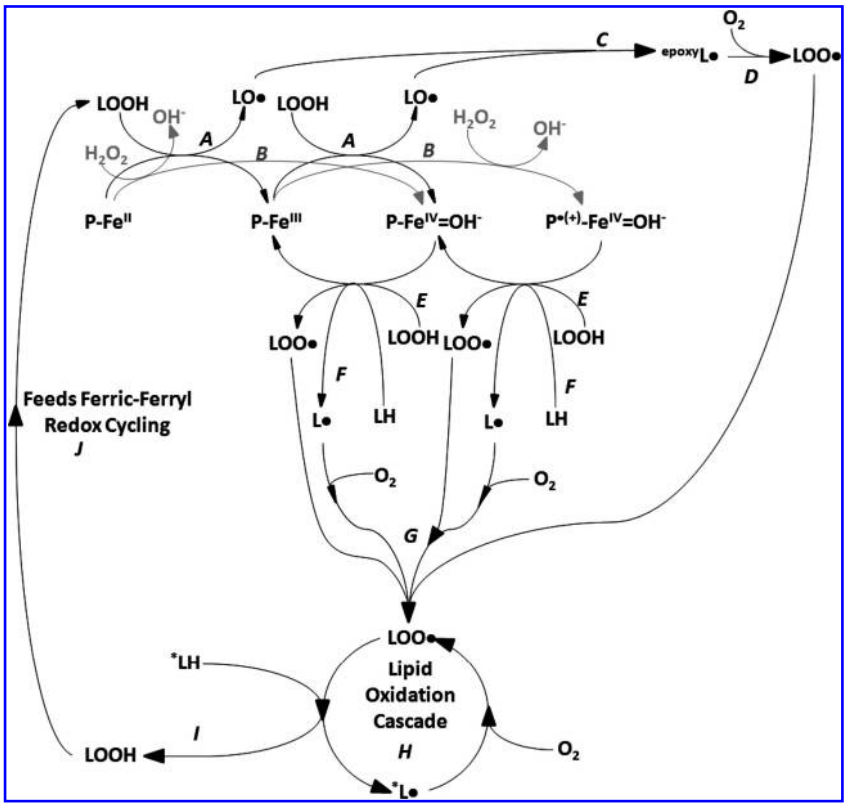

FIG. 4. Simplified pseudo-peroxidase reactions of hemoproteins with lipids. In addition to the reactions with H_2O_2, Mb or Hb will catalyze a variety of reactions with lipids and lipid hydroperoxides. Lipid hydroperoxide (LOOH) will react with ferrous or ferric hemoprotein in one-electron steps to generate ferric or ferryl, respectively, plus a lipid alkoxyl radical (LO•, **A**). Hydrogen peroxide, a two-electron acceptor, forms ferryl from ferrous or ferryl plus a cationic protein radical ($P^{\bullet+}$, **B**, in grey) from ferric; the protein radical quickly deprotonates to lose its cation charge. This alkoxyl radical, if formed near a *cis-cis* pentadiene π bond system [like that contained in polyunsaturated fatty acids (PUFAs) such as arachidonate, linoleate, or docasahexenate], will rearrange to form an epoxy-alkyl radical (epoxyL•, **C**). Alternatively, it will abstract a hydrogen from another lipid to form an alcohol (not shown). Oxygen will then react with epoxyL• to form a lipid peroxyl radical (LOO•, **D**). Both the radical and ferryl heme iron will react with lipid hydroperoxides to generate ferric heme and lipid peroxyl radicals (**E**) or will oxidize lipids to generate lipid alkyl radicals (L•, **F**) (220). The lipid peroxyl radicals, generated from step E or from oxygen reacting with lipid alkyl radicals (**G**) will react with further lipids (*LH) and oxygen to form a cycle of oxidation (**H**), resulting in generation of lipid hydroperoxides (**I**), plus many other lipid oxidation products such as the isoprostanes and electrophiles. The lipid peroxides generated by this cycle can feed the redox cycling of the hemoprotein (**J**).

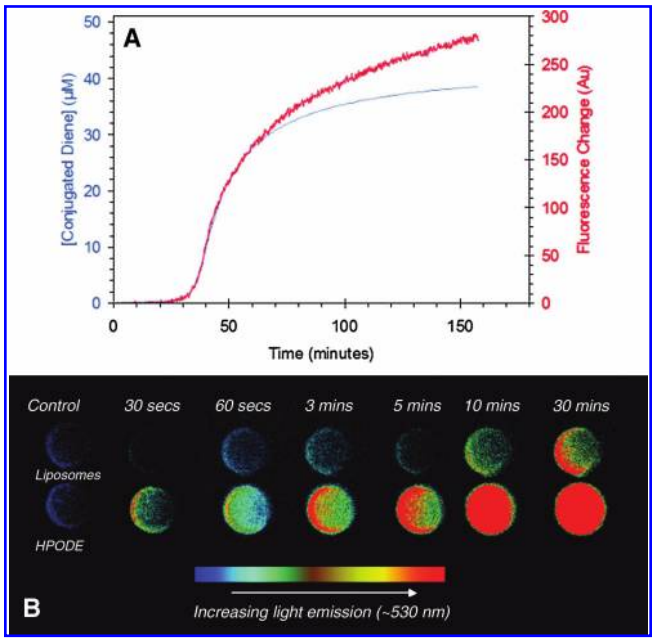

FIG. 5. Singlet oxygen formation as a by-product of hemoglobin-induced oxidation of lipids and lipid peroxides. (**A**) The reaction of ferric Hb (1 μM) with extruded unilamellar asolectin liposomes (~100 Å diameter, 250 μg/ml, 100 mM sodium phosphate, pH 7.4, containing 100 μM DTPA) as a function of time. After a lag period, lipid oxidation cascades with the formation of lipid-based conjugated dienes that can be followed in the UV spectrum (234 nm). In a separate experiment, performed under identical conditions, a singlet oxygen fluorescence sensor (singlet oxygen sensor green, 250 nM) was added to the reaction. (**B**) Formation of singlet oxygen in samples of reaction in Petri dishes between Mb and liposomes (upper data set) or Mb and the lipid hydroperoxide 13S, 9-*cis*, 11-*trans* octadecadienoic acid (HPODE, lower data set). Images were taken by a cooled charge-coupled device and adapted from the publication by Flors *et al.* (84). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

lipids. However, small concentrations of lipid peroxides in the liposome preparation react with the globin to form ferryl heme and lipid radical species. This process is initially slow, with low steady-state concentrations of ferryl. At a critical concentration of ferryl heme and radical concentration, the lipid oxidation cascades, rapidly oxidizing the lipid molecules, resulting in the overall increase in conjugated diene that can be followed in the ultraviolet region of the spectrum (Fig. 5A), the time period until the cascade event can be shortened or eliminated by a bolus addition of hydrogen peroxide or lipid hydroperoxide. Alternatively, aging the liposomes in oxygen over several hours/days also promotes a more rapid cascade of lipid oxidation because of enhanced lipid hydroperoxide content. Removal of the low levels of peroxide contained in the liposome preparation by reductants such as ebselen can prevent oxidation of the lipids in a dose-dependent manner. Addition of Mb that has been pre-degraded by hydrogen peroxide does not induce rapid lipid oxidation, indicating that iron, released from the heme does not induce significant lipid oxidation reactions under these conditions (Reeder, unpublished data). One of the by-products of lipid oxidations is singlet oxygen (Eq. 10) (118). A reactive oxygen species, $^1O_2$ closely follows the formation of conjugated dienes (Fig. 5A), as observed through interaction of a $^1O_2$ fluorescence sensor (Singlet Oxygen Sensor Green, by Invitrogen). The formation of $^1O_2$ and conjugated diene only deviate in the latter stages of the reaction, perhaps because of the slower oxidation of unsaturated lipids that do not form conjugated dienes. Furthermore, the reaction between lipid hydroperoxides and Mb, the mechanism of which has been previously described (220), also generates $^1O_2$ (Fig. 5B) (84). The formation of $^1O_2$ is potentially damaging to biologic systems and can induce further oxidation reactions (53). It will rapidly oxidize further lipid molecules and any organic compound containing carbon–carbon double bonds to form hydroperoxides or endoperoxides. In proteins, $^1O_2$ will specifically oxidize cysteine, histidine, methionine, and tryptophan residues (118). In plant photosystems, if the chlorophyll triplet states are not quenched by photochemistry or carotenoids, they will interact with triplet oxygen to produce $^1O_2$ (194). Environmental stresses can lead to inhibition of electron transport, resulting in the production of $^1O_2$, which has been implicated in damage to the D1 protein of the photosystem II reaction center, and up-regulation of the expression of genes involved in the molecular defense response of plants against photooxidative stress (150). If stress is prolonged, chlorophyll bleaching can also result (14). It should be noted that lipid membranes without extensive unsaturated fatty acid side chains, such as phosphotidyldimyristoyl, can still be readily oxidized by Mb or Hb, showing no change in conjugation, but will show oxidation products such as malondialdehyde, which can be detected by using the TBARS colormetric assay (thiobarbituric acid–reactive substances) (115, 118) or by $^1O_2$ formation by using the fluorescence sensor (Reeder, unpublished data).

1. Isoprostanes. The result of the oxidation of lipid, either as free fatty acids or esterified to the membrane, is the formation of a large series of products, some of which have potent physiologic properties. One such class of compounds that have recently seen significant attention is that of the isoprostanes (isoPs). Isoprostanes are prostaglandin-like molecules that are derived from the noncyclooxygenase oxidation of lipids containing eicosatetraenoic acid chains (180). Although often described as nonenzymatically derived, the pseudo-enzymatic property of Mb and Hb is one of the initiators of isoPs formation, as shown by the *in vitro* formation of isoPs by the reaction of Mb and low-density lipoproteins (LDLs) (176). Other known initiators of isoPs are reactive oxygen species (228, 230, 237) and labile iron (295). Free radical–catalyzed oxidation of lipids *in vitro* resulted in the formation of many isomers of isoPs. One class of isoPs identified, known as F_2-isoPs or 8-epi prostaglandin F_2, induces reduction in renal blood flow and glomerular filtrate when injected into rat peripheral veins at doses of 0.5 to 2.0 μg/ml (180). The formation of isoprostanes or their metabolites is now widely accepted as one of the more reliable approaches to assess lipid peroxidation reactions *in vivo* (177, 230). Many free radical processes can initiate lipid oxidation, with both labile iron (the so-called free iron) and respiratory hemoproteins capable of initiating isoPs formation *in vitro* and *in vivo* (176, 238). However, once initiated, the conversion of a lipid alkyl radical to isoPs can become autocatalytic. One possible

mechanism of their formation, initiated by Mb or Hb, is presented in Fig. 6.

The formation of isoPs arises from the oxidation of omega-6 eicosatetraenoic acid fatty acid side chains of membrane lipids. However, the prevalence of these side chains differs in different tissues. Whereas eicosatetraenoic acid, a 20-carbon chain fatty acid with four *cis* double bonds is common in most vertebrate cell membranes, it is less common in brain tissue. Docosahexaenoic acid, like eicosatetraenoic acid, is an unsaturated fatty acid abundant in brain tissue and the retina. Docosahexaenoic acid is an omega-3 fatty acid with 22 carbons and six *cis* double bonds and comprises 40% of unsaturated fatty acids in brain tissue. The resulting oxidation of docosahexaenoic acid generates various products, including isoP-like compounds known as neurosprostanes. F_4-neuroprostanes have been detected in normal human cerebrospinal fluid, with levels in patients with Alzheimer disease significantly higher than those in age-matched controls (229).

Interaction of F2-IsoPs with thromboxane A_2 receptors, as well as the stimulation of endothelin-1 expression on endothelial cells, induces phosphorylation of p38 mitogen-activated protein (MAP) kinase, which regulates cell contraction (132). The mechanism of this well-characterized posttranslational modification of a protein can be found in most standard biochemistry textbooks and is a typical pathway for the induction of a cellular effect, resulting in smooth muscle contraction and increased hypertension (174).

2. Electrophilic-responsive element. Lipid oxidation products from the radical-mediated oxidation of lipids by hemoproteins can generate cell-signaling molecules other than isoprostanes. The mechanism of isoprostanes-induced vasoactivity is well known through the indirect effect on MAP kinases; however, a novel pathway for cellular responses has recently come to light that does not include phosphorylation of MAP kinases. It was previously observed that the oxidation of LDL in atherosclerotic lesions generates a broad spectrum of conjugates between fragments of oxidized fatty acids and apolipoprotein B (197). One hypothesis for the formation of these adducts is that the uncontrolled generation of free radicals leads to the conversion of lipids from compounds with no reactivity toward nucleophiles to those, by virtue of electrophilic carbon centers, that are capable of forming stable adducts with nucleophilic centers on proteins: the thiol groups of the surface expose cysteine residues (131, 248, 273).

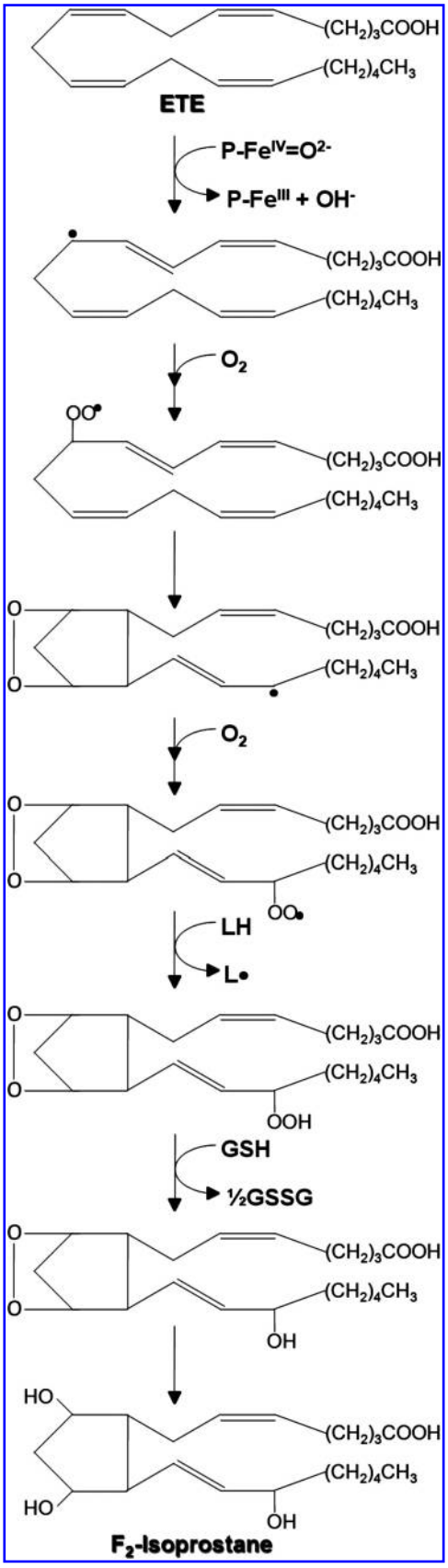

FIG. 6. Proposed mechanism of hemoprotein-induced formation of isoprostanes from eicosatetraenoic acid (arachidonic acid). Eicosatetraenoic acid (ETE), free or phosphorylated in lipid membranes, is vulnerable to oxidation by ferryl Mb or Hb. Abstraction of a hydrogen from the lipid diene forms an alkyl radical with reacts with oxygen to for a peroxyl radical, rearranging to form an epoxide alkoxyl radical. After a further reaction of oxygen, the epoxide peroxyl radical then (*1*) loses the peroxyl radical, perhaps through termination with another peroxyl radical or by propagation with another lipid or protein, (*2*) reduces the peroxide by reaction with the hemoprotein or reductant such as GSH, and (*3*) cleaves the epoxide dioxygen. The isoprostanes product is one of a vast series of lipid oxidation products that have vasoactive properties (129, 163, 228, 230, 235).

Isoprostanes are electrophilic lipids, some of which contain a carbon-centered electrophile on the cyclopentenone ring (*e.g*, A_2 and J_2 isoprostanes) (57, 131, 248). Other electrophilic lipids capable of forming protein adducts include prostaglandins and are, therefore, part of the normal inflammatory response; with posttranslational modifications being regulated by enzymatic processes (*e.g*., cyclooxygenase). The proteins modified by electrophiles have been collectively termed the electrophile-responsive element (EpRE) (52).

Cysteine residues have the potential to control selective cell-signaling pathways, because thiol chemistry offers the possibility of modification by structurally diverse species, including those derived from oxidized lipid and peroxides or nitric oxide (59, 63). It has been proposed that electrophilic lipids may induce cellular responses by triggering signaling pathways through the EpRE. These redox signaling pathways are distinct from phosphorylation pathways and play a regulatory role in cell differentiation and apoptosis (198), as well as adaptation to stress (*e.g*., heme oxygenase). It has been established that electrophilic lipid oxidation products are found at sites of inflammation, including the artery wall, and that they can activate both anti- and pro apoptotic signaling pathways, depending on concentration (158). The loss of control of these signaling pathways, likely through uncontrolled redox chemistry, is thought to contribute to the pathogenesis of atherosclerosis (119). On exposure of the cell to electrophiles, the EpRE is activated through electrophilic lipids binding to the cysteine rich Keap1, dissociating it from NF- E2–related factor-2, which plays a major role in transcriptional activation of antioxidant-responsive element-driven genes (157, 188). Downsteam effects include increased expression of NADPH quinone oxidoreductase and glutamyl cysteine ligase (137, 159) . It was demonstrated that the electrophilic lipids induce the transcriptional regulation of antioxidant enzymes through activation of the EpRE at low concentrations (52,159). At higher concentrations, however, these electrophilic lipids induce apoptosis. Understanding how oxidative-stress reactions control the switch between cytoprotection and apoptosis is of fundamental importance in the field of cell redox signaling.

The effect of electrophilic lipids generated from heme protein redox chemistry on the EpRE is not well understood, and their role in diseases is currently unclear. However, as oxidation of lipids by Mb/Hb generates electrophilic lipids, a link must be present and demands future exploration. It is also likely that different cell types will respond differently to different levels of oxidative damage, either engaging an antioxidant response or inducing apoptosis.

VII. Heme-to-Protein Cross-Linked Globins, a Marker for Peroxidatic Activity of Globins

In the 1950s, George and Irvine (93) described the capacity of Mb and Hb to function as peroxidases, reacting with hydrogen peroxide to form the ferryl oxidation state. This reaction can be followed by gross observation through the formation of a transient red species when peroxide is added to the brown ferric species at neutral or alkaline pH values, with the eventual return of the original brown ferric species. However, at acidic pH values, the result of the reaction with peroxides is a rapid formation of a permanent green coloration. This was interpreted to result from irreversible damage to the heme moiety. Two decades later, work by Fox et al. (87) reported that the green heme could not be separated from the protein by standard acid-solvent extraction techniques. The heme is normally "dissolved" in the hydrophobic heme pocket, with an iron–histidine bond connecting the heme to the protein, a bond that is acid labile. Thus, the inability to separate the green heme from the protein meant that the heme had become covalently bound to the protein through a mechanism of radical-mediated damage to the heme (87). Later, a more-detailed examination of the covalently bound heme reported that the heme was bound to a nearby osine residue (Tyr-103, sperm whale, human) through a bond between the aromatic ring of the tyrosine and the β-meso carbon of the heme (49).

These data were recently challenged by our laboratory, first by observing that the optical properties of the covalently bound heme are consistent with the conversion of the b-type Fe-protoporphyrin-IX into a d-type chlorin heme, in which one of the pyrrole rings has been converted to a pyrroline ring (219). This loss in the conjugation of one of the pyrrole rings was not consistent with the previously suggested structure. Second, mutation of the redox active tyrosine, reported as the link between the heme and protein into a redox-inactive phenylalanine, does not affect the ability of the protein to form the covalent link, casting doubt on the assignment of this tyrosine as the linker (215). It has been reported that the α chain of Hb forms a covalent bond between the heme and Ser-138 when challenged with peroxide at neutral pH, although the heme group seems to be severely damaged with the loss of iron (139). It has also been noted that the distal histidine (His-64) is essential for the formation of a covalent bond between the heme and protein in Mb (215). Hydrogen bonding between the distal histidine and peroxide bound to the heme iron (compound 0) facilitates the peroxide oxygen–oxygen bond breakage. Thus, the absence of the distal histidine interferes with the kinetics of radical production but not the eventual radical species that are detected (28, 62). Covalent linking between the heme and proximal histidine has also been reported, but the halogenated compound required to generate this species ($BrCCl_3$) means that it is unlikely to form *in vivo*. In addition, the color of the covalently linked heme retains its characteristic b-type spectrum (192).

Despite the confusion as to the identity of the amino acid that links the heme to the protein, the mechanism of its formation is somewhat clearer (Fig. 7). Key to the mechanisms of the formation of a covalent link is the effect of pH on the extent of heme-to-protein cross-linking. Evidence indicates that the protonated form of the oxoferryl heme, known to be highly reactive and radical-like in nature (221), is required to initiate cross-linking (219), explaining its pH dependence. The protein-based radical is also a key component in the formation of the covalent bond between the heme and protein. Allowing the protein-based radical to migrate away from the heme before the oxoferryl iron becomes protonated (through a pH jump) leads to inhibition of heme-to-protein cross-linking (219). Therefore, three ingredients are required for the formation of the heme-to-protein cross-link: (a) a protein-based radical, (b) a ferryl heme iron, and (c) a protonation event, likely protonation of the oxoferryl. The heme-to-protein cross-linked forms of Mb or Hb are very stable compounds (49, 87), and we have identified them in urine, kidney, and cerebrospinal fluid (see Section VIII, later). As the ferryl heme

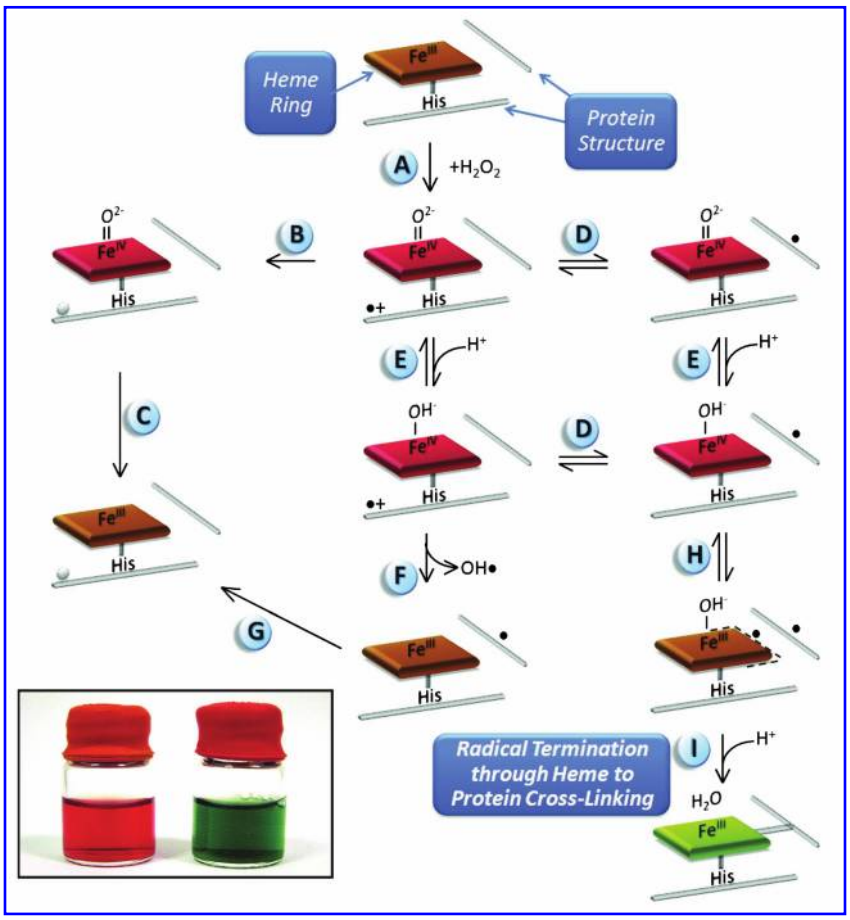

FIG. 7. Basic mechanism of peroxide-initiated formation of heme to protein cross-links. Ferric myoglobin [Fe(III), brown] or hemoglobin reacts with peroxide to produce the ferryl oxidation state [$Fe(IV)=O^{2-}$, red, step A] plus a protein-based cationic radical (•+). At high pH, the ferryl species is stable, but no heme-to-protein cross-linking is formed. The protein radical has time to quench before the ferrylMb eventually autoreduces (steps B and C), potentially resulting in various covalent protein modifications (*). The charge of the cation radical is quickly lost through deprotonation and can migrate to an amino acid residue [reported to be a tyrosine (49) or histidine (215)], close to the porphyrin ring (step D). Protonation of the oxoferryl at low pH (step E) destabilizes the ferryl species, being equivalent to a ferric heme iron plus a radical. This may result in the formation of ferric heme plus a hydroxyl radical (step F), which will enviably dissipate (step G). Alternatively, the protonated ferryl may result in the formation of a ferric heme with delocalized porphyrin radical, step H. Both the porphyrin radical and the radical on the nearby amino acid side chain react in a termination step, forming a protein-porphyrin covalent bond (step I). Heme-protein cross-linking will occur only if the protein radical and protonated oxoferryl species are present (219). Inset: Carbon monoxide ferrous forms of horse myoglobin (*left*) and purified heme-to-protein cross-linked myoglobin (*right*). The green coloration of heme-to-protein cross-linked myoglobin is less intense in its ferric form. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

iron and protein radical are direct products of the reaction between hemoproteins and peroxides, the formation of a heme-to-protein cross-link can be used as a marker for its previous peroxidatic activity *in vivo* without the need to detect the inherently reactive and unstable ferryl heme iron directly.

VIII. Hemoglobin and Myoglobin Redox Activity *In Vivo*

A. Hemoglobin redox activity under physiologic conditions

Under physiologic conditions, the peroxidatic chemistry of Mb and Hb is kept to a minimum by an extensive antioxidant defense system. Being compartmentalized in the erythrocyte or mycocyte, the hemoprotein can be micromanaged with heme reductases, glutathione, ascorbate, uric acid, *etc.*, all contributing to keep the globin in the physiologically active ferrous oxidation state and to sequester any radical species that are generated before severe damage is done. Nevertheless, even with this extensive antioxidant defense system in place, evidence suggests that globins undergo redox chemistry with peroxides under normal physiologic conditions. Examination of the Hb from fresh red blood cells reveals that approximately 1% of the Hb is in the physiologically inactive ferric oxidation state. The autooxidation of Hb is evidently a factor in the formation of ferric Hb; however, redox chemistry through the reaction of Hb with peroxide could also account for the amount of ferric protein in RBCs.

Evidence that the Hb peroxidatic activity occurs under physiologic conditions arises from the detection of free radicals in the blood. The signature of these free radicals, as detected with EPR spectroscopy, is identical to Hb-based free radicals generated from the *in vitro* reaction of Hb and hydrogen peroxide (265). This suggests that Hb participates in pseudoperoxidatic activity *in vivo*. The migration of free radicals from other sources could also account for the detection of free radicals on Hb; however, it is difficult to envisage where these radicals could be generated within the RBC, if not from Hb itself.

Free radical formation on the protein inevitably leads to the free radical damage of the protein and heme. Free radical damage to the heme moiety has also been observed in blood. (278). This modified form of the heme can be achieved *in vitro* through the reaction of the globin with peroxides, forming a d-type heme in which one of the pyrrole ring is transformed to a pyrolline ring by free radical damage (261). This modified form of the heme protein is green in appearance (similar to the color of heme-to-protein cross-linked Mb; Fig. 7, inset), but

normally does not exceed a fraction of 1%. Under normal conditions, ~1 in 2,000 hemes is in this modified form. The levels of this damaged hemoprotein are also seen to be elevated during exercise, indicating that enhanced levels of stress lead to further oxidative damage of the heme (278). After exercise, the levels of this d-type heme quickly revert to baseline or subbaseline levels, indicating that that a mechanism exists for its removal or degradation or repair *in vivo*. The mechanism for this process is currently unknown, but its existence implies that the presence of these modified hemes is not desired.

B. Hemoglobinopathies, oxidative stress, and malaria

Current research on the redox activity of Hb overwhelmingly supports the conclusion that Hb redox chemistry can play a role in damaging oxidative reactions whenever the hemoprotein becomes separated from its antioxidant-rich environment. However, in some conditions, enhanced levels of oxidative stress may be beneficial to the organism, such as infection of the host erythrocyte by the malaria parasite.

Malaria is a devastating disease that affects 300–660 million people a year, with threats of infection to 2.2 billion (256). Infection by the protozoan parasite *Plasmodium falciparum*, one of the most common and damaging malarial parasites, induces oxidative stress in both the host and parasite, which can be beneficial or pathologic, depending on the levels of reactive oxygen species, such as superoxide or peroxide (209). The malaria parasite matures within the erythrocyte, in which Hb is the main protein and consequently the main source of nutrient to the parasite (107). The parasite ingests the Hb by means of pinching off vesicles from the cytostome region of the protozoan and breaking down the Hb in a digestive vacuole to provide amino acids for its growth and maturation (269, 293). Heme released from the Hb degradation accumulates within the digestive vacuoles as hemozion, insoluble crystalline particles (247). The amount of Hb broken down by the parasite is extensive; with heavy malarial infection in an adult human, an estimated 100 g of Hb may be proteolyzed in a single cycle (107). Phagocytation of hemozoin is known to cause toxicity to the monocyte, inducing the oxidation of polyunsaturated fatty acids by a nonenzymatic, free radical mechanism, and thus may play a role in increased cytoadherence, vascular permeability, chemotaxis, and immunodepression in malarial infection (243). Heme oxygenase-1 (HO-1) activity has recently been reported to protect against noncerebral forms of malaria. In mice, the expression of HO-1 suppresses the prooxidant effects of free heme, preventing it from sensitizing hepatocytes to undergo TNF-mediated programmed cell death by apoptosis (244). In addition, the antimalarial properties of heme antistacking compounds such as the quinolines are well known.

It is recognized that certain hemoglobinopathies can function to protect the host from malarial infection, particularly if the host has the heterozygous genetic trait rather than the medical complications accompanying the full disease. Genetically protected individuals include those with Hb S (sickle cell anemia), thalassemia (both α and β), Hb E (a single-point βGlu26Lys mutation also associated with thalassemia), as well as glucose-6-phosphate dehydrogenase deficiency (75). Such individuals show increased survival after malarial infection, which explains the relation to the prevalence of these traits in populations with high frequencies of malarial infection (224). Individuals with the Hb E trait (prevalent in Southeast Asia) have a significantly decreased antioxidant level, as measured by radical cation decolorization assay (195). This decrease in antioxidant capacity can be partially reversed by vitamin E supplementation (196). A lack of glucose-6-phosphate dehydrogenase decreases blood glutathione concentrations, which in turn increase the susceptibility of erythrocytes to oxidative damage. Such alterations in the redox equilibrium are believed to be a mechanism that protects the host from the malaria parasite (21). The redox equilibria relation between host and parasite is complex; however, these hemoglobinopathies have provided insights into this balance. The response of the host immune system to malarial infection involves phagocytosis, inducing peroxide production, which also contributes to the pathology of the disease. The production of redox-active byproducts from Hb degradation, namely free heme or iron, together with hydrogen peroxide, confers oxidative insult on the host cell. However, the parasite also supplies antioxidants to the host and possesses an efficient enzymatic antioxidant defense system (21). Enhanced oxidative stress after the administration of prooxidants slows or prevents infection, showing that the malaria parasite is susceptible to superoxide and peroxide (21, 209). The Hb of genetically protected individuals is more prone to oxidation from the physiologically activated ferrous to the inactive ferric form, mainly because of enhanced rates of autoxidation (7, 123, 182). This in turn generates reactive oxygen species, which can lead to oxidation of erythrocyte membranes (60) and cross-linking or binding of spectrin and band-3 (72). Enhanced lipid oxidation in hemoglobinopathies has also been proposed to be a key mechanism in protecting the host against malarial infection. It has been proposed that an enhanced interaction between Hb and erythrocyte membranes induces mechanisms that (a) decrease invasion of the erythrocyte by *P. falciparum*, (b) impair parasite survival and development within the erythrocyte, and (c) accelerate infected erythrocyte clearance by phagocytosis (75).

The details of the mechanism(s) of hemoglobinopathies that protect against malaria are complex and still not fully resolved. However, it is evident that the enhanced levels of oxidative stress, either directly or indirectly related to Hb redox chemistry, may be a key component of this mechanism of protection in some hemoglobinopathies.

Blackwater fever is a complication of malarial infection, mainly attributed to *P. falciparum* but also associated with others such as *P. vivax* (142). Characterized by extensive hemolysis, hemoglobinuria, and kidney failure, Blackwater fever can often be triggered by the use of antimalarial drugs or synchronous hemolysis caused by an unusual hyperparasitemic state (74). Few epidemiologic studies have been conducted on the causes of toxicity during Blackwater fever; however, the clinical complications are similar to those of early HBOC studies and rhabdomyolysis-associated acute renal failure, which is associated with the ferric-ferryl redox chemistry of intact hemoproteins. To the author's knowledge, no studies have been conducted to confirm the mechanism of toxicity and whether ferryl Hb reductants may be used as therapeutic agents. In the treatment of malaria, iron chelation therapy has been used as an anti malarial strategy; however, the mechanism of action appears to be the formation of toxic complexes with iron rather than the withholding of iron (167).

C. Toxicity in the kidney: The redox activity of myoglobin after rhabdomyolysis

The involvement of respiratory heme proteins in the pathogenesis of oxidative damage has, until recently, been extremely difficult to prove and quite controversial, mainly because of the transient nature of the high oxidation state of Hb or Mb, making ferryl detection *in vivo* extremely difficult. Previous studies have tried various techniques to identify ferryl Mb *in vivo* and *ex vivo*, but definitive proof of Mb redox cycling has only been achieved from isolated rat hearts that were challenged with lipid peroxides. The ferryl heme was detected by using sulfide to trap the ferryl. However, this trap is too unstable and toxic to be used *in vivo* (284). Thus, the link between ferryl Mb or Hb and pathologic conditions *in vivo* was an extremely tenuous one.

The formation of a heme-to-protein cross-link was previously looked on as simply an *in vitro* curiosity, with no apparent relevance to physiologic or pathophysiologic conditions. This was until our discovery of the heme-to-protein cross-linked form of Mb in the kidney of animal models of rhabdomyolysis through HPLC analysis of urine and kidney tubule washouts (176), and later, in the urine of human patients diagnosed with rhabdomyolysis-associated acute renal failure (128). Rhabdomyolysis is the breakdown of muscle tissue, releasing muscle enzymes into the bloodstream. Rhabdomyolysis can occur by many mechanisms, with crush injuries (*e.g.*, trauma) and drug abuse (*e.g.*, alcohol, cocaine, ecstasy) being the commonest causes in Western countries. Other causes of rhabdomyolysis include ischemic injuries (*e.g.,* compression, sickle cell), viral (*e.g.*, influenza), bacterial (*e.g.*, legionella, malaria), prescription drugs (*e.g.*, statins), toxins (*e.g.*, animal venom, poisonous plants/fungi, carbon monoxide), hypothermia, hyperthermia, electric shock (*e.g.*, lightning strike), intense exercise (*e.g.*, marathon runners, military training), metabolic disorders (*e.g.*, hypothyroidism, diabetic ketoacidosis), genetic disorders (ATP deficiencies, sickle cell crisis), and shaken-baby syndrome. Because of the relatively small size of myoglobin (~17 kDa), it is filtered through the glomerulus into the kidney, where it is excreted predominantly in the ferric oxidation state (176). In approximately one third of patients diagnosed with rhabdomyolysis, acute renal failure follows. This kidney dysfunction is attributed to both intense renal vasoconstriction and renal tubular necrosis (15, 22, 113). The vasoconstriction was previously shown to result from the formation of lipid oxidation products, such as the IsoPs, in the kidney glomerulus (127, 128, 176). IsoPs, as described previously, are derived by nonenzymatic free radical–mediated oxidation of lipids (180). Significantly, IsoPs have also been found to be esterified to the renal lipid membranes in the kidney, demonstrating that lipid oxidation is generated *in situ* (176). Moreover, the formation of IsoPs esterified to the monolayer of LDL can be generated *in vitro* through the incubation of ferric Mb with LDL (176).

The presence of heme-to-protein cross-linked Mb in the urine and kidney after rhabdomyolysis was a definitive link between ferryl Mb and the mechanism of a disease state. As a heme-to-protein cross-link can be formed only when the heme protein has been redox cycled, it is convincing evidence that the protein had been in the ferryl oxidation state sometime in its recent history (219). Thus, it follows that Mb was acting as a pseudo-peroxidase *in vivo*, generating free radicals and inducing oxidative damage to lipids, resulting in the production of isoprostanes and electrophilic lipids, among others (Fig. 8). Critically, heme-to-protein cross-linked Mb has been found to be much more toxic compared with native protein. Oxidation of LDL by heme-to-protein cross-linked Mb was 5 times greater than that of native protein (280). Therefore, not only is heme-to-protein cross-linked myoglobin a marker of Mb-related oxidative damage, but it also contributes to the oxidative damage itself by enhancing lipid oxidation reactions. The heme-to-protein cross-linked form of Hb does not seem to possess enhanced toxicity compared with native protein, although it does not show any decrease in toxicity (Reeder, unpublished data).

Thus a "vicious cycle" of kidney damage appears to be occurring, with small amounts of ferryl Mb formed by intrinsic levels of peroxide, either hydrogen peroxide or lipid hydroperoxides, resulting in the generation of small amounts of lipid radicals (Fig. 8). These lipid radicals induce propagation reactions, generating more lipid peroxides and more ferryl Mb. IsoPs induce vasoconstriction, restricting blood flow and hence oxygen delivery to the kidney, resulting in acidosis. The decrease in pH accelerates the peroxidatic activity of Mb, leading to greater lipid radical production and higher levels of ferryl heme. Heme-to-protein cross-links are formed that themselves have an enhanced peroxidatic activity. Thus, radical-generation and lipid-oxidation cascades ultimately lead to acute renal failure. Currently, the role of EpRE in acute renal failure is unclear; however, it is evident that any antioxidant defense role that the EpRE plays is insufficient to prevent renal damage or that EpRE-signaled apoptosis may result from extensive electrophilic lipid production.

D. Toxicity in the brain: The redox activity of hemoglobin following subarachnoid hemorrhage

A hemolytic disease that bears striking similarities to acute renal failure after rhabdomyolysis is that of delayed vasospasm after subarachnoid hemorrhage (SAH). A head trauma or aneurysm can lead to bleeding into the cerebrospinal fluid (CSF) between the arachnoid membrane and pia mater surrounding the brain. This form of stroke is fatal in ~50% of cases, with the largest percentage of fatalities occurring before the damaged blood vessels can be repaired through surgical intervention (275). However, many survivors experience neurologic damage caused by delayed vasospasm. Restriction of the blood flow to the brain resulting from vasospasm causes ischemic brain injury, occurring in approximately one third of patients diagnosed with SAH, with >50% of patients displaying delayed ischemia showing permanent neurologic damage (77). Like the contamination of the kidney with Mb, the CSF is contaminated with Hb. With a reduced antioxidant capacity, oxidative damage occurs that may lead to the formation of IsoPs in the CSF (235). Also, like that of rhabdomyolysis, we have shown by HPLC that the respiratory hemoprotein in the CSF is modified such that a percentage of the protein is in the heme-to-protein cross-linked form, demonstrating that Hb undergoes redox cycling in the CSF (Fig. 9) (218, 223).

A correlation between the elevation of CSF free fatty acids and a worsened outcome after stroke, traumatic brain injury, and subarachnoid hemorrhage has been reported (206). F_2-IsoPs (129) and F_4-neuroprostanes have been detected in

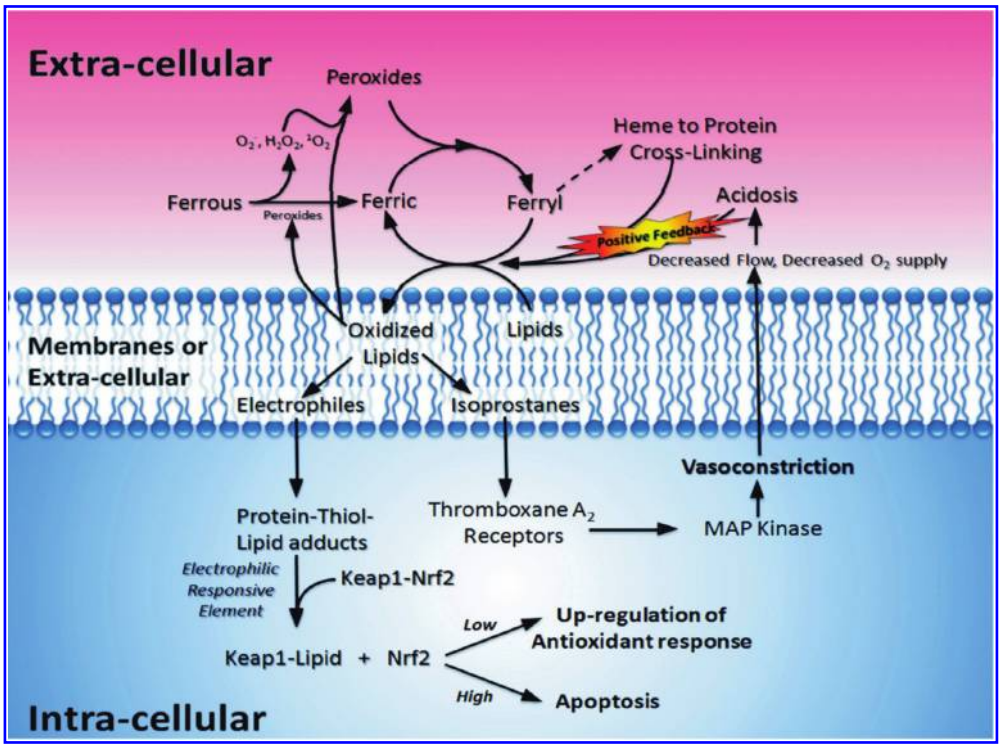

FIG. 8. Summary of the main reactions of hemoglobin with lipids. Ferric ferryl redox cycling of Mb or Hb can occur whenever the protein is outside its normal antioxidant-rich environment. This includes the kidney glomerulus after rhabdomyolysis (128, 176, 218, 222), the cerebrospinal spinal fluid after subarachnoid hemorrhage (218, 222), or potentially, the blood plasma after sickle cell anemia or the addition of hemoglobin-based oxygen carriers. These oxidative conditions generate lipid oxidation products, including isoprostanes, that cause vasoconstriction, decreasing the blood and oxygen supply (129, 176, 180, 207). This can induce acidosis (not in blood plasma) that enhances hemoprotein oxidative chemistry and induces heme-to-protein cross-linking in a positive-feedback mechanism (127, 176, 219, 223). Electrophilic lipid can induce protein thio-lipid adduct formation that, depending on conditions, can upregulate the antioxidant defense or induce apoptosis (52, 159). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

the CSF of patients after SAH (207). The correlation demonstrates oxidative damage to docosahexaenoic acid in brain tissue after SAH and suggests that F_4-neuroprostanes in CSF could be a good predictor for the outcome of SAH compared with F_2-isoprostanes at early time points (129). Some studies infer that suppression of oxidative stress in the CSF after SAH could be beneficial (163). Others support therapies to suppress cyclooxygenase and lipoxygenase activities (206).

No studies correlated IsoPs neuroprostanes with Hb redox chemistry in the CSF. However, the evidence that Hb undergoes redox cycling, known to generate isoprostanes from fatty acids *in vitro*, together with the evidence that oxidative damage to unsaturated fatty acids leads to compounds linked to pathologies associated with SAH, strongly support the hypothesis that Hb, through its peroxidatic activities in the CSF, is directly responsible for free radical damage, resulting in lipid oxidation and hence inducing delayed vasospasm, ischemia, and ultimately neurologic damage.

E. Targets for therapeutic intervention

In both acute failure after rhabdomyolysis and delayed vasospasm after SAH, the kidney/CSF becomes acidotic. Alkalinization therapy to prevent acute renal failure involves increasing the pH through bicarbonate infusion (22, 296). This therapy is used only after acute renal failure, as the patient must be closely monitored for potentially serious side effects of bicarbonate infusion, including myocardial arrhythmias (22). Therefore, this is considered by some to present too great a risk to use as a therapy. Until recently, the mechanism for this therapy was unknown. Alkalinization therapy did not assist Mb clearance to unblock the kidney renal tubules, as shown by studies that demonstrated that alkalinization treatment did not affect Mb deposition in the kidney (176). However, the pH was found to be critical in the mechanism of the peroxidatic activity of Mb (see later). Thus, alkalinization treatment appears to work by detoxifying Mb and short-circuiting the vicious cycle of lipid oxidation and acidosis catalyzed by Mb ferric-ferryl redox cycling (Fig. 8). It is hoped that this can be a beginning in the development of therapeutic compounds that inhibit Mb/Hb redox cycling and hence attenuate renal dysfunction and delayed vasospasm. Alkalization treatment is not an option for delayed vasospasm after SAH because of the extensive regulation of bicarbonate concentrations in the CSF to maintain tight control over the pH. The hypothesis is that compounds that can inhibit or short-circuit the cycle of lipid oxidation can potentially be used as treatments for preventing acute renal failure after rhabdomyolysis and potentially delayed vasospasms after SAH.

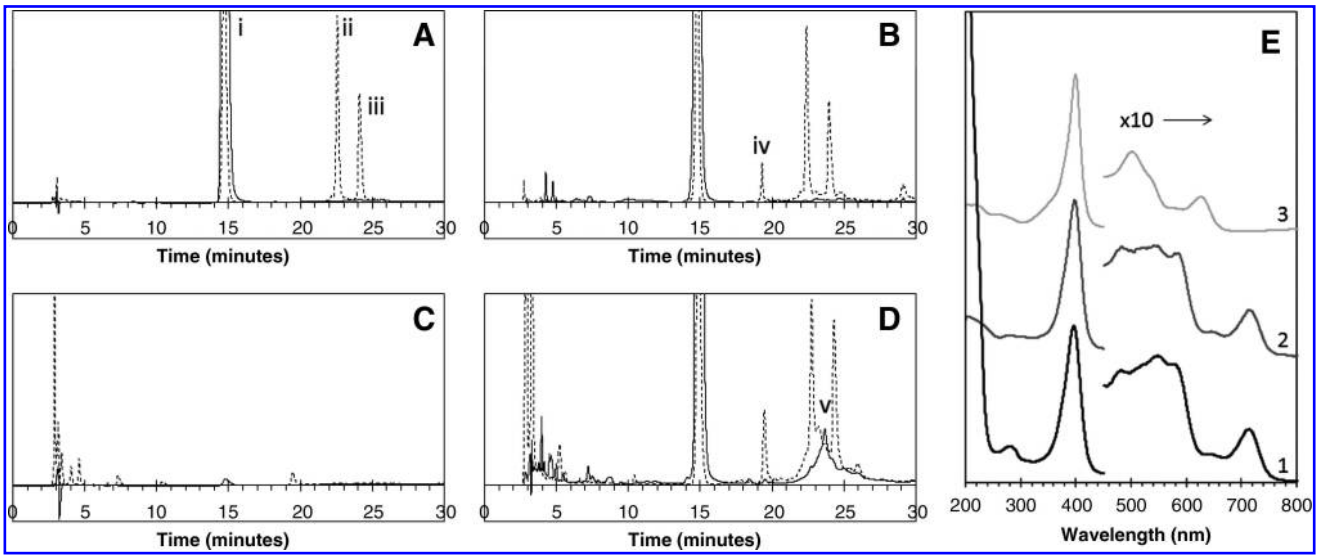

FIG. 9. HPLC of samples from blood and cerebrospinal fluid measured at 280 nm (*dotted line*) and 400 nm (*solid line*). (**A**) HPLC of purified human Hb. Because of the acid solvent of the HPLC, the heme and protein components separate. Peak (i) is the heme component, exhibiting absorption at both 280 and 400 nm. Peaks (ii) and (iii) are the β chains and α chains, respectively, both absorbing at 280 nm but not 400 nm. (**B**) Lysed whole blood. Hb components can clearly be seen with the addition of peak (iv), representing plasma human serum albumin. (**C**) CSF of hospitalized patient without evidence of subarachnoid hemorrhage. Virtually no Hb can be observed, with some heme (peak i) likely due to small contamination of CSF with blood when sample was taken. (**D**) CSF of patient diagnosed with subarachnoid hemorrhage. The heme and apoprotein components of Hb are present with the addition of many smaller heme peaks between 4 and 13 min and a large peak (v), with absorbance at both 280 and 400 nm, representing heme-to-protein cross-linked Hb. (**E**) Optical spectra of CSF heme components. Spectrum (1, black) is heme-to-protein cross-linked Hb with a distinctive 720-nm band and the protein bands present in the UV. The visible section of this spectrum is identical to a d-type "chlorin" heme (2, dark grey), also possessing a 720-nm band. Both spectra can be observed after peroxide addition to pure Hb. The 720-nm band is absent in undamaged b-type heme (Fe-protoporphyrin IX) (3, light grey). The 720 nm is present only under the conditions of the HPLC and not when the protein is folded under neutral pH values. Conditions for HPLC are described in reference (219).

Two accepted treatments are available to ameliorate the effects of delayed vasospasm after subarachnoid hemorrhage. Nimodipine is a calcium channel blocker that reduces hypertension, and so limits the effects of the vasospasm. If the symptoms of delayed vasospasm develop despite the use of calcium channel blockers, then patients are treated with aggressive hypervolemic, hypertensive, and hemodilution therapy. If the proposal that free radical damage results in lipid oxidation is correct, then nether of these current therapies targets the root cause of vasospasm. A recent study showed that acetaminophen, when introduced to an animal model of rhabdomyolysis, significantly improves renal functioning and prevents oxidative damage both *in vitro* and *in vivo* (26). With no appreciable capacity to function as an iron chelator (see next section), acetaminophen may function to prevent oxidative damage by Mb, as it has been shown to be an efficient ferryl Mb reductant, preventing lipid oxidation reactions and inhibiting the formation of heme-to-protein cross-links *in vitro*. Because of the high redox potentials of ferryl Mb (and Hb, ~1.4 V), a wide range of possible ferryl heme reductants remains to be explored as therapeutic agents. The advantage of such therapies designed to decrease ferryl heme-induced oxidative damage is that they may prevent or at least limit oxidative damage before the lipid-oxidation cascade occurs, and, if nontoxic, could be given as a treatment before the first signs of rhabdomyolysis, instead of waiting until extensive kidney damage (or delayed vasospasm) has occurred, as with the current treatments.

IX. Hemoproteins *versus* Labile Iron

The hypothesis that intact hemoprotein can play a major role in the induction of oxidative damage to tissue after a myolytic or hemolytic event, such as rhabdomyolysis or SAH, is still a controversial subject. Many believe that it is not the intact protein but the iron, released from degraded protein, that initiates oxidative damage. In this section, the evidence for iron-induced damage is critically examined and contrasted with the evidence for hemoprotein involvement in oxidative-damage reactions.

The toxic effects of iron overload are well known, ever since the understanding that excessive iron from regular blood transfusions used to treat patients with thalassemia major causes tissue damage, eventually leading to organ failure and death. The current use of iron chelators such as desferrioxamine (deferoxamine, desferal), deferriprone (ferriprox), and deferasirox (exjade) are essential in the removal of excess iron and the prevention of iron deposition. Thalassemia is an inherited autosomal disease in which the patients have a limited capacity to produce $\alpha_2\beta_2$ terameric Hb because of insufficient synthesis of either the α or β subunit, leading to abnormal Hb and anemia. Regular transfusions of blood or packed erythrocytes can increase life expectancy. However, iron overload can result from prolonged transfusions, requiring concurrent iron-chelation therapy to increase iron excretion. Congestive cardiac failure is a primary cause of mortality in thalassemia patients with inadequate chelation therapy (146, 147).

Degradation of both Hbs, through processes such as redox cycling or interaction with hemoxygenases, yields labile iron that can induce oxidative chemistry through Fenton chemistry (Haber–Weiss reaction):

$$Fe^{(II)} + H_2O_2 \rightarrow Fe^{(III)} + OH^{\bullet} + OH \qquad \text{Eq. 12}$$

$$Fe^{(III)} + H_2O_2 \rightarrow Fe^{(II)} + OOH^{\bullet} + H^{+} \qquad \text{Eq. 13}$$

Hydroxyl radicals ($OH^{\bullet}$), the product of heterolytic cleavage of the peroxide dioxygen bond by iron, are extremely powerful oxidants that will react with most biomolecules at diffusion-controlled rates (55). Thus, it will diffuse only a short distance before reacting and causes great damage within a small radius of its site of production. Therefore, hydroxyl radicals in living systems have an extremely short half-life and thus are difficult to detect directly or indirectly (251). After a myolytic/hemolytic event such as rhabdomyolysis or SAH, Mb and Hb are separated from their antioxidant-rich environment and can be degraded to release the iron through a number of possible processes, including heme oxygenase activity and extended redox cycling. The toxicity of this iron has been examined through the use of iron chelators as potential therapeutic agents to attenuate damage caused through Fenton chemistry. In numerous studies, the introduction of iron chelators to the disease animal model has yielded improvements in the condition, such as increased renal function after rhabdomyolysis (245, 296, 297) or protection against delayed vasospasm after subarachnoid hemorrhage (13, 279). Therefore, these studies have led to the conclusion that labile iron, either from the hemoproteins themselves or from other sources, presents a major factor in the pathogenesis of acute renal failure after rhabdomyolysis or other posthemolytic disease conditions.

The view that labile iron rather than intact hemoproteins is largely responsible for oxidative damage under pathologic conditions may be challenged. It is has been long known that desferrioxamine affects the reaction between peroxides and the intact hemoproteins through a mechanism of removal of the ferryl species through reduction (61). Desferrioxamine is also a substrate for peroxidases (179) and can sequester free radicals (117, 144). More recently deferiprone, another chelator developed to treat iron overload, has been identified as an efficient reductant for both ferryl Mb and ferryl Hb (217). Therefore, these iron chelators can affect hemoprotein-induced oxidative chemistry in addition to labile iron-induced oxidative chemistry. With such a large repertoire of actions that can affect oxidative reactions, the interpretation of the results of many studies showing that desferrioxamine, or other iron chelators, ameliorate oxidative damage is ambiguous with respect to the initiator of the cellular damage. This is especially significant in diseases in which respiratory hemoproteins may be involved. Under these circumstances, both intact and degraded forms of the heme protein, from which iron may be liberated, could potentially play a role in the mechanism of oxidative damage.

After rhabdomyolysis, heme oxygenase-1 (HO-1) expression is often observed to be elevated (185). HO-1 degrades the heme and removes it from circulation, the result of which is the release of free iron, carbon monoxide, and biliverdin (145). The physiologic effect of this reaction has been studied in animal models of HO-1–deficient mice and overexpressed HO-1 in rats. Heme oxygenase-1 (HO-1) knockout mice have a greatly decreased ability to degrade heme groups. These mice are also more susceptible to complications after induced rhabdomyolysis, exhibiting lower renal function and higher cellular damage (186). This normally nonfatal model of rhabdomyolysis was associated with a high mortality rate with the HO-1–knockout mice. Experiments in which HO-1 was overexpressed in rats by using a double-hemorrhage model of SAH inhibited arterial contractions induced by Hb and decreased vasospasm (189). Therefore, these experiments showed the indispensability of HO-1, attenuating hemoprotein-induced oxidative damage, and in doing so, suggesting a lesser role for labile iron than was previously believed.

Current medical procedures to treat rhabdomyolysis-associated renal dysfunction involve volume-replacement therapy to "wash out" the kidneys, or, in some cases, to administer bicarbonate to increase blood/urine pH (22, 296). These treatments are used only when the patient is hospitalized, and bicarbonate treatment, only when the patient is in intensive care. Thus, the treatments attempt to restore function to the kidney only when serious damage has already occurred. The hope is that treatments like low-molecular-weight ferryl reductants, such as acetaminophen, could be given at diagnosis of rhabdomyolysis or before, when rhabdomyolysis is only suspected. Thus, early intervention with ferryl heme reductants would limit kidney damage by decreasing lipid oxidation by Mb (or iron) and thus preventing lipid-oxidation cascades and tissue damage. Additionally, although the blood–brain barrier poses a greater challenge for the delivery of such therapeutic agents, it is hoped that delayed vasospasm after a subarachnoid hemorrhage could also be targeted by ferryl heme reductants.

X. Ferryl Heme Protonation Gates Globin Toxicity

The ferryl form of hemoglobin, as initiator of a large repertoire of prooxidant reactions, is an important species to understand. Even with extensive study over the past few decades, new information about the behavior of the ferryl species is coming to light. The activity of Mb and Hb in markedly pH-dependent reactions, such as the consumption of peroxides (220), LDL oxidation to form vasoactive isoprostanes (176), and heme-to-protein cross-link formation (219), are all considerably enhanced at acidic pH values. As discussed previously, these reactions are believed to contribute to cellular damage under conditions of oxidative stress. Therefore, as these reactions proceed more rapidly under acidic conditions, the toxicity of the globin will be also be enhanced. Indeed, bicarbonate treatment to ameliorate the effects of Mb deposition in the kidney after rhabdomyolysis prevents acidosis and has been linked to a decrease in the oxidative damage of renal tissue by Mb (176). The pH profiles of all these reactions seem to be connected to the stability of the ferryl heme. At acidic pH values, the ferryl heme is significantly more unstable, obtaining an electron from its environment to reform the ferric species. This change in activity has been assigned to the protonation of the ferryl heme itself (221).

$$P\text{-}Fe^{(IV)}{=}O^{2-} + H^{+} \rightarrow P\text{-}Fe^{(IV)}\text{-}OH^{-} \qquad \text{Eq. 14}$$

Using rapid-scan stopped-flow methods to discriminate between slower protein conformational changes and rapid

protonation events, it has been possible to examine the spectrum of the protonated ferryl hemoprotein even at pH values that would cause protein denaturation. The ferryl forms of both Mb and Hb display an optical transition with pK_a about 4.7, directly correlating ferryl protonation status with Hb/Mb ferryl stability and reactivity (252). In classic peroxidases such as cytochrome P450, the ferryl pK_a is likely to be modulated by a carboxylate side-chain hydrogen bonded to the proximal imidazole group (126); this added negative charge should increase proton affinity and shift the pK_a toward more-basic values. By contrast, the proximal imidazole/histidine ligand in Mb and Hb is hydrogen bonded to a neutral backbone carbonyl group, implying a smaller proton affinity.

The instability of the protonated ferryl species enables this species to be considered as possessing a "radical-like" nature. The protonated ferryl form quickly extracts an electron, making a radical species, either from the hydroxide to form a hydroxyl radical, or from the porphyrin/protein:

$$\text{P-Fe}^{(\text{IV})}\text{-OH}^- \equiv \text{P}^{\bullet+}\text{-Fe}^{(\text{III})}\text{-OH}^- \text{ or P-Fe}^{(\text{III})} + \text{OH}^\bullet \quad \text{Eq. 15}$$

Both hydroxyl or porphyrin radical species will propagate, oxidizing substrates such as lipids or initiating further protein radical formation, heme modifications, heme-protein cross-linking, and protein conformational changes. These modifications result in irreversible changes in the optical properties of Mb and influencing the consequent reactivity of myoglobin toward substrates, for example, enhancing NADH oxidation (191) or enhancing peroxidatic activity.

XI. The Importance of Tyrosine in the Oxidation of Substrates

The ferryl heme iron of Mb or Hb, and the protein-based radical generated with the ferryl, can oxidize a series of compounds, as described earlier. Tyrosine residues contained within the hemoproteins seem to play additional roles that attenuate its functionality, particularly in relation to its peroxidatic activity. Able to function as redox cofactors, tyrosine residues have been shown to facilitate comproportionation reactions to use high concentrations of oxyferrous Mb or Hb to remove ferryl heme (see earlier). Tyrosine residues also assist ferryl autoreduction, facilitating electron transfer through Tyr-103 and Tyr-151 of sperm whale Mb and possibly Tyr-42 of the human Hb α chain (156). Some -osine residues, such as Tyr151 in sperm whale, are also involved in the formation of dimers and oligomers, by using the protein radical to enable covalent cross-linking between Mb chains (155), although an equivalent tyrosine residue is not present in human Hb.

We recently reported that the kinetics of the reaction of ferryl with exogenous substrates can be modulated through a tyrosine residue positioned close to the heme but also on the protein surface (214, 217, 227). The electron transfer from the reductant to the heme can thus occur in one of two distinct pathways (Fig. 10). The first is the direct transfer between the reductant and the heme. For this to occur, the reductant must access the heme pocket, making the kinetics of ferryl reduction highly dependent on the physical properties of the reductant (*i.e.*, size, hydrophobicity). The second pathway shows a much higher affinity, yet the rate of electron transfer is largely independent of the reductant used. In Mb, removal of a specific tyrosine residue by site-directed mutagenesis (Tyr-103, horse, sperm whale, human) effectively deletes this pathway (214). This tyrosine on the G helix is close to the heme iron, but also on the protein surface (Fig. 11A). Thus, the tyrosine acts as a redox-active intermediate, transmitting electrons (or, of course, radicals in the reverse direction) to the ferryl heme iron from compounds in the bulk solution. In the absence of this tyrosine, the reduction by compounds such as ascorbate or urate is much slower, with some reductants up to 4 times slower. Critically the effect of tyrosines on the kinetics of ferryl Mb reduction is optimal at concentrations of reductant in the low micromolar range (*i.e.*, at concentrations that can be readily achieved *in vivo* with administration of drugs or with normal or reduced antioxidant capacities). According to the Marcus theory of electron transfer, the rate of electron transfer between reductant and heme iron should be much greater than that observed. The slow observed kinetics of ferryl heme iron reduction (although much faster than in the absence of the tyrosine) may reflect a multistep electron-transfer mechanism. Alternatively, a small percentage of the protein may be in the form that allows rapid electron transfer and is in equilibrium with the inactive state. It has been proposed that protonation or deprotonation steps are required for electron transfer to occur, having pK value(s) far removed from the pH at which the experiments were conducted (214). Thus, only a subpopulation of protein (*e.g.*, with a protonated oxoferryl heme iron or a deprotonated tyrosine) will show a high transfer rate, giving a low overall observed rate of electron transfer.

This through-protein electron pathway is also observed in human Hb. However, only the alpha chain shows the enhanced ferryl reduction rate when exogenous reductants are added. The β chain does not show this enhanced reduction, and, in the presence of low concentrations of ascorbate, is reduced $\leq$12-fold slower than the α chain (216). Thus, the kinetics of ferryl reduction in the Hb α chain by ascorbate is similar to that of Mb possessing Tyr-103 equivalent, but the chain shows similar reduction kinetics to Mb with this tyrosine removed. Although neither chain possesses a tyrosine on the G helix equivalent to Mb, the α chain does have a tyrosine on the C helix at position 42 that occupies almost the same spatial coordinates as the tyrosine of Mb, allowing a redox connection between the heme iron and the protein surface (Fig. 11B). The β chain of Hb, however, does not have this redox-active amino acid, but instead, a redox-inactive phenylalanine is at position 41, equivalent to position 42 in the α chain (Fig. 11C). Although the relevance of this through-protein electron-transfer pathway to *in vivo* cellular toxicity is currently unclear, it is proposed that the toxicity of proteins can be ameliorated by this electron-transfer pathway and that modulating this pathway through site-directed mutagenesis may be a starting point for the rational design of the next generation of Hb-based blood substitutes (216).

The presence of the through-protein electron-transfer pathway seems to be conserved in many species of the animal kingdom. With a database of $>$200 full sequences of myoglobin from various species known, it is now possible to examine the occurrence of Tyr-103 (G4, human equivalent) throughout the animal kingdom. All mammals possess Tyr-103 in their Mb, with a few exceptions noted in the marsupial family, where this tyrosine becomes a phenylalanine residue [*e.g.*, red kangaroo (274), duck-billed platypus]. Avian and

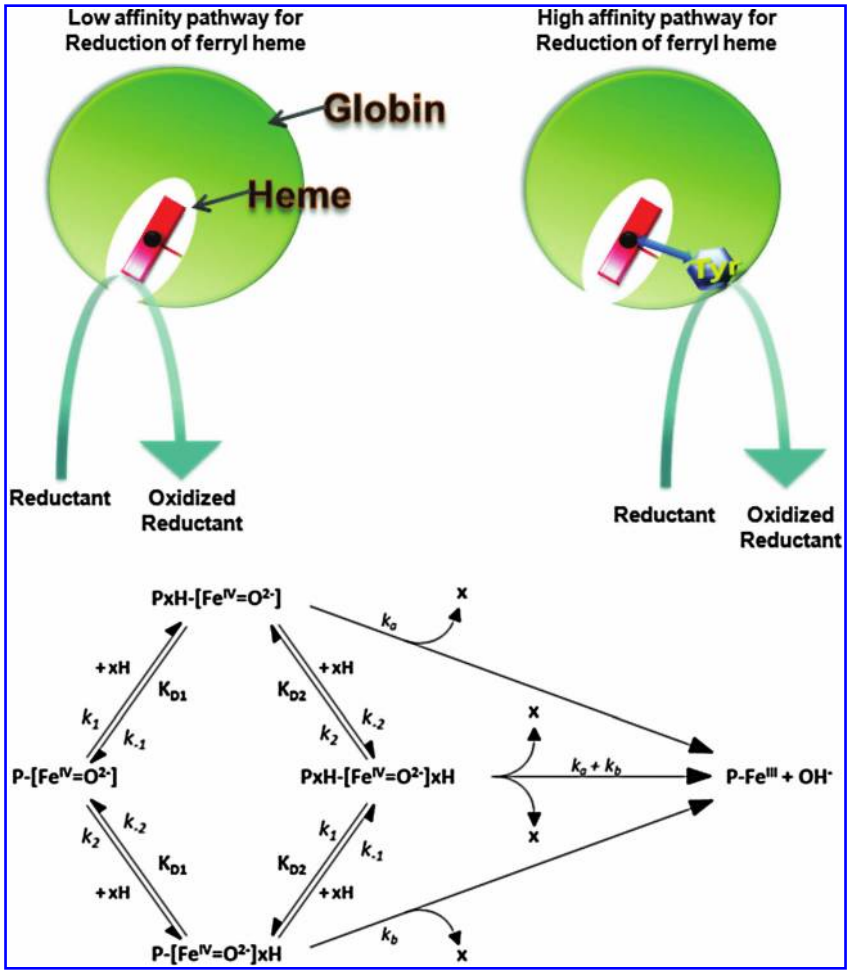

FIG. 10. Two-site model for reduction of ferryl hemoprotein. *Upper left:* The low-affinity pathway involves direct electron transfer between the reductant and the heme, making the rate constant for electron transfer highly dependent on the reductant used. *Upper right:* The high-affinity pathway involves electron transfer through the protein, involving a tyrosine residue that acts as redox-active intermediate, facilitating electron movement from the reductant to the heme (214, 216, 217). *Lower figure*: Reductant (xH) binds to two possible sites on myoglobin in its ferryl oxidation state {P-[Fe(IV)=O^{2-}}, where P denotes protein) with affinities KD1 and KD2. Only from these two sites can electron transfer from the reductant to the ferryl iron take place. The high-affinity binding site is situated at, or close to, a surface-exposed tyrosine residue {PxH-[Fe(IV)=O^{2-}]}, allowing the transfer of an electron through the protein to the ferryl heme iron to generate the ferric protein [P-Fe(III)]. The low-affinity site is situated in the heme pocket, allowing electron transfer directly between the reductant and the ferryl heme {P-[Fe(IV)=O^{2-}]xH}. This model also allows both sites to be filled by reductant {PxH-[Fe(IV)=O^{2-}]xH}. It is assumed that the binding on one site will not affect the affinity of binding to the other site. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

reptile myoglobin sequences show a Tyr-103 equivalent, but this tyrosine is absent in fish, mollusks, and other invertebrates. This indicates that the appearance of this tyrosine took place ~250–350 million years ago, perhaps after the late Devonian or Permian–Triassic extinction events. For tetrameric hemoglobins, the α-chain Tyr-42 equivalent (C7, human) appears to be highly conserved throughout the animal kingdom (Fig. 12, upper panel). However, equivalent position on the β chain (C7, position 41 in humans) is a phenylalanine in mammals, avians, and some reptiles, but a tyrosine in fish and molluscs (Fig. 12, lower panel). Site-directed mutation of the C7 tyrosine in α human Hb decreases cooperatively and in-

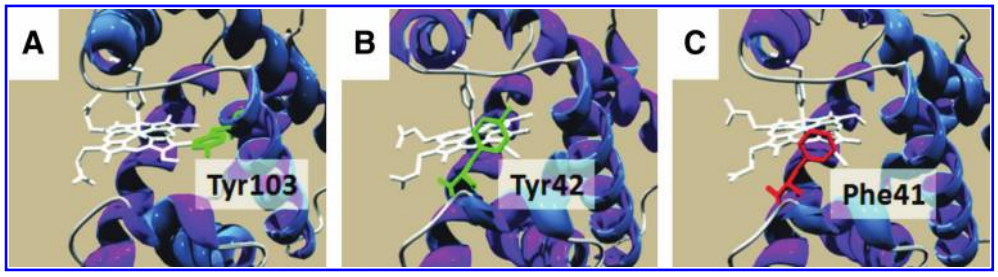

FIG. 11. Tyrosine residues as redox cofactors in globins. Tyr-103 (G4) of horse Mb is close to the heme and is surface-exposed, making it ideal to act as an electron conduit from exogenous reductants to the ferryl heme iron (**A**). Human hemoglobin α chain has a tyrosine in approximately the same position (Tyr-42, C7) (**B**); however, the C7 residue in human hemoglobin β chain is a redox-inactive phenylalanine (**C**). The proximal histidine of the heme group (white) is shown above the plane of the heme. Crystal structures used were 1A3N for human hemoglobin and 1WLA for horse myoglobin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

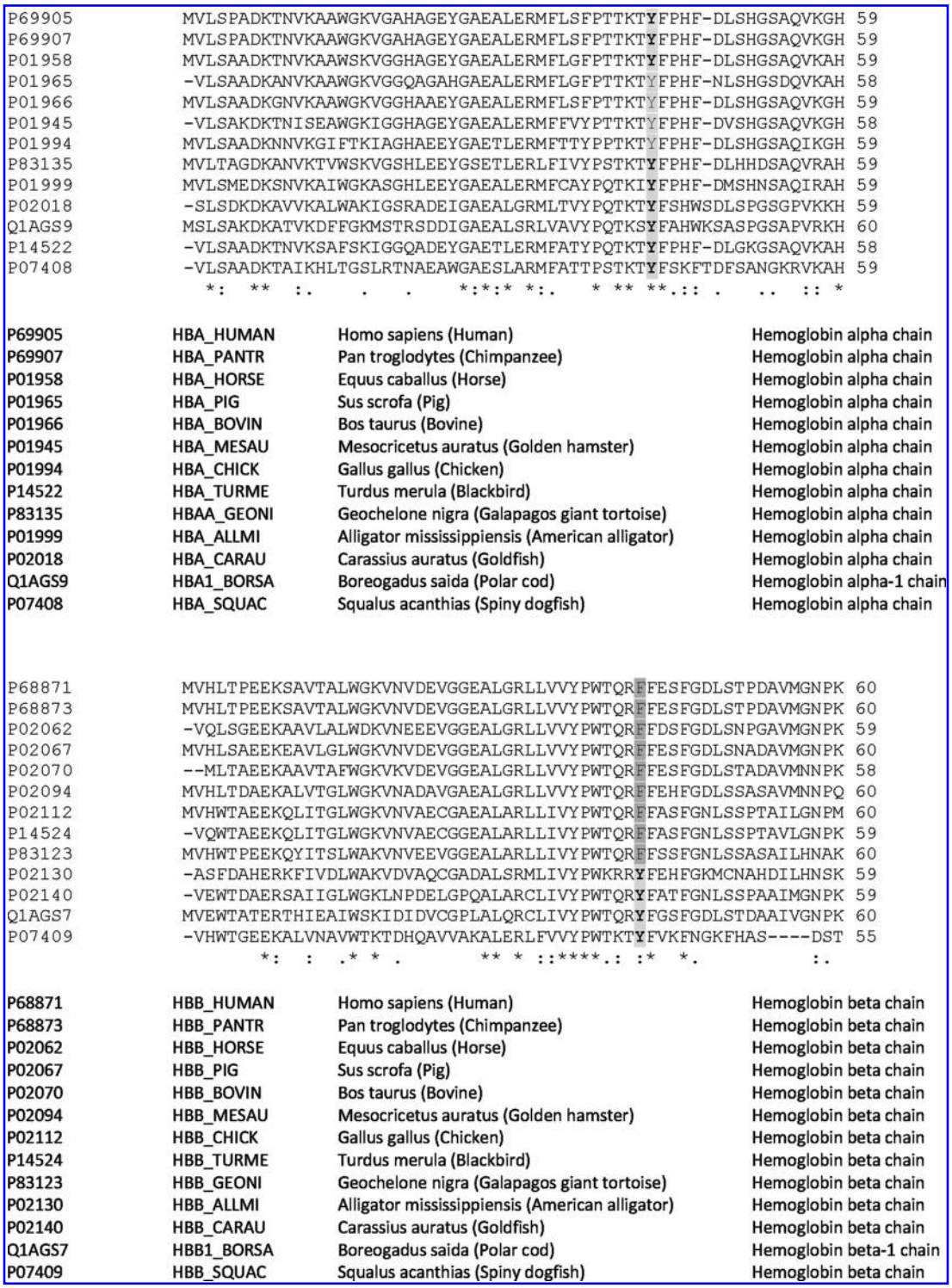

FIG. 12. (*Upper*) **Sequence alignment of a hemoglobin α chain, N terminus.** Tyrosine C7 (Tyr-42, human) is highly conserved. Sequences obtained from UniProt (www.uniprot.org.). (*Lower*) Sequence alignment of a hemoglobin β chain, N terminus. Phenylalanine C7 (Phe-41, human) is largely conserved throughout mammals and birds, but is a tyrosine in amphibians and fish, and split between tyrosine and phenylalanine in reptiles. Sequences obtained from UniProt (www.uniprot.org).

creases oxygen affinity (134). A natural variant of the β chain of human Hb with the phenylalanine C7 position replaced with tyrosine, known as Mequon, reported no phenotype anomalies until drug administration after a viral infection caused a hemolytic crisis with Heinz body formation, followed by complete recovery (42). Drug administration alone did not show hematologic abnormalities, and it was concluded that the Mequon mutation may have disturbed the heme environment, rendering it susceptible to oxidative denaturation in the presence of infection. Whether these

evolutionary changes in the G4, C7 tyrosines are random mutations that subsequently had no major impact on the function of the globins, or are driven by environmental conditions to give a physiologic advantage, requires further phylogenic and biochemical investigation.

XII. Hemoglobin and Its Use as an Artificial Blood Substitute

The development of viable blood substitutes has been a goal over a period of many decades. Blood transfusion is generally a safe practice; however, concern is voiced about hidden diseases that could permeate the transfusion population, as HIV did in the 1980s. The cost of screening blood is becoming ever higher, with more and more tests required to declare the donation "safe" from diseases such as HIV, hepatitis, West Nile virus, and even the common cold (which could seriously affect critically ill patients). Thus, the cost of comprehensively screening donations is prohibitive in developing countries and is becoming prohibitive in developed countries. The population that is able to donate blood is ever decreasing, with an aging population, and more people are unable to donate blood because they take medication for "modern" diseases such as high blood pressure, diabetes, and allergies. A viable, safe blood substitute would eliminate such problems and revolutionize the blood-transfusion industry. Advantages of a safe blood substitute include universal compatibility, long-term storage, guaranteed sterility, and immediate availability. Such a product would have a particular use in rapid deployment to patients in extreme, life-threatening situations outside the hospital, such as trauma. This has also led to interest in its potential use in situations of battlefield trauma.

Perfluorocarbons and hemoglobin-based oxygen carriers (HBOCs) have been the focus of considerable interest, in terms of both scientific research and commercial investment. HBOCs are developed primarily for use in the emergency resuscitation of trauma patients and for surgical procedures (4). However, the vast majority of HBOCs developed over the past 20–30 years have failed during clinical or preclinical trials. Only one hemoglobin-based product is commercially available to date (Oxyglobin by Biopure), which is licensed only as a veterinarian product. Many comprehensive reviews are available on the viability, clinical trials, and suspected problems of blood substitutes, and reading is recommended (3, 5, 39, 56, 287). What follows is an outline of the history of HBOCs, the current issues with the first and later generations of HBOCs, and what the redox chemistry of Hb, described throughout this review, could contribute to the understanding of the deleterious mechanisms of HBOCs and hence to the rational design of new HBOCs.

A. The rational design of hemoglobin-based oxygen carriers

Infusion of cell-free Hb causes an increase in systolic and diastolic blood pressures, decreased heart rate, and extensive renal damage, affirming that the erythrocyte serves important protective functions, protecting the Hb from degradation in the circulation and shielding the tissue from toxic side reactions of Hb (8). Under physiologic conditions, extracellular Hb from erythrocyte lysis is efficiently sequestered by binding to haptoglobin, with binding constants of $\geq 10^{15}$ M (133). The binding of haptoglobin to Mb is very much weaker, with reported binding constant of 8.5×10^{6} M (236). Thus, the sequestering capability of haptoglobin for Mb is insufficient to prevent renal toxicity after rhabdomyolysis. Levels of haptoglobin in the blood can be used as a measure of erythrocyte lysis (18). The haptoglobin pathway to remove lysed Hb safely from erythrocytes may become overwhelmed by infusion of cell-free Hb. Consequently, the Hb is removed by excretion through the kidney in its $\alpha\beta$ dimeric form. The kidney renal tubules become damaged, decreasing urine output and creatinine clearance, and eventually leading to acute renal failure (239). The mechanism of renal damage has not been extensively studied. However, in the author's opinion, renal damage is likely to arise from the same biochemical mechanisms as Mb damage to the kidney after rhabdomyolysis (see Section VIIIB).

The first generation of HBOCs, developed during the 1980s and 1990s, was aimed at eliminating renal toxicity through prevention of Hb dimerization, thus increasing its vascular retention by decreasing renal clearance and preventing haptoglobin depletion (287). Various techniques of maintaining the tetrameric form of Hb were tested. These included α-α chain dimerization by 3,5-bis(dibromosalicyl) fumarate (DBBF) (283), production of genetically cross- linked Hb (*e.g.*, rHb1.1) (165), surface decoration with polyethylene glycol (PEG) (2), or dextran (268), or encapsulation of the hemoglobin in particles such as liposomes (234). These methods prevented Hb dimerization, and hence the modified Hbs were found in the tissues rather than the urine.

Various clinical trials revealed that the first generation of HBOC products induced increased blood pressure and decreased heart rate, mild to severe chest and abdominal pain; some studies observed increased pancreatic enzymes (211). Clinical and preclinical trials observed that the HBOCs were less effective than standard saline treatments, leading to the conclusion that these HBOCs were not viable products (240, 255). It was discovered that much of the O_2 was released before the capillaries, such that oxygen tensions in the capillaries were ~20–30 mm Hg, at the steep slope of the Hb-saturation curve. This meant that too much O_2 was being delivered to the arterioles, which may be a trigger for vasoconstriction. The problem with modifying the Hb molecule, such as cross-linking, PEGylation, and genetic engineering, is that the O_2 affinity tends to be increased, with a concurrent decrease in cooperativity and the Hill coefficient. This was initially considered detrimental to HBOCs effectiveness, and efforts were made to retain the O_2 affinity of the HBOCs. According to Winslow, the assumption that HBOCs required low oxygen affinities was misplaced, as tissue po_2 levels can decline to only a few mm Hg without engaging anaerobic metabolism (288, 290). Therefore, any HBOC would release its oxygen, regardless of the affinity of the carrier, and thus the affinity for oxygen is not considered a critical factor in the development of HBOCs.

These and similar studies also led to the conclusion that nitric oxide (NO) sequestration was a major factor in the adverse effects observed in the first generation of HBOCs (76). Examination of the fluid dynamics of erythrocytes revealed that a "null space" existed close to the endothelium, free from erythrocytes. Thus, erythrocytes keep Hb a safe distance from the endothelium and hence away from NO (4, 130). This revelation seemed, at first, to be a fatal blow to the develop-

ment of cell-free HBOCs, as prevention of NO binding to Hb also meant preventing O_2 binding, eliminating the primary function of HBOCs. However, site-directed mutagenesis studies explored the molecular basis of NO binding in the heme cavity, leading to mutations that decreased the affinity of Mb for NO without significant diminution of its oxygen binding (6, 79). It was concluded that such mutant Hbs were less vasoactive than either αα-Hb or rHb1.1 (289).

An innovation to combat enhanced hypertension after introduction of HBOCs is the polynitroxylation of the HBOC. Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl), a cell-permeable hydrophilic nitroxide that protects against oxidative stress, causes dose-related hypotension, accompanied by accelerated heart rate and increased skin temperature (116). This protective effect has been associated with an increase in endothelial NO concentrations (299) and as a SOD mimetic, although other SOD mimetics such as 3-carbamoyl-PROXYL did not affect blood pressure in animals (116). The covalent addition of Tempol to αα cross-linked Hb adds antioxidant activity to the HBOC, as observed in studies *in vitro* and in an animal hemorrhage model (41). Resuscitation of the animal after 30 or 60 min after the hemorrhage with saline or Ringer's lactate did not improve base deficit (bicarbonate loss, indicative of metabolic acidosis), systemic hemodynamics, or regional blood circulation. Although albumin, αα cross-linked Hb, and polynitroxylated αα cross-linked Hb did improve these parameters, only the HBOCs improved survival time with the polynitroxylated HBOC, proving to be less hypertensive than other HBOCs as measured by increases in both cardiac output and systemic vascular resistance (41). Similar results have been noted with the polynitroxyation of other HBOCs (40). Therefore, this approach to add antioxidant activity directly onto HBOCs, may improve the efficacious of HBOCs as resuscitative fluids.

It was recently discovered that a technique for chemical polymerization of Hb with O-raffinose, a periodate-oxidized ring, opened trisaccharide, caused nonspecific modifications, most noticeably to cysteine groups. This induced a rigid protein structure that locked Hb in the T-state and resulted in heme instability (27, 140). The effect of these modifications is an increase in microvascular damage and permeability compared with other chemically modified Hbs in rats (16), although it is not yet known whether these specific modifications are responsible for the microvascular toxicity (5). Many such modifications have been shown to influence normal dynamic conversion between R and T states of Hb and heme stability (9, 276). It is important to note, however, that some modifications, such as nonspecific modification of bovine Hb with glyceraldehydes to form polyHb, do not appear to destabilize heme binding relative to native Hb, as these do not modify critical amino acids, such as cysteine or methionine residues (16). Nonetheless, heme stability and nonspecific modifications have been key issues with a number of HBOCs, a topic that requires particular in-depth evaluation in the future development of HBOC products (5). Notwithstanding the advances in the understanding of mechanisms of prevention of NO reactivity, nonspecific modifications, and heme instability, HBOCs that satisfy these criteria still exhibit increased levels in hypertension (1, 5, 38). Focus has begun to turn to the peroxidatic activity of Hbs and the role that these mechanisms play in inducing hypertension and other toxic reactions.

B. The redox chemistry of hemoglobin-based oxygen carriers: Inducers of oxidative stress?

Despite the extensive effort engaged in the formation of safe HBOCs, and various claims that certain new-generation products do not induce hypertension, no product has yet found approval for human use from the U.S. Food and Drug Administration (FDA). The publication of a meta-analysis of HBOCs in 2008 concluded that HBOC use is linked to a significantly increased risk of myocardial infarction and mortality (184). Currently, a number of issues require resolution:

1. Chemical modifications of Hb may enhance the ability of Hb to be involved in redox chemistry.
2. Free Hb close to the vasculature and sources of NO may result in the creation of an oxidative environment.
3. The inherent toxicity of Hb due to its ability to participate in redox chemistry when outside its protected environment of the red blood cell must be addressed (4).

Thus attention, while still mainly focused on NO chemistry, may be slowly turning toward oxidative stress chemistry.

Concerns that reactive oxygen species are being formed, through the autooxidation of oxyferrous Hb, have been previously examined. Enhanced autooxidation through chemical and engineered modifications has been linked to enhanced toxicity. Autooxidation of ferrous Hb to ferric Hb is an inherent problem that leads to the formation of superoxide (O_2) and forming the ferric (met) form of Hb, facilitating the release of heme from the protein and inducing hydrogen peroxide formation from superoxide dismutation (Eq. 3). Subsequently, HBOCs were produced that include superoxide dismutase and catalase covalently linked to Hb through chemical modifications to decrease ROS formation, resulting from Hb autooxidation. Efforts are also under way to produce such protein complexes through chimeric "fusion" proteins, with Hb and superoxide dismutase expressed as a single polypeptide chain (112). Such products have been shown to decrease hydroxyl radical production and to scavenge free radicals in rat models of ischemia/reperfusion injury (69, 213) and prevent ferryl Hb formation *in vitro* when challenged with hydrogen peroxide (70, 112). The one drawback with these Hbs linked with antioxidant proteins is that the reaction between lipid hydroperoxides and Hb, shown to be a significant pathogenic mechanism after hemolytic events, is relatively unaffected by the presence of superoxide dismutase and catalase.

Acellular Hbs are particularly susceptible to oxidation and hence denaturation, heme loss, and free radical production. Oxyglobin and other HBOCs have reported redox potentials similar to those of native Hb (between −150 and −50 mv) (78). A negative redox potential limits the reduction of Hbs by plasma-component antioxidants such as glutathione, β-NADH, and ascorbic acid, interfering with the physiologic role of the HBOCs. The annelid Hb from *Lumbricus* has a reported redox potential of about +100 mv, thereby enhancing the reductant capacity of plasma antioxidants compared with other HBOCs and preventing autoxidation, even by classic oxidizing compounds, such as ferricyanide (78, 122).

Addition of ferrous, and to a lesser extent, ferric HBOCs to endothelial cell cultures does not induce cytotoxicity; however, Hb and HBOCs added in the ferryl oxidation state show

a high degree of cytotoxicity (68, 71). Bolus additions of hydrogen peroxide and Hb to cells lead to glutathione depletion (67). In ischemia/reperfusion models of endothelial cells in culture, the formation of ferryl Hb was linked to cellular lipid oxidation (172). The effects of H_2O_2-induced ferric-ferryl redox cycling of both Hb and diaspirin cross-linked HBOC on endothelial cells are changes in cellular morphology, a decrease in soluble reduced thiols, induction of G_2/M cell-cycle arrest, and apoptotic cell death (68), although another report found that modified HBOCs, but not native Hb, induced apoptosis in the presence of H_2O_2 (108). These effects can be counteracted by using catalase to remove the peroxide, cyanide to block Hb redox chemistry (locking the Hb in the ferric oxidation state), or addition of antioxidants such as ascorbic acid to remove ferryl Hb and to scavenge free radicals (5). This free radical chemistry, as described earlier, can induce oxidative damage to the cell membrane. Studies have documented induction of HO-1 as a cellular response to high oxidation states of Hb, as well as other proinflammatory responses. HO-1 transcription is markedly increased when Hb or HBOCs are added to endothelial cells (92). Extracellular addition of Hb and polymerized Hb to rats and guinea pigs caused HO-1 induction, as well as ferritin production (46). Higher levels of ascorbic acid were also observed in rats, but not guinea pigs, because of their ability to generate their own ascorbic acid, like guinea pigs and humans. The result was the detection of ferrous and ferric iron deposits in rats, whereas only ferric iron was observed in guinea pigs. This implies that differences in endogenous antioxidant defenses lead to different renal handling of Hbs. HBOCs also influences cellular response pathways to hypoxia, affecting the expression of hypoxia-inducible factor (HIF-1). Ferrous HBOCs enhance HIF-1 expression in a time course that correlates with ferric HBOC accumulation (294). Addition of ferric HBOCs to hypoxic endothelial cells doubles the expression of HIF-1 and HO-1, with detection of ferryl Hb in the cell media resulting from endogenous peroxide consumption. This effect on HIF-1 expression was partially reversed through addition of NO, without affecting HO-1 expression (294). It has been proposed that the expression of HIF-1 may be reliable method of measuring the tissue oxygenation and redox status of HBOCs under cell-culture conditions and may be a clinically relevant method of assessing HBOC toxicity (294).

One of the main problems associated with assessing oxidative stress *in vivo* was reported to have been the lack of a method to monitor the oxidative chemistry of Hb (5). As described earlier, heme-to-protein cross-linking can be used as a biomarker of oxidative damage, observed in kidney and urine after rhabdomyolysis and in the CSF after SAH (Section VIII). However, the necessity for the hemoprotein to be in the protonated oxoferryl state means that little heme-to-protein cross-linking is observed under neutral pH conditions. Heme-to-protein cross-linking in blood, even under conditions of potential oxidative stress such as animal-transfusion models, has not been detected (Reeder, unpublished data). Noncovalently bound, oxidatively modified d-type chlorin hemes have been detected in humans under conditions of mild stress (278) and with HBOCs (Hb-Dex-BTC and Oxyglobin) added to endothelial cells (92). However, the highest percentages of damaged hemes detected were in stock preparations of HBOCs and decreased rapidly after shear stress to endothelial cells, suggesting that stress conditions facilitate the apparatus to repair or remove oxidatively modified heme groups (92). The reactivity between Hbs with oxidatively modified heme groups and HO-1 is not currently known. Induction of HO-1 may, however, act as an oxidative stress marker for evaluation of different HBOCs and may provide insight into the HBOCs' toxicity mechanisms (294). Iron release from HO-1 activity has been expressed as a concern, as it has been shown that the iron chelators desferrioxamine reverse vasoconstriction after α cross-linked Hb addition to rabbit hearts and to decrease the coronary vasomotor response to acetylcholine (168, 181), Additionally, desferrioxamine was found to inhibit partially tissue toxicity after transfusion of polymerized bovine Hb to hamsters, decreasing the microvascular and oxygenation changes (47). Both studies concluded that the chelators partially ameliorated HBOC toxicity through inhibition of iron-mediated free radical processes. Referring to Section IX of this review, an alternative evaluation for the results of desferrioxamine amelioration of HBOC toxicity could be offered through the capacity of desferrioxamine to function as a reductant for ferryl Hb (61, 222) and its free radical scavenging ability (117, 144, 179).

The removal of cell-free Hb from the extracellular environment through binding to haptoglobin is an efficient mechanism to remove safely cell-free Hb from circulation. The Hb-haptoglobin complex binds to monocyte and macrophage scavenger receptor CD163, mediating internalization of the complex into an endosomal compartment for controlled degradation (151). Receptor CD163 was found to play a key role in inflammatory and wound responses. The Hb-haptoglobin complex binds to CD163 through a cysteine-rich receptor domain region (170). Haptoglobin-knockout mice display no decreased levels of plasma Hb clearance (162), and neither do humans with anhaptoglobinemia (154). Therefore, a lower-affinity binding of haptoglobin-free Hb exists so that, when haptoglobin levels are depleted, Hb may still be degraded safely by macrophage internalization, albeit at a reduced rate (241). This biphasic nature of CD163 receptors has led to the finding that some chemically modified HBOCs (*e.g.*, α-α cross-linked Hb) have a greater affinity for CD163 receptor than native Hb (241). Thus, HBOCs may be "tailored" to have specific clearance rates to minimize toxicity by reducing the duration of the HBOC at sites of local tissue injury and inflammation, where HBOC stability could be compromised (5). Haptoglobin has been shown to have significant antioxidant properties. Although the full extent of the protective functions is current relatively unexplored, haptoglobin has been shown to protect LDL oxidation against Hb-induced oxidation *in vitro* (18). Studies also show that haptoglobin plays a physiologically important role in the amelioration of tissue damage by hemoglobin-driven lipid peroxidation, with haptoglobin-knockout mice showing a greater susceptibility to oxidative damage and a decreased ability to repair or regenerate damaged renal tissues (162). This suggests that HBOCs precomplexed with haptoglobin-like molecules may be a significant protector against oxidative damage. However, it also was recently reported that the Hb–haptoglobin complex retains its peroxidatic activity, reacting with peroxide to generate covalent cross-linking between the Hb and haptoglobin-forming aggregates (141). Although these aggregates are taken up by macrophages more rapidly than are nonaggregated complexes, the engulfed aggregates activate superoxide production, leading to enhanced intracellular

oxidative stress, which may lead to microvascular vasoconstriction. In contradiction to these results, *in vivo* experiments carried out in guinea pigs and dogs showed unequivocally that the Hb-haptoglobin complex reduced the blood pressure, inducing vasodilatation rather than vasoconstriction (25).

Specific modifications to Hb have been observed under oxidative conditions. As described earlier, when challenged with hydrogen peroxide, the heme may become covalently linked to the protein, possibly through a histidine residue under conditions of acidosis and a serine residue under more-neutral pH conditions. In addition, specific irreversible modifications to the protein have been observed in human Hb. These included oxidation of sulfur-containing amino acids including βMet-55, βCys-93, and βCys-112, all being oxidized to the methionine sulfoxide and cysteic acid forms, respectively, and βTrp-15 was found to oxidize irreversibly to oxyindolyl and kynureninyl products (139). Surprisingly, however, no modifications to amino acids in the α subunit were observed. This suggests an explanation for the observation that, under oxidative conditions, β-chain cross-linking is more resistant to peroxide-mediated damage than α-chain cross-linking (5, 190). Such modifications to the β chain could potentially be useful as markers of oxidative stress after infusion of HBOCs, however, more studies are required to examine the effects of HBOC Hb modifications to these oxidative modifications and whether such changes can be detected under stress conditions *in vivo*.

From the information presented here, with an intense focus of research into the generation of safe HBOCs and solving the problems that have arisen from previous and current HBOCs is still a formidable challenge. An extensive base of knowledge exists on the formation of lipid oxidation products from Mb and Hb redox chemistry from *in vitro* experimentation and, more recently, from pathological studies *in vivo* after posthemolytic and myolytic events. As such, the toxicity of Hb outside its normal antioxidant-rich environment of the erythrocyte is inherent. It is surprising, therefore, that currently only marginal focus exists on the effects of the redox chemistry on lipid oxidation and the broad spectrum of cellular responses that such chemistry can evoke, including hypertension. Studies on the formation of lipid oxidation products, such as the potent vasoactive isoprostanes, are relatively straightforward experiments; yet few studies have yet been reported. Additionally, new methods for determining oxidative modifications to both the protein and heme moieties of Hb can inform on the extent of Hb redox chemistry under transfusion conditions. Until such experiments are fully explored, the evidence that the oxidative chemistry of Hb may play a major role in the adverse effects of HBOCs will remain circumstantial. Of course, the proposal that HBOCs induce toxicity through oxidative reactions may be open to question. However, if HBOC toxicity is, at least in part, due to oxidative reactions, then new proposals for engineering better HBOCs with decreased redox activity, or adding therapeutic cofactors to suppress redox chemistry, can be offered.

Further studies on the heme-pocket geometry of HbI and HbII of the clam *L. pectinata* (described in Section VIB) have found that the reactivity of HbI to peroxides is much greater than that of HbII. This also coincides with a slow NO association and fast NO dissociation with ferrous iron for HbII, as well as a low autoxidation rate (73). Mutation of the HbI heme pocket Phe-B10 to a tyrosine residue to emulate the Tyr-30 (B10) of HbII results in a reduced reactivity of HbI for peroxides, decreasing the kinetics of the appearance of ferryl HbI eightfold to levels comparable to those observed with HbII. This mutation also decreases the autoxidation rate of HbI by a similar extent and reduces the NO association of HbI for NO by twofold, although residue Gln (E7) contributes largely to the distal control of NO binding (73). Therefore, it is possible to control the reactivity of HBOCs toward NO affinity, autoxidation, and reactivity with peroxides through engineering of the heme-binding protein pocket. These changes in the intrinsic reactivity of the hemoprotein can be combined with other rational design suggestions, such as the addition of heme to protein electron-transfer pathways (Section XI) or binding haptoglobin/haptoglobin mimetics. With the addition of more-traditional modifications, such as PEGylation, polymerization, or compartmentalization, a compelling rationale exists for the generation of future HBOCs with further decreased levels of intrinsic toxicity.

XIII. Conclusions

In conclusion, the heme-containing respiratory proteins Mb and Hb have the ability to function as pseudo-enzymes, reacting with NO, sulfides, and peroxides. The redox activity of these hemoproteins seems to be inherent to the hexacoordinate hemoproteins of Ngb, Cygb, and nonsymbiotic plant Hbs, which appear to incorporate the role of redox enzyme in their physiologic function. As these hexacoordinate enzymes pre-date their better-known pentacoordinate counterparts, their redox chemistry appears to be evolutionarily conserved. Under physiologic conditions, vertebrate Hb can influence the scavenging or delivery of NO to endothelial cells, causing changes in vasoactivity. The physiologic functions of some plant Hbs, particularly nonsymbiotic AHb1, seem to be related to NO regulation under hypoxic stress. Therefore, it is not unreasonable that vertebrate globins may also express some of these same functions, although the physiologic relevance of these reactions is still under debate.

Under pathologic conditions, Hb and Mb redox activity can lead to changes in the vasoactivity through NO depletion or by oxidizing lipids to form vasoactive products, such as the isoprostanes and electrophilic lipids. The formation of a covalent bond during ferric–ferryl redox cycling under conditions of acidosis has provided a biomarker for the previous peroxidatic activity of Mb and Hb *in vivo* and has shown that this chemistry is routine when the hemoproteins are outside their normal antioxidant-rich environment. The role that Mb and Hb play in the pathogenesis of posthemolytic/myolytic disease states is still unclear, with evidence that labile iron may also contribute to free radical production, although this evidence is in contention. The failure of HBOCs to develop into safe, viable blood substitutes has led many now to believe that the cytotoxic effects observed with previous and current generations of HBOCs are due, at least in part, to their redox chemistry. A new understanding of the mechanisms by which these hemoproteins behave under oxidative conditions is leading to therapeutic interventions and targeted changes in the rational design of HBOCs, detoxifying the ferryl form of the protein, and hence limiting the oxidative the damage that they cause.

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Abbreviations Used

AHb1/AHb2 = arabidopsis class 1/class 2 hemoglobin
Cygb = cytoglobin
ecHbs = erythrocruorins
EpRE = electrophilic responsive element
Hb = hemoglobin
HBOC = hemoglobin-based oxygen carrier
HO-1 = heme oxygenase-1
H_2O_2 = hydrogen peroxide
Lg = leghemoglobin
Mb = myoglobin
Ngb = neuroglobin
NO = nitric oxide
nsHbs = nonsymbiotic hemoglobins
P•+ = protein cation radical
PEG = polyethylene glycol
SAH = subarachnoid hemorrhage
SNO-Hb = *S*-nitroso-hemoglobin
trHbs = truncated hemoglobins

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