

ARS focused edition "REDOX CHEMISTRY OF GLOBIN-ASSOCIATED DISORDERS"

FORUM REVIEW

Title: **Redox and peroxidase activities of the hemoglobin superfamily – relevancy to health and disease.**

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Abbreviated title: "Functional roles of the hemoglobin superfamily"

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## Abstract

*Significance:* Erythrocyte hemoglobin and myocyte myoglobin, although primarily oxygen carrying proteins, have the capacity to do redox chemistry. Such redox activity in the wider family of globins now appears to have important associations with the mechanisms of cell stress response. In turn an understanding of such mechanisms *in vivo* may have a potential in the understanding of cancer therapy resistance and neurodegenerative disorders such as Alzheimer's. *Recent Advances:* There has been an enhanced understanding of the redox chemistry of the globin superfamily in recent years, leading to advances in development of hemoglobin-based blood substitutes and in hypotheses relating to specific disease mechanisms. Neuroglobin and cytoglobin have been linked to cell protection mechanisms against hypoxia and oxidative stress, with implications in the onset and progression of neurodegenerative diseases for neuroglobin and cancer for cytoglobin. *Critical Issues:* Despite the advances in the understanding of the redox chemistry of globins, the physiological roles of many of these proteins still remain ambiguous at best. Confusion over potential physiological roles may relate to multifunctional roles for globins which may be modulated by surface exposed cysteine pairs in some globins. Such roles may be critical in deciphering the relationships of these globins in human diseases. *Future Directions:* Further studies are required to connect the considerable knowledge on the mechanisms of globin redox chemistry *in vitro* with the physiological and pathological roles of the globins *in vivo*. In doing so, new therapies for neurodegenerative disorders and cancer therapy resistance may be targeted.

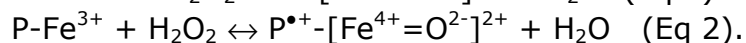
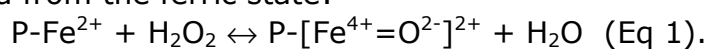
## Introduction

The redox chemistry of hemoglobin has long been considered an undesired side reaction, leading to the formation of radicals, vasoactive compounds and cytotoxicity. Yet this redox chemistry is an inherent nature of the biochemistry of all globins. Recent research into the redox activity of hemoglobin and investigations into the nature of the biological activity of other members of the hemoglobin superfamily have presented insights into the diversity and complexity of globin-associated redox chemistry. This redox chemistry relates not only to pathological mechanisms, that some might consider to be the main role of such reactions, but also potential physiologically relevant functions. What follows is a brief introduction to the redox chemistry of the hemoglobin superfamily and the relationship between this chemistry and physiological and pathological mechanisms that have been developed over the past few decades.

The mechanisms of the redox activity of globins have relevance in bacteria, plant and animals and the understanding of proteins from one class of globins may inform on the redox functions of others. This may be of particular relevance to the understanding between potential links between the redox activity of vertebrate globins and mechanisms of disease and cell protection. For example, there has been a realization of the importance of the redox chemistry of cytoglobin (Cygb) in cancer therapy resistance and neuroglobin (Ngb) in protection against neurodegenerative diseases. However the mechanisms that control possible redox functions of these globins remain unclear. Potential multifunctional roles of globins may provide an answer to conflicting reports concerning redox functions of globins. Mechanisms that can modulate the activity of the globins *in vivo*, such as oxidation of cysteines, will be explored. In addition, true multifunctional globins such as dehaloperoxidase (DHP) will be examined to give insights into the potential mechanisms of protein function modulation *in vivo*.

### Peroxidase activity of hemoglobin and myoglobin in disease.

During the pioneering work on the mechanisms of peroxidase activity conducted during the 1950s and 60's, myoglobin (Mb) and hemoglobin (Hb) provided an ideal model system for study. The ease of obtaining significant quantities of the protein and the slow reaction between H<sub>2</sub>O<sub>2</sub> and these respiratory heme proteins provided an opportunity to follow the reaction in detail (35,36,57). The reaction between Mb or Hb and H<sub>2</sub>O<sub>2</sub> was termed (and frequently still is) a "pseudo-peroxidase" activity as it was not considered that these reactions were relevant *in vivo*. However, many now consider globins to be true peroxidases with specific biological roles for this activity. This reaction generates a hyper valent ferryl heme iron from both ferrous and ferric oxidation states with a protein based radical if initiated from the ferric state:



Where P represents protein and P<sup>•+</sup> a protein cationic radical. For peroxidases such as ascorbate peroxidase and catalase, this radical is stabilized on the

porphyrin or nearby amino acid. For Mb and Hb this reactivity occurs mainly under uncontrolled pathological conditions with the radical migrating throughout the protein or onto unintended substrates, with the ferryl (equivalent to compound II) also able to initiate oxidation of substrates such as lipids. For reviews on this subject see (86,97). The reaction between Mb or Hb and lipid peroxides also generates ferryl heme iron and radical species. However, being a one electron acceptor, the radical products of lipid-based peroxides are the lipids themselves, leading to a potential cascade of lipid oxidation reactions and potent cell signaling molecules (49,68,96).

The redox chemistry of Mb or Hb is suppressed within the myocyte and erythrocyte respectively and generally only become apparent following myocyte injury and/or hemolysis. This is particularly relevant for erythrocytes due to the constant process of hemolysis in the blood stream. There are numerous systems for dealing with this undesired redox chemistry in the blood plasma including haptoglobin (Hp) and hemopexin (24,98). These proteins sequester free hemoglobin and free heme respectively, providing a defense against hemoglobin-mediated oxidative damage during intravascular hemolysis. The binding of alpha-beta dimers of Hb to Hp also appear to inhibit the damaging effects of Hb peroxidase activity, not by preventing the primary reactivity of the globin with peroxidase, but by limiting the reactivity of the high oxidation state of the protein (17). Here the ferryl heme iron is stabilized, increasing the steady state of ferryl heme iron. Ferryl stabilization is accompanied by an increase in the amount of the peroxide-induced tyrosine free radical on the beta chain ( $\beta$ Tyr145). Thus Hp may function to protect against vasculature damage by preventing oxidation of species that initiate undesirable free radical chain reactions without altering the reactivity of Hb with small exogenous ligands such as  $O_2$ , NO and  $H_2O_2$  (17). Extensive hemolysis can overwhelm the Hp and heme sequestering proteins and Mb, released from muscle damage (rhabdomyolysis), cannot be sequestered by Hp, leading to kidney damage (68). The redox activity of alpha Hb can also be suppressed when bound to its chaperone protein alpha hemoglobin stabilizing protein (67). The decrease in peroxidase activity results from the change in heme coordination from penta- to hexa- when alpha Hb is bound to the chaperone protein (30,44). Thus the chaperone prevents oxidation of the protein, and hence protein destabilization, before formation of  $\alpha_2\beta_2$  or  $\alpha_2\gamma_2$  tetramers.

The high oxidation state of the iron ( $Fe^{4+}$ ) is difficult to detect directly *in vivo* due to the transient nature of the hyper-valent ferryl heme iron and protein-based radical generated concurrent with ferryl heme. In addition the reactions they can potentially promote, that of free radical-mediated oxidation, can be sourced from a variety of alternative reaction systems such as Fenton chemistry or cyclooxygenase enzymes. Nonetheless, isolated ischemic rat hearts showed significant oxidation of Mb to the ferryl state by residual  $H_2O_2$ (3). The ferryl heme iron and protein radical are highly oxidizing compounds, damaging proteins, DNA and small molecules, however lipids are particularly vulnerable to oxidation. This prooxidant activity of Hb is linked to a number of disease states including atherosclerosis (84). The formation of ferryl Mb or Hb may not be preventable, with ferryl Hb associated free radical observable in normal blood by

EPR spectroscopy (110). However, the ferryl iron and the protein radical can be safely dealt with in the environment of the myocyte or erythrocyte by various electron donating antioxidant agents such as ascorbate and urate (18). It is of note that iron chelators such as desferrioxamine and deferriprone, ameliorators of oxidative damage, can efficiently reduce the ferryl heme iron to the ferric in addition to their prevention of Fenton chemistry by labile iron (16,90). Therefore it is difficult to determine the mechanism of any ameliorative effects obtained from the use of such chelators *in vivo*. That is, whether the mechanism of the antioxidant effects of these chelators are by donation of electrons to the high oxidation states of hemoglobin or by preventing the Fenton chemistry of non-heme iron.

A covalent link between the heme and protein is formed from heme undergoing a ferric-ferryl redox cycle under acidic conditions (14,88,94). Although noted as a stable green-colored by-product of ferric-ferryl redox cycling over half a century ago, it was considered primarily as a curiosity with no obvious biological relevance (35). However, as a stable and specific product of Mb/Hb redox cycling, it had the potential to fulfil the role as a marker of their previous redox chemistry *in vivo*. Heme-to-protein cross-linked Mb has been detected following rhabdomyolysis (muscle damage) in the kidney of animal models and urine of human patients (48,49,68). Such covalent modifications of Hb has also been detected following subarachnoid hemorrhage in the cerebrospinal fluid (91). This is direct evidence of the involvement of these globins in redox chemistry, which appears to occur whenever the globin becomes separated from its normal reductive environment. Potent vasoactive lipid oxidation products, such as isoprostanes, are generated during acute kidney injury following rhabdomyolysis and during delayed vasospasm in the cerebrospinal fluid. Isoprostanes can be formed *in vitro* by the peroxidase activity of Mb/Hb on cell tissue or low density lipoproteins (8,68,92). Hence globin redox chemistry is a likely origin of these vasoactive compounds *in vivo*, particularly when combined with the evidence that the globins undergo redox cycling by the cross-linking in the same disease tissue. It should be noted that Fenton chemistry can also lead to production of vasoactive products (130,131) and hence the significance of the finding of such heme to protein cross-links *in vivo* is debatable.

Other covalent modifications are noted in human Hb peroxidase redox activity. Both adult and fetal show extensive oxidative modifications to  $\alpha$  and beta/gamma chains, although the  $\beta/\gamma$  chain are more prone to oxidative modifications than that of  $\alpha$  chain (55). In a variant of Hb ( $\gamma$ -Val67 to Met, Hb Toms River) incorporation of labeled oxygen from  $H_2^{18}O_2$  to the methionine converts the amino acid into asparagine (106). Irreversible oxidation of  $\beta$  Cys93 occurs, up to two thirds of  $\beta$  Cys93 is oxidized with  $\sim 10$  fold excess of  $H_2O_2$  (55). This modification is even more extensive in sickle cell Hb. Other 'hotspots' of amino acid modifications include  $\beta$  Trp15, Trp37, Met55 and Cys112, as well as  $\alpha$  Tyr24, Met32, Tyr42, Met76 and Cys104 (55). These structural instabilities from such modification can lead to heme loss and oxidative damage to mitochondria.

Ferryl Hb also has important reported effects on inflammation and cell adhesion molecules. Heme released from damaged globins is a powerful pro-

oxidant, qualifying it as a danger-associated molecular pattern (DAMP) molecule, increasing the activation of Nrf2 and HO-1 (125). However, Hb itself has reported pro-inflammatory effects, targeting vascular endothelial cells, inducing the expression of pro-inflammatory genes in endothelial cells, including activation of the NF- $\kappa$ B family of transcription factor cell adhesion molecules such as E-selectin, Icam-1, and Vcam-1 (104). This leads to effects on the actin cytoskeleton, disrupting the endothelial cell monolayer. Furthermore, actin filament length is also affected in red blood cells, with ferryl Hb decreasing the actin polymerization in cells infected with *Plasmodium falciparum* malaria *in vitro* (20). This effect is enhanced with sickle cell Hb S or Hb C potentially preventing the parasite from creating its own actin cytoskeleton within the host cell cytoplasm, giving a possible mechanism of protection against the malarial parasite. The ferryl form of Hb S also induces oxidative stress in lung epithelial cells over that of HbA, attributed to the longer lived ferryl species of Hb S (55). However both HbA and HbS induce HO-1 and superoxide production with impairment of mitochondrial oxygen consumption.

The redox chemistry of Hb is also relevant in the development of hemoglobin-based oxygen carriers (HBOCs) used as potential blood substitutes or oxygen therapeutics. Numerous attempts have been made at generating a stable, non-toxic form of cell-free hemoglobin with limited success to date. However, these studies have focusing on preventing kidney damage (through increasing the size and stability of the protein structure by polymerisation, encapsulation or pegylation) and preventing scavenging of nitric oxide (NO) (through site-directed mutagenesis) (2,119). These aspects of HBOC chemistry are undoubtedly important factors in generating safer HBOCs, however, the peroxidatic activity of Hb and HBOCs have only recently been considered to be potential factor in taming HBOCs (1,2). One strategy to limit the redox chemistry of HBOCs *in vivo* is to enhance the ability of naturally occurring antioxidants in the blood to remove the cytotoxic ferryl form of the hemoglobin by introducing electron transfer pathways to the engineered HBOC (*vide infra*).

### **Through-protein electron transfer pathways.**

Through-protein electron transfer pathways (TPETPs) have the ability to accelerate the reduction of the ferryl oxidation state of globins, enhancing the safer removal of the cytotoxic forms of hemoglobins by physiological concentrations of antioxidants such as ascorbate, urate or even low concentrations (micromolar) of iron chelators such as deferiprone. We first reported these TPETPs to be naturally occurring in Mb of some species (87), noting the potential implications for therapeutic intervention in oxidative stress and for engineering more effective blood substitutes. These pathways, incorporating redox active tyrosines (Tyr103 in human/horse/sperm whale Mb) are able to pass electrons from the reductant to the heme iron more efficiently than direct access to the heme pocket (Figure 1). Human hemoglobin alpha subunit contains such a TPETP with a redox active tyrosine ( $\alpha$ -Tyr42), but the beta subunit equivalent position is occupied by a redox-inactive phenylalanine ( $\beta$ -Phe41) and hence has no TPETP. The position of Tyr42 in Mb and Tyr103 in  $\alpha$

Hb are in similar spatial positions (even though from different helices) and thus appear to perform similarly with regard to rates of electron transfer. Mutagenesis can introduce such pathways into the beta chain or remove them from the alpha chain, affecting the kinetics of electron donation of the reductant to the heme iron accordingly (89). Substitution with other redox active residues such as tryptophan do not show TPETP activity (89). At physiological concentrations of ascorbate ( $\sim 50 \mu\text{M}$ ) proteins with the TPETPs can effectively enhance ferryl iron reduction up to 12 fold over those without the pathway (89). Other serum antioxidants such as uric acid as well as phenols, hydroxyurea and iron chelators such as hydroxypyridinone CP20 (deferriprone) can also act as ferryl reductants utilizing the TPETP (18,87,89,90).

A study using a model Mb from *Aplysia Fasciata* (sometimes referred to as *Aplysia Limacina*, a junior synonym of *Aplysia Fasciata*) showed that the addition of a single tyrosine residues (Phe98Tyr) to generate a TPETP interfacing the heme iron and external environment can enhance ferryl reduction up to twelve hundred times, two orders of magnitude faster than those found in human Hb (93). With the inclusion of a distal histidine (V63H) to more closely mimic the redox chemistry of human Hb, the half times of ferryl reduction are altered from tens of minutes seen with the V63H *Aplysia* Mb variant (and human Hb) to sub-second half times with the V63H/F98Y *Aplysia* Mb mutations (93). Currently the reason for the two orders of magnitude difference of the electron transfer rates of engineered *Aplysia* Mb from *A. Fasciata* and human Hb are unknown. However, it is a current goal of research to incorporate such high rate TPETPs into recombinant human hemoglobin as a potential HBOC (103). It is not clear whether these mechanisms of ferryl reduction relate to globins where the high oxidation state of the globin may have physiological relevance. It is noteworthy however that some globins such as Hell's Gate globin-I, a thermophilic protein structurally similar to Ngb in *Methylobacterium inferorum* have multiple tyrosine residues within the heme pocket (80,112). Whether this high tyrosine content relates to thermostability or redox activity remains to be seen.

### **Physiological role for the redox chemistry of neuroglobin and cytoglobin in health and disease?**

It is evident that the redox chemistry of vertebrate Hbs and Mbs, primarily involving the peroxidase activities of these proteins, relates to conditions of pathology where such redox activity is undesirable. However the redox activity of globins appears to be of potential benefit, particularly to cell survival under conditions of stress. In the last two decades the family of globins have expanded considerably, with particular interest in human globins Ngb and Cygb and the potential functions and relations to cell protection in disease states. Both Ngb and Cygb are up-regulated under hypoxia suggesting potential oxygen binding/sensing, NO scavenging or nitrite reductase functions. However, both proteins also appear to play a role in oxidative stress mechanisms and maybe involved in cell signaling or cell protection. Recent research has led to the proposal of numerous potential biological functions under normal cellular conditions and in stress responses. Due to this large and diverse literature there

is currently uncertainty with regard to the primary function of these proteins (28,72,81,86,101,123). The molecular mechanisms of are still poorly understood, particularly with respect to their functionality *in vivo*. What follows focuses on the current opinions relating to the biological activity of Ngb and Cygb that have been advanced over the last few years.

### **Neuroglobin and Neuroprotection.**

Discovered in 2000, Ngb is the third globin to be found in humans after Hb and Mb (12). A hemoglobin with typical 3-on-3  $\alpha$ -helical fold of both Mb and Hb, Ngb differs from these protein by way of the geometry of the heme iron coordination. In the absence of exogenous ligands, the distal histidine is directly coordinated to the iron, making the ferric and deoxy ferrous heme iron low-spin hexacoordinate, coordinated by both proximal and distal histidines. The oxygen binding proteins Hb and Mb are high spin pentacoordinate in the absence of exogenous ligands, with the distal histidine unable to form a bond with the iron and instead forms a hydrogen bond to stabilize oxygen binding (65,74). Nonetheless Ngb has an affinity for oxygen of  $\sim 1$  torr, similar to that of Mb (12,81) with a proposed function of the temporary oxygen storage (25). The physiological function of Ngb may be related to its ability to react rapidly with NO in nervous tissue, functioning as an NO dioxygenase enzyme (Figure 2). This could be important in maintaining the function of neuron mitochondria, preventing mitochondrial poisoning by NO (54,118). However, this reactivity requires the Ngb to be in the oxyferrous form with high concentration of oxygen in neuronal cells, a supposition that is highly debated (4,9,12,25,116). An alternative view is that Ngb may function as a nitrite reductase, generating NO under conditions of oxidative or hypoxic stress (Figure 2) (83,114), similar to that proposed for Hb under similar conditions (19,37,63). Nitrite reductase activity has been proposed to underlie neuroprotective signaling under hypoxic conditions through NO production from nitrite (114), although Hb nitrite reductase activity is greatest when the protein is at its p50 for O<sub>2</sub> saturation due to allosteric modulation (37,38). It has also been proposed that Hb can form dinitrogen trioxide (N<sub>2</sub>O<sub>3</sub>), dissociating into NO and NO<sub>2</sub> (37,51). Such an application for this biochemistry of Ngb has not been elucidated, but as an uncharged molecule N<sub>2</sub>O<sub>3</sub> can diffuse through the cell membrane which could potentially act as a transport for NO to the endothelium (13,51).

Low concentrations of Ngb in brain neurons associated with increased risk of Alzheimer's disease (111). However, Ngb has also been shown to be upregulated in Alzheimer's disease (108). It has been proposed that Ngb prevents cytochrome c from forming apoptosomes, averting neuron cell death (85). Another proposal is that Ngb functions as an oxidative-stress responsive sensor, with oxidized ferric Ngb inhibiting GDP dissociation from the alpha subunit of heterotrimeric G protein, thereby acting as a guanine nucleotide dissociation inhibitor (GDI) (122,124). The GDI activity of Ngb correlated with its neuroprotective activity during oxidative-induced structural changes to alpha G protein (123). However, it is of note that Human Ngb but not Zebrafish Ngb exhibits such GDI activity (123), suggesting a more complex and diverse nature of this protein.



Whatever the biochemistry of Ngb *in vivo*, it is clear that the primary function of this highly conserved protein is related the protection of neuronal activity (10) and that more research is required for a more complete understanding of the molecular mechanisms of this protein *in vivo* and its potential role in protection against stress-related cell damage and neurodegeneration.

### **Cytoglobin, tumor suppression and cancer therapy resistance.**

Cygb is the fourth globin to be found in humans and discovered in 2001. Initially referred to as stellate cell activating protein (56) and histoglobin (115), the heme pocket structure of Cygb has similar in nature to Ngb, possessing a hexacoordinate iron ligation in the absence of exogenous ligands (22). However, the distal histidine coordination of Cygb appears to be weaker than observed in Ngb, with partial heme penta-coordination observed in the structure of one of the Cygb subunits of the homodimer (22). This pentacoordinate-like nature of the Cygb can be further enhanced by increasing the distal histidine off-rate, observed in the monomeric form of the protein with an intramolecular disulfide bond (7). There have been many suggested functions of Cygb. Cygb has been proposed to act as an oxygen diffusion proteins similar to Mb, based on observations of increased expression of Cygb in heart and muscle in amphibians that lack Mb (126). Cygb has also been proposed to function as an NO scavenger, from observed high rates of NO consumption and its fast re-reduction by ascorbate and cytochrome P450 reductase (64). This proposal of NO consumption has also been enhanced by increased NO levels and decreased intracellular nitrate production from partially silenced Cygb (41). However, the most intriguing property of the protein appears to relate to cell protection against oxidative stress and potential relationship with carcinomas. However, this is a double-edged-sword as Cygb appears to function not only as a tumor suppressor but also protects hypoxic tumors against therapy. The potential role of Cygb in cancer suppression and hypoxic tumor protection has been recently reviewed (15).

Promotor methylation downregulates Cygb expression as observed in oral cancer (100), in head and neck squamous cell carcinoma (101) and in non-small cell lung carcinoma (127), indicating that the gene for Cygb functions as a tumor suppressor. Furthermore, methylation of the gene for Cygb is able to discriminate patients with and without lung cancer, using biopsies and sputum sample (102). Cygb deficient mice has been found to promote carcinogenesis in lung and livers when exposed to carcinogens such as diethylnitrosamine (113). Cygb loss may contribute to tumor recurrence and a worse prognosis in gliomas and may serve as an independent predictive factor for prognosis of glioma patients (128).

Expression of mRNA for Cygb was found to correlate with hypoxia inducing factor 1-alpha (HIF-1 $\alpha$ ) in squamous cell carcinoma (101) indicating that expression of Cygb is regulated by hypoxia. Increased expression of this gene correlates with clinicopathological measures of a tumor's biological aggression (101). The Cygb gene also has a protective role for proliferating keratinocytes under normoxic conditions and protects against exogenous hydrogen peroxide

oxidative insults (59). This study shows that  $\Delta$ Np63, a p63 transcription factor protein, targets the *Cygb* gene leading to upregulation of *Cygb* and inhibition oxidative stress-induced apoptosis in keratinocytes. This also present in lung and breast cancer cell lines, indicating that *Cygb*-mediated scavenging of reactive oxygen species may also have a role in epithelial tumors and lung cancers. Furthermore, in human lung cancer data sets, the interaction of  $\Delta$ Np63 with *Cygb* gene significantly predicts reduction of patient survival (59). From the studies of *Cygb* and cancer prevention and promotion it is clear that *Cygb* has a bimodal tumor suppressors and oncogene functions (73).

Although the relationship of *Cygb* and with tumors has seen the greatest attention in recent years, other properties of *Cygb* also involve protection of the liver from fibrosis. Stemming from the protein's original naming in 2001 (56), *Cygb* is expressed in stellate cells but not fibroblasts (70), and as such the expression of *Cygb* can distinguish stellate cells from portal myofibroblasts in damaged liver. Furthermore, overexpression of *Cygb* in rat hepatic stellate cells inhibits their differentiation into myofibroblast-like cell phenotypes when challenged with oxidative stress (129). Similarly *Cygb* anti-fibrosis activity on rat livers decreased thioacetamide-induced liver fibrosis, which was proposed to involve apoptosis induction from activation of the caspase cascade (46).

### **Controlling protein function through cysteine oxidation.**

As described above, the biochemistry of the heme can potentially promote the scavenging or formation of NO as observed *in vitro* for Hb, Ngb and *Cygb* (Figure 2). In addition to this biochemistry surface-exposed cysteine residues have the capacity to generate complexes with NO, forming S-nitrosothiol. This has been observed for human Hb *in vitro* binding NO to the cysteine on the beta chain of human hemoglobin ( $\beta$ Cys93) to form Hb-SNO (39). The relevance for Hb-SNO for hypoxia response remains a widely debated subject and only discussed in brief here (37,39,53,79,121,132). Roles for this NO binding chemistry have been suggested to be a mechanism for the regulation of hypoxic vasodilation. This cysteine is highly conserved in the mammal and avian species (and is also present in fetal Hb as  $\gamma$ Cys93), so much so that the only other amino acids that are as highly conserved as  $\beta/\gamma$  Cys93 are a histidine and phenylalanine involved in heme stabilization (132). The relevance of hemoglobin forming and carrying NO relates to conditions of hypoxia, with the hypothesis that hypoxic conditions trigger the release of NO to induce a vasodilation response. Indeed mice with a  $\beta$ Cys93Ala mutation appear to have deficient hypoxic vasodilation and enhanced mortality with transient hypoxia (132). Thus  $\beta$ Cys93 appears to have an antioxidant role, protecting mice against lipopolysaccharide-induced reactive oxygen species and hypertension (121).

Like Hb, Ngb and *Cygb* also possess Cys residues that are surface exposed and have the potential to form SNO complexes. *In vitro* data shows that *Cygb* and Ngb can form SNO complexes, for example when reacting with nitrite under anaerobic conditions (83), but the relevancy of this chemistry *in vivo* has yet to be explored in detail. However, the cysteines of Ngb and *Cygb* are in pairs, promoting the view that the formation of disulfide (cystine) bonds are important in functional regulation of the proteins. Such dicysteine bridges can be

intermolecular, forming dimers (dimer<sub>S-S</sub>), intramolecular, forming monomers (monomer<sub>S-S</sub>) or reduced to free sulfhydryl (monomer<sub>S-H</sub>). These dicysteine bridges can alter the structure and ligand binding properties of the active site of both Ngb and Cygb and in doing so potentially regulate its function *in vivo*. Both Ngb and Cygb show significant dependence of their biophysical properties on the redox state of their surface-exposed cysteines (120). These cysteines pairs (Cys38/Cys83) in human Cygb (Figure 3) are highly conserved throughout mammals, but not in other vertebrates. Cys46/Cys55 in human Ngb (Figure 3) is more widely found in other vertebrates. Numerous studies have shown that these cysteine pairs affect both exogenous and endogenous ligand binding to the heme iron, with supposition that the redox state of the cell modulates the ligand affinities of these proteins (7,42).

Recombinant expression of Ngb primarily generates the protein with a disulfide bond between Cys46 and Cys55 (monomer<sub>S-S</sub>) located on the CD loop region of the protein (42). The protein in this monomer<sub>S-S</sub> form shows high NO dioxygenase and nitrite reductase activities but negligible peroxidase-like activities (71,114). This, in part, has reinforced the view that Ngb functions primarily as cytoprotective role against NO-induced impairment of mitochondrial metabolism (105). However, the state of the cysteine pair also appears to modulate nitrite reductase activity, with an enhanced formation of NO when the protein in the monomer<sub>S-S</sub> conformation and proposed to be a stress responsive mechanism (114). Breaking the disulfide bond leads to a large decrease in the oxygen affinity of Ngb, linked to a decrease in the histidine dissociation rate (43), Figure 4. Stabilization the hexacoordinate form with in the monomer<sub>S-H</sub> protein has also been proposed to be the mechanism of slowing the nitrite reductase activity of Ngb (114). The crystal structure of the monomer<sub>S-S</sub> form of Ngb was difficult to generate, with original published crystal structures of a double cysteine mutant (82). Molecular dynamic simulations suggested that the formation of a disulphide bond changes the CD loop from strand to a helix structure, structuring the active site and acting as a redox control for Ngb function (69). The predicted structural changes of the CD loop was confirmed with a recent publication of the structure of the wild type protein in the monomer<sub>S-S</sub> form (40). Ngb is presumed to be monomeric *in vivo*, however, dimers have been reported from studies using retinal neurons (77) and that such dimers are likely to be disulfide-linked (60). Hence elucidating conditions where the protein is monomer or dimer and the effects of these conformations on cell signaling pathways is of particular importance when considering the physiological role of the protein.

Recombinant expression of Cygb in *E.coli* tends to lead to the formation of mixture of three forms of conformations of protein depending on the redox state of two surface exposed cysteine residues (Cys38 and Cys83) – (i) dimer<sub>S-S</sub> with two intermolecular disulfide bridges spanning Cys38 on one protein to Cys83 on the other protein and *vice versa* (ii) monomer<sub>S-S</sub> protein with the intramolecular disulfide between the B and E helices and (iii) monomer<sub>S-H</sub> protein with free sulfhydryl (7). Current crystallographic data on recombinant Cygb shows either dimer<sub>S-S</sub> conformation (e.g. PDB structure 1UMO (22)) or monomer<sub>S-H</sub> conformation with free sulfhydryl (e.g. PDB structure 3AG0 (66)) or Cys to Ser

mutations (e.g. PDB structure 1V5H(107)). To date no structures of Cygb with intramolecular disulfide bond have been published.

Monomeric<sub>S-S</sub> Cygb show significantly different carbon monoxide (CO) and cyanide (CN) ligand affinities compared to monomer with free sulfhydryl or with cysteine mutations, showing that ligand binding in both ferrous and ferric oxidation states of the protein are affected by the redox state of the cysteine residues (117). The dissociation rate for the distal histidine is 1000 fold higher with the monomer<sub>S-S</sub> protein compared to monomer<sub>S-H</sub>, (6), Figure 4. Thus the monomer<sub>S-S</sub> form of Cygb although still primarily bis-his hexacoordinate in the absence of exogenous ligands, possesses properties that are more pentacoordinate-like compared to the monomer<sub>S-H</sub> protein. Furthermore, the monomeric<sub>S-S</sub> form of Cygb shows higher peroxidase-like activities compared with other forms of the protein, oxidizing lipids and lipid membranes more efficiently (7). In contrast with this, the dimeric protein with intermolecular disulfides or monomer with free thiol shows poor lipid peroxidase activity, decreased ligand-binding kinetics and a ridged hexacoordinate nature (6,7).

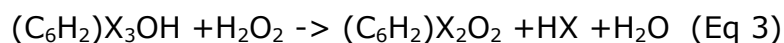
Crystal structure of dimer<sub>S-S</sub> Cygb places the two sulphur atoms some 12 Å distant (1V5H) (107), suggesting a significant shift in the B/E helices to accommodate the formation of a disulfide bond between the two residues. Comparison to crystal structure of Cygb with free sulfhydryl (2DC3) (66) shows the two cysteine sulfurs to be some 7 Å closer (Figure 3D). These effects on structure are likely to affect the heme pocket geometry on the distal side, however, no structural information of the monomer<sub>S-S</sub> form of the protein is yet available other than the ligand binding and reactivity effects described above. Nonetheless the question remains what is the form of Cygb *in vivo* and is this modulated by the redox environment of the cell?

We have previously reported that the conformer state of these cysteines also influences lipid-induced structural changes to Cygb (95). This effect is observed only in the ferric oxidation state of Cygb and only with the protein possessing an intramolecular dicysteine bond (monomer<sub>S-S</sub>) between Cys38 and Cys83. There is no effect of lipids on ferrous Cygb or ferric Cygb dimer<sub>S-S</sub> or monomer<sub>S-H</sub> (6,7). Thus Cygb appears to have a precise response to molecules such as lipids in an oxidized cell environment. Whether this distinctive mechanism of altering protein function *in vitro* has relevancy *in vivo* is currently unknown. However, the interaction between Cygb and fatty acids and lipids is similar to the finding that cytochrome c interacts with the lipid cardiolipin, a mechanism that relates to caspase-induced apoptotic response to mitochondrial damage (75,78). It is possible that the interaction between Cygb and lipids (or other similar molecules) has important biological and clinical roles. This, together with the observations that the cysteines affect the binding properties of the protein suggests a hypothesis that Cygb forms an essential signaling element between the cell's mechanism for sensing stress and its response through upregulation of anti-stress proteins (7,95). This hypothesis is summarized in Figure 5. Here the oxidation state of the two surface cysteines is key to the protein function. In an oxidizing environment, the formation of an intramolecular disulfide bond alters the biochemical properties of the protein through changing the protein heme pocket structure and hence changes to its redox activity. One consequence of

these changes in protein function, namely a high lipid peroxidase activity, would lead to production of lipid-based oxidation products. Like that of the formation of isoprostanes generated from Hb and Mb under conditions of oxidative stress, lipid oxidation would have consequences on cell signaling. This potentially links observations that hypoxia and oxidative stress upregulate Cygb production with an anti-oxidant response via activation of pathways such as the electrophilic responsive element. Electrophilic lipids such 4-hydroxynonenal, prostaglandins and isoprostanes are known to activate antioxidant responses such as through the Nrf2/ARE (antioxidant responsive element) pathway, inducing upregulation of antioxidant proteins or alternative stress responsive pathways (e.g. NF-κB) (58,61). This mechanism of linking stress conditions to antioxidant responses could in turn be linked with pathological roles of the protein, including the observed correlation between increased Cygb gene expression and a hypoxic tumor's biological aggression (101) or the cell protection mechanism of upregulated Cygb in hypoxic cancer cells protecting hypoxic tumors from chemotherapy and radiotherapy (101). The scheme depicted in Figure 5 is based primarily from *in vitro* data, and *in vivo* observations, any direct link between the two is currently unconfirmed and requires further research.

### **Dehaloperoxidase: The multifunctional hemoglobin.**

When considering the potential functions of mammalian proteins such as Ngb and Cygb information about potential physiological functions can be gained from examination of similar globins from other organisms such as bacteria and annelids. Phylogenetic studies on globins have provided insights into potential functions, particularly when the globin is localized in specific organs or is found in selected [tissue](#) (for recent reviews see (11,99)). However, most of these studies focuses on vertebrate globins and one class of globin has been largely overlooked until recently. The annelid protein dehaloperoxidase (DHP) shows remarkable diversity in its capacity to have multiple functional activities (33). Despite its name, the structure of DHP has much more in common with the hemoglobin superfamily than it has with the peroxidase superfamily, hence DHP is often referred to as dehaloperoxidase-hemoglobin. DHP has a biologically relevant peroxidase activity, despite having a typical globin fold structure, converting tri-halogenated phenols to quinone products using hydrogen peroxide:



X=I, Br, Cl or F.

DHP has the highest known peroxidase activity of any globin. Found in *Amphitrite ornate*, the peroxidase activity is believed to stem from evolutionary pressure for the organism to deal with toxic brominated and chlorinated compounds produced from the ecosystem in shallow coastal mudflats and marine estuaries (31-33,62). Two isoform of the protein; DHP A and DHP B differ by 5 amino acids (21,23), but have considerably different peroxidase activities. The peroxidase activity of DHP A is lower by one order of magnitude than that of horseradish peroxidase, but higher by one order of magnitude than sperm whale Mb (76). DHP B is the dominant isoform and has three times the peroxidase activity compared to DHP A (33). The peroxidase mechanism

involves classic peroxidase activity with the trihalogenated phenol oxidized by the radical generated with the hyper-valent ferryl iron. The trihalophenol can be oxidized by compound I, taking the radical from the porphyrin, or by compound ES, with the radical believed to be associated with a series of tyrosine residues (27,29). The second electron comes from the ferryl iron of compound II to form ferric DHP and quinone product. Additionally DHP has further activity revealed with peroxygenase activity, with oxygen atom transfer to substrate following ferryl formation, as well as oxidase activities, with dioxygen consumption in the absence of peroxide (5).

The oxygen affinities for DHP A and B are 4.2 and 2.0 torr respectively (33,109), hence DHP appears to have affinities for oxygen in the range for usefully delivery of oxygen to the organism. Indeed DHP A and B are the only hemoglobin genes identified in *A. ornata* (45). Sometimes referred to as the 'dehaloperoxidase paradox' the function of DHP as an O<sub>2</sub> carrier and a peroxidase depends on the oxidation state of the heme iron (34). A structural comparison of the heme pocket of DHP compared to erythrocyte Hb and Cygb (Figure 6) shows small differences, specifically in the flexibility of the distal histidine and the number of aromatic residues in the distal pocket. While DHP has features that appear to be unique to the family of globins, both the structure and properties of the protein supports the possibility, indeed a probability, of multifunctional roles of other globins, particularly those of Cygb and Ngb.

#### Summary and future directions

It is evident that the redox chemistry of hemoglobins, once considered an *in vitro* curiosity, have real physiological and pathological roles. DHP is a true multifunctional protein, binding oxygen but also performing peroxidase activities vital for the organisms' survival. Mb, although primarily an O<sub>2</sub> binding protein has a secondary function as an NO dioxygenase, protecting mitochondria from respiratory poisoning by NO. Some view Hb to have a role for carrying NO as SNO-Hb, but like Mb definitely shows redox chemistry when outside its normal antioxidant environment. These proteins have, or appear to serve, multifunctional roles. It is not a great leap in faith to propose that other heme proteins such as Ngb and Cygb may also have multi-functional properties, involving ligand binding and stress response mechanisms. However, the detailed mechanisms of these roles *in vivo* have yet to be fully explored, particularly in respect to linking the *in vitro* reactivities of the proteins to gene expression studies and biochemical studies of the properties of the proteins *in vitro*.

The recent discovery in 2012 of a fifth globin in humans, androglobin (Adgb), extends the interest in globin redox chemistry and function even further. With a 1667 amino acids sequence, Adgb comprises a calpain-like domain, a hemoglobin domain and an IQ calmodulin-binding motif (50). The discovery of a role of Adgb as an oncogene, promoting cell proliferation has important implications in cancer research (52), however, very little is known about this multi-domain protein. Phylogenetic studies have gained insights into the potential functions of some globins, particularly when appearing in specific organs and vertebrate species (11,99). Nevertheless many of the physiological functions of globins are yet to be identified, or the observed *in vitro* functions

verified *in vivo*. The current interest of the role of globins such as Ngb in neurodegenerative disease and Cygb cancer therapy resistance make deciphering these mechanisms of globins a critical objective for developing an understanding of these diseases and potentially discovering novel targets for therapy.

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#### Author Disclosure Statement

BJR has patents and patent pending pertaining to use of through-protein electron transfer pathways for use in hemoglobin-based blood substitutes.

#### Abbreviations used:

ARE = antioxidant responsive element

Cygb = cytoglobin

DAMP = damage-associated molecular pattern

Dimer<sub>S-S</sub> = dimeric protein with intermolecular disulfide bridges

DHP = dehaloperoxidase

γ-GCL = gamma-glutamylcysteine synthetase

GDI = guanine nucleotide dissociation inhibitor

Hb = hemoglobin

HBOCs = hemoglobin-based oxygen carriers

HIF-1α = hypoxia inducing factor 1-alpha

HO-1 = heme-oxygenase-1

Hp = hemopexin

ICAM-1 = intercellular adhesion molecule 1

Mb = myoglobin

Monomer<sub>S-S</sub> = monomeric protein with intramolecular disulfide bridge

Monomer<sub>S-H</sub> = monomeric protein with free sulfhydryl

Ngb = neuroglobin

NO = nitric oxide

NQO1 = NAD(P)H dehydrogenase

NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells

Nrf2 = nuclear factor erythroid-derived 2- related factor 2



SOD = superoxide dismutase

TPETP = through protein electron transfer pathways  
Vcam-1 = vascular cell adhesion protein 1

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