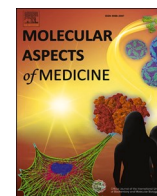




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The peroxidatic activities of Myoglobin and Hemoglobin, their pathological consequences and possible medical interventions

Michael T. Wilson, Brandon J. Reeder^{*}

School of Life Sciences, University of Essex, Wivenhoe Park, Colchester, Essex, CO4 3SQ, UK

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ABSTRACT

Under those pathological conditions in which Myoglobin and Hemoglobin escape their cellular environments and are thus separated from cellular reductive/protective systems, the inherent peroxidase activities of these proteins can be expressed. This activity leads to the formation of the highly oxidizing oxo-ferryl species. Evidence that this happens *in vivo* is provided by the formation of a covalent bond between the heme group and the protein and this acts as an unambiguous biomarker for the presence of the oxo ferryl form. The peroxidatic activity also leads to the oxidation of lipids, the products of which can be powerful vasoconstrictive agents (e.g. isoprostanes, neuroprostanes). Here we review the evidence that lipid oxidation occurs following rhabdomyolysis and sub-arachnoid hemorrhage and that the products formed from arachidonic acid chains of phospholipids lead, through vasoconstriction, to kidney failure and brain vasospasm. Intervention in these pathological conditions through administration of reducing agents to remove ferryl heme is discussed. Through-protein electron transfer pathways that facilitate ferryl reduction at low reductant concentration have been identified. We conclude with consideration of the therapeutic use of Hemoglobin Based Oxygen carriers and how the toxicity of these may be reduced by engineering such electron transfer pathways into hemoglobin.

1. Introduction

When I (MTW) arrived in Rome in February 1971 as a postdoctoral fellow I knew very little about myoglobin (Mb) or hemoglobin (Hb). I was working on the cytochromes so, although familiar with heme proteins, I was not well acquainted with the globin family. The laboratory (Istituto di Chimica Biologica) in Rome was a world leader in the study of Hb and Mb so I found myself surrounded by experts in the field, including many visitors from all around the world who flocked to work in Rome. How fortunate for me to have had the opportunity to learn so much in such a short time and from teachers as eminent as Eraldo Antonini and Maurizio Brunori. The time I spent in Rome was the happiest of my scientific career and it was when I really learnt how to do experimental research. In the summer of 1971, there was a buzz of excitement in the laboratory heralding the arrival of the first copy of “The Book” (Antonini and Brunori, 1971) I remember how impressed I was that such a wealth of knowledge could be compiled and the experimental aspects so well integrated with the theoretical.

Following my fellowship in Rome I returned to the UK and somewhat later developed an interest in the properties of Hb and Mb and “The

Book” has been ever present in my laboratory and until the present it is still frequently consulted. It is still considered as the Bible for Mb and Hb biochemistry.

This review is concerned with an aspect of the biochemistry of Mb and Hb that did not, however, feature in The Book because there the emphasis was on the physiological roles of Mb and Hb rather than the pathophysiology of these proteins. Here we consider the deleterious peroxidatic effects of Mb and Hb that become apparent when these proteins are released from their cellular environments i.e. the myocytes and the erythrocytes and enter the tissues of the organism. When enclosed in their cells the hemes are maintained in the ferrous state that is a requirement for them to act as oxygen storage or transport proteins, as oxygen has affinity for and bind only to this state to form oxy complexes. Furthermore, these cells contain a range of antioxidant defense mechanisms that protect them from oxidative damage. A problem arises however once the heme proteins escape their cellular compartments and enter spaces that lack reductive and defense systems. This problem stems from the oxidation of the heme oxy complexes by the bound oxygen (autoxidation) that can no longer be reversed by reduction within the cell. The product of autoxidation are ferric heme (Fe^{3+}) Hb or Mb and

^{*} Corresponding author.

E-mail addresses: wilsmt@essex.ac.uk (M.T. Wilson), reedb@essex.ac.uk (B.J. Reeder).

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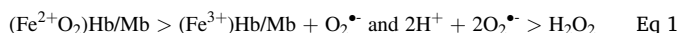
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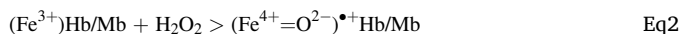
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the superoxide anion, that may dismute to generate hydrogen peroxide (Eq (1))



It is the further reaction between the ferric protein and H_2O_2 , generated either from the autoxidation reaction or from macrophage activity that initiates peroxidatic activity and tissue damage. Eq 2



The product of this reaction is an oxyferryl heme iron and a radical cation that quickly migrates to a residue on the protein forming $(\text{Fe}^{4+}=\text{O}^{2-})^{\bullet+}\text{R}$. This may then deprotonate to yield the species $(\text{Fe}^{4+}=\text{O}^{2-})^{\bullet}\text{R}$, the neutral radical which is EPR detectable. It is the consequences of the formation, within the tissues, of these highly oxidizing ferryl and ferryl-induced radical species that is the subject of this review.

2. Evidence that the oxyferryl radical cation is formed *in vivo*

Giulivi and Davies reported the presence of a ferryl heme species in human red blood cells exposed to a steady flux of H_2O_2 , showing that the anti-oxidant systems of the cell could be overwhelmed (Giulivi and Davies, 1994). That this can happen under normal conditions within humans was demonstrated by Svistunenko et al. (1997). In this study samples of venous blood from healthy adults were drawn, frozen and examined by EPR spectroscopy. The EPR spectra clearly showed the presence of a protein based radical at $\sim 1 \mu\text{M}$ concentration. This radical was identical in line-shape and power saturation behavior to that generated *in vitro* through the reaction of Fe(III)Hb (metHb) with H_2O_2 . Additionally, chromatographic and mass spectroscopic analysis of Hb showed that an oxidatively modified chlorin product of heme can be detected in healthy adults and that this can significantly increase during acute oxidative stress (Vollaard et al., 2005). These studies demonstrate that even within the protective environment of the red blood cells and the plasma sufficient peroxide and metHb are present to react and yield the characteristic EPR protein radical signal, oxidative damage to the heme and by implication the oxyferryl species.

The evidence for the occurrence of the oxyferryl species and radical in animal and human disease states, such as acute kidney dysfunction (previously called acute renal failure) following rhabdomyolysis, is very strong as there is a biomarker that unequivocally proves that these oxidants are formed under conditions in which the proteins escape their cellular environments. Rhabdomyolysis is a condition in which Mb is

released from myocytes either through mechanical injury (crush injury) or chemical/drug induced lysis of muscle cells (Briner et al., 1986; Bywaters and Beall, 1941). In humans crush injury often results from earthquakes, exposure to explosions, e.g. bombing campaigns, and it can also be a consequence of drug overuse, intensive exercise, etc (Atef-Zafarmand and Fadum, 2003; Hampel et al., 1983; Nepali et al., 2017; Sinert et al., 1994; Su et al., 2009). A distinctive feature of rhabdomyolysis is that patients who survive the initial mechanical trauma and begin to heal then succumb to kidney failure some days later. The urine of these patients is coloured by myoglobin, released from myocytes, and as a molecule with MWt of 17000 Da it passes through the kidney into the post glomerular space and then to the bladder. Examination of this Mb shows that in transit it has undergone a remarkable transformation. In its native state within the myocyte the heme and protein constituents of holo-myoglobin are not connected to each other through a covalent bond and may easily be separated at acid pH using organic solvents that dissolve the heme (Antonini and Brunori, 1971; De Duve, 1948). A significant fraction of the myoglobin recovered from the urine is however radically altered, namely the heme and protein are covalently linked and cannot be separated by HPLC (Fig. 1) (Holt et al., 1999; Reeder et al., 2002a, 2002b). How has this transformation come about? *In vitro* studies have shown that the ferryl species, formed from the reaction between Fe(III) Mb and H_2O_2 (Eq (2)), can react further. The protonated ferryl species $(\text{Fe}^{4+}=\text{OH}^+)^{\bullet+}\text{R}$ may be considered to have partly the character $(\text{Fe}^{3+}=\text{OH}^+)^{\bullet+}\text{R}$ i.e. a transient ferric-hydroxyl radical species. The highly reactive hydroxyl radical, OH^{\bullet} , abstracts a hydrogen atom from the porphyrin ring producing a radical on the porphyrin that terminates with the radical on the protein to form a covalent bond (Reeder et al., 2002b, 2007; Silaghi-Dumitrescu et al., 2007). Formation of this bond alters the conjugated system of the porphyrin converting it to a d type heme with a spectrum different from that of the original b type heme. In fact the CO complex of the covalently bound heme is bright green (Reeder, 2010).

The fact that covalent heme-to-protein bonds are formed as a consequence of rhabdomyolysis, and that the crosslinked product has the same spectral properties as those of the crosslinked protein formed *in vitro* substantiate the view that formation *in vivo* is through the reaction of Fe(III) Mb with H_2O_2 in the post glomerular space of the kidney, where there is no protective anti-oxidant system (Boutaud et al., 2010; Holt et al., 1999; Reeder et al., 2002a). Thus, the cross-link between the protein and the heme constitutes an unambiguous biomarker for peroxidatic activity of the holo-protein.

Given the above one may enquire whether there are other chemical transformations that result from this activity. Examination of the urine

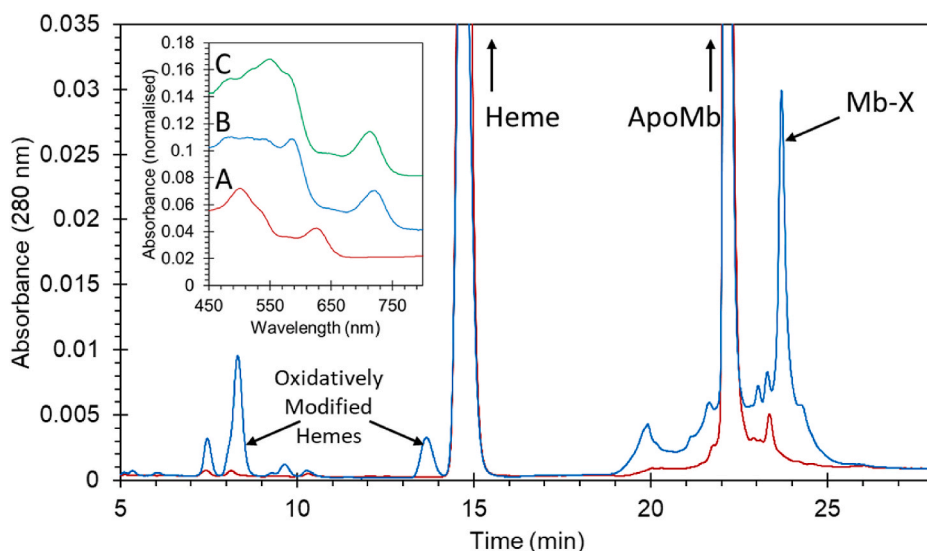


Fig. 1. Reverse-phase HPLC of myoglobin (red line) and oxidatively modified myoglobin (blue line). Myoglobin shows heme B (~ 14.7 min) and apoMb (22.1 min) only. Peroxide-damaged Mb shows additional oxidatively modified hemes (7–14 min) and a heme that is covalently attached to the globin (peak maximum 23.7 min). Inset: HPLC optical spectra of unmodified heme (A, red) oxidatively modified 'd-type' free heme (B, blue) and covalently bound cross-linked heme (C, green). Spectra are offset for clarity. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of human patients suffering from rhabdomyolysis and rat models of this condition revealed the presence of a range of lipid oxidation products (Boutaud et al., 2010; Gois et al., 2016; Holt et al., 1999; Moore et al., 1998; Shanu et al., 2013). Of particular interest was the occurrence of a class of oxidized lipids termed isoprostanes formed from the hydrocarbon chains of membrane phospholipids in the glomerular membrane. Indeed, these oxidized hydrocarbon chains were also found in the membrane still attached to the phosho head group proving that they were produced *in situ* in the kidney and had not been formed elsewhere and excreted through the kidney (Moore et al., 1998). The isoprostane formed from arachidonic chains, termed F_2 isoprostane, is a molecule with potent vasoconstrictive properties (Fukunaga et al., 1993; Morrow et al., 1992) and the discovery of this in the kidney prompted the proposal that the formation of this through the peroxidatic action of Fe(III) Mb is responsible for blood flow restriction and hence kidney failure (Moore et al., 1998). This hypothesis has been strengthened through further studies carried out *in vitro* in which the oxidation of lipids, catalyzed by Mb, was followed through conjugated diene formation or through singlet oxygen production (Flors et al., 2006; Reeder, 2010). These studies elucidated a complex mechanism initiated by H_2O_2 or lipid peroxide and thereafter propagates through a radical chain reaction (a simplified version of which is given in Fig. 2). This mechanism involves a catalytic redox cycle between Fe(III) and FeIV=O. Such a cascade of isoprostane formation, it may be appreciated, would severely restrict blood flow through the capillaries of the glomerulus and therefore have a very deleterious effect on kidney function. Furthermore, Reeder et al. have shown that X-linked Mb is considerably more pro-oxidant than native Mb potentially making the situation worse (Reeder and Wilson, 2005a). The peroxidatic activity of Mb is strongly enhanced at lower pH values, an effect we propose to arise from direct protonation of the ferryl species to generate the highly oxidizing $FeIV=OH^+$ ($FeIII(OH)^+$) species (see above) (Reeder et al., 2002b; Silaghi-Dumitrescu et al., 2007). Thus, once Mb has entered the post glomerular space a vicious cycle is established in which isoprostanes are

formed that restrict blood flow, this in turn leads to anaerobiosis of the tissue and the production of lactic acid that then lowers the pH which itself now enhances the catalytic activity of Mb. This is illustrated in Fig. 2.

It is relevant in this context to note that one of the first and most common medical interventions following rhabdomyolysis is infusion with bicarbonate solutions (Better and Stein, 1990; Zager, 1989). We suggest that this procedure elevates the post-glomerular pH and thus lowers the fraction of protonated oxoferryl and renders $FeIIIMb$ less harmful and protects the kidney (Moore et al., 1998; Silaghi-Dumitrescu et al., 2007).

A similar oxidative mechanism has also been associated with Hb. Examination of cerebral spinal fluid from patients who have suffered subarachnoid hemorrhage reveals that a fraction of the Hb, freed from the protective antioxidant system of the erythrocyte through lysis of these cells in the cerebral fluid, have heme covalently bound to the α and β chains (Reeder et al., 2002a). Again *in vitro* studies have demonstrated that this bond is formed through the reaction of the ferric heme with peroxides (Reeder and Wilson, 2005b). There is another strong parallel between what happens in the kidney and what happens in the cerebral spinal fluid, namely, in the CSF lipid oxidation yields potent vasoconstrictors, the neuroprostanes, analogous to the isoprostanes found in the kidney. It has been proposed that the neuroprostanes are responsible for the delayed vasospasm that follows hemorrhage and which is the secondary cause of fatality following hemorrhage (Corcoran et al., 2011; Hsieh et al., 2009).

3. Strategies to intervene in the peroxidatic activity of Mb and Hb

From the above account it may be appreciated that major pathological events can follow the escape of Mb or Hb from their cellular environments and migration into spaces where antioxidant defenses are insufficient to protect against peroxidatic activity. Central to this activity is the redox cycle in which the $FeIIIMb/Hb$, formed by auto oxidation of the oxy forms or from macrophage activity, reacts with H_2O_2 to form the ferryl species. This latter is returned to the ferric form by oxidizing biomolecules in the vicinity e.g. protein (forming the cross-linked biomarker) or lipid (forming the isoprostanes and neuroprostanes). This catalytic cycle may be short circuited by the addition of reductants that react with the ferryl species, generating the ferric form or potentially the physiologically active ferrous form, before it has time to oxidize lipids.

In order to intervene medically in this catalytic cycle, it is essential to find efficient and safe reductants. Reeder et al. have studied the mechanism of reduction of ferryl Mb and Hb using a wide range of reductants and have demonstrated that all reductants so far examined transfer electrons to the ferryl heme through two quite separate pathways (Reeder et al., 2008a, 2008b, 2008c). These are: 1) Through the heme edge that is partially exposed to the reductant approaching from the bulk solution and 2) through a surface tyrosine residue that acts as a quasi-redox cofactor, accepting an electron from the reductant and donating it to the ferryl heme. As there are two independent sites each reacting with the reductant, one might expect that the time courses would reflect this by exhibiting two exponential phases. In fact, this need not necessarily be so. If at all times throughout the reaction the binding reactions are at equilibrium, meaning that the association and dissociation rate constants for binding are much faster than the electron transfer rate constants, then all the ferryl species are reduced to the ferric state together and follow a single exponential time course. The single rate constant for reduction of the ferryl species, k_{obs} , does, however, carry the information that two processes are involved. The dependence of k_{obs} on reductant concentration follows the sum of two hyperbolae reflecting the two hyperbolic binding processes.

The evidence for these pathways is provided in Fig. 3A in which the time course for the reduction of sperm whale ferryl Mb is shown at a

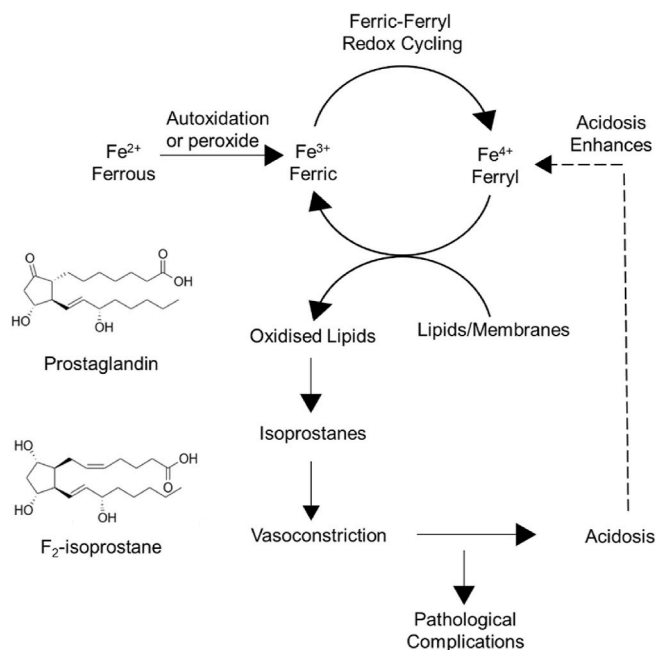


Fig. 2. Scheme showing the basic mechanism of ferric-ferryl redox cycling in lipid-based oxidative damage. Ferryl heme iron (and also protein-based radicals) can oxidize lipid membranes to initiate formation of a series of isoprostanes (15- F_2 Iso-P shown) with similar vasoactive properties to prostaglandins. Often resulting in acidosis due to the reduced supply of oxygen to the tissue, the lowered pH enhances and amplifies lipid oxidation in a vicious cycle.

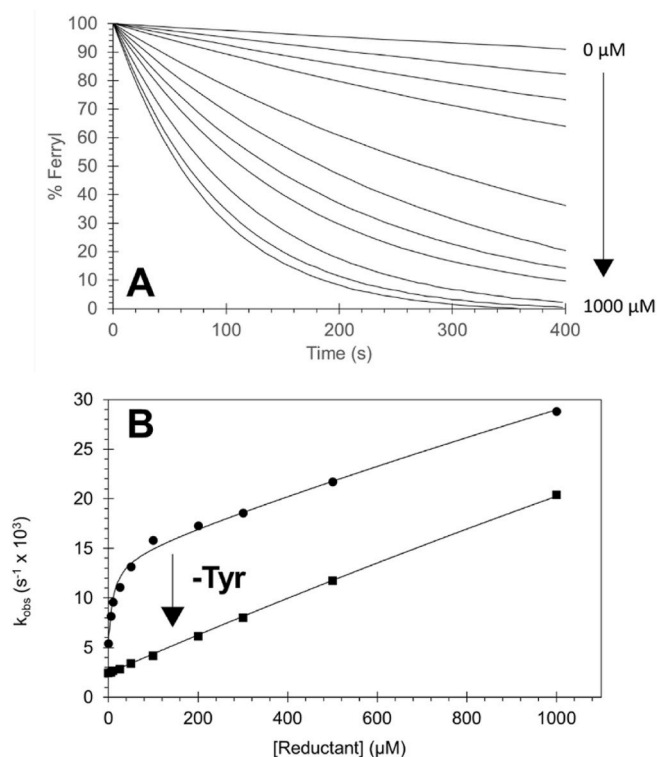


Fig. 3. (A) CP20 acts as a reductant for ferryl myoglobin (sperm whale), similar to urate and ascorbate, reducing the protein to the ferric oxidation state. (B) The kinetic profile of the reduction of ferryl myoglobin by CP20 (circles). The profile is fitted to a two hyperbola representing the two possible electron pathways. The through-protein pathway can be eliminated through mutation of the myoglobin to remove a redox active tyrosine between the heme iron and external environment (Tyr103 to Phe, squares).

number of reductant concentrations. Here the reductant CP20 (1,2-dimethyl-3-hydroxypyrid-4-one) known commercially as deferiprone, it is both a reductant and an iron chelator given orally to remove iron in cases of iron overload e.g. following blood transfusions for Thalassemia (Hoffbrand, 1995; Zanninelli et al., 1997). These time courses fit well to single exponentials for Mb (Reeder et al., 2008a, 2008c) and double exponentials for Hb (Reeder et al., 2008b). The rate constant for reduction given by these fits increase with increasing reductant concentration and this dependency is shown in Fig. 3B. These data can be fitted as the sum of two hyperbolic curves (Reeder et al., 2008b). A full mechanistic analysis reported by Reeder et al. (2008a) demonstrates that this behavior is expected if the reductant can bind at two independent sites on the protein and each site can donate an electron to the ferryl heme (Reeder et al., 2008a, 2008b; Silkstone et al., 2016a, 2016b). This analysis allows the assignment of K_D values (equilibrium dissociation constants for the binding reaction) to the sites and rate constant for the electron transfer rate within the reductant/protein complex.

Substituting Tyr103 for a Phe in sperm whale Mb abolishes the high affinity site leaving the lower affinity site still operational (see Fig. 3B), indicating that this residue (~5 Å from the heme) acts as part of a through-protein electron transfer pathway. The behavior shown in Fig. 3 is essentially the same for all reductants so far examined.

Furthermore, for a given protein the maximum electron transfer rate constant for the high affinity site is the same for all reductants confirming that once the site is fully occupied by the reductant the electron transfer rate constant depends on the nature of the protein and not the reductant. The affinity for this site for reductants does, however, depend on the nature of the reductant as may be expected, as binding involves specific interactions between the reductant and the protein. This behavior is illustrated in Table 1 where it is seen that for Equine Mb the

Table 1

Values for dissociation constant (K_D) and rate constant maxima for the high-affinity site for iron chelators and related compounds and reductants at pH 7.4. Values for K_{D1} and k_{max1} were calculated from the fitting kinetic data to a double rectangular hyperbola function. ND denotes not determined as the time courses of the high and low-affinity electron transfer pathway could not be separated. “—” denotes no high affinity pathway as the protein contains no tyrosine residues. CP02 (1-ethyl-3-hydroxypyrid-2-one), CP20 (1,2-dimethyl-3-hydroxypyrid-4-one), CP358 (1,6-dimethyl-2-(N-4',N-propylsuccinamido) methyl-3-hydroxypyridin-4-one).

Myoglobin	Compound	K_{D1} (μM)	k_{max1} (s^{-1})
Equine	CP02	9.7	0.96×10^{-2}
	CP20	110	1.17×10^{-2}
	CP358	330	1.19×10^{-2}
	Maltol	74	1.27×10^{-2}
	8-Hydroxyquinoline	47	1.15×10^{-2}
	1-Hydroxypyridine 2-one	ND	ND
	Hydroxyurea	340	1.25×10^{-2}
	p-Cresol	ND	ND
	Phenol	ND	ND
	Ascorbate	0.8	1.20×10^{-2}
	CP20 (V63H)	—	—
Aplysia	CP20 (V63H/F42Y)	26	4.47×10^{-1}
	CP20 (V63H/F98Y)	446	4.30

maximum rate constant (k_{max1}) remains $\sim 0.01s^{-1}$ irrespective of the chemical nature of the reductant used while the values of K_D vary widely.

Electron transfer through the second lower affinity site is also strongly affected by the size, shape and charge of the reductant, because the reductant must partially enter the heme crevice to access the heme edge prior to electron transfer (Reeder et al., 2008a). The two pathways are illustrated schematically in Fig. 4.

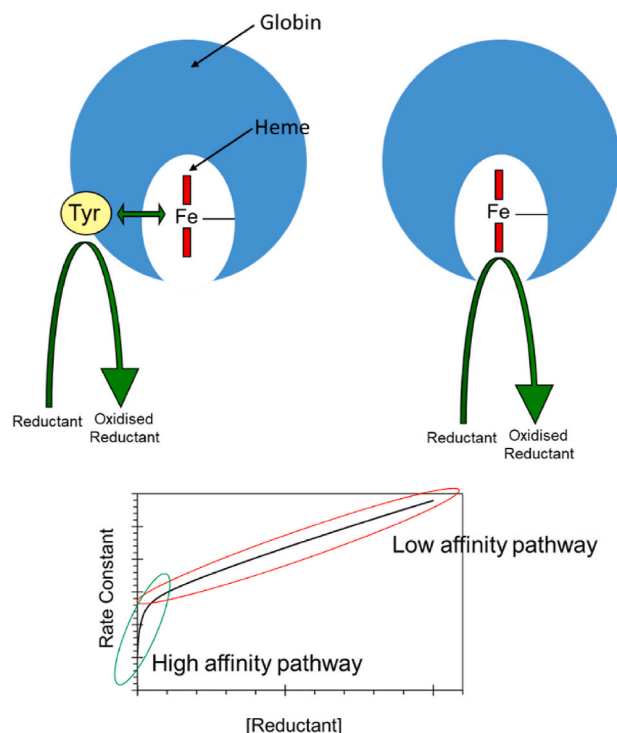


Fig. 4. Two site model for reduction of ferryl globin. Through-protein electron transfer via redox active tyrosine (top left panel) results in a high affinity pathway enhancing ferryl reduction at low concentrations of reductant (lower panel circled in green). Direct access to the heme pocket (top right panel) results in a lower affinity electron pathway (lower panel circled in red). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

To confirm the conclusions drawn above regarding the role of surface tyrosines in ferryl heme reduction the Mb from *Aplysia faciata* (sea hare) was studied (Reeder et al., 2012; Svistunenko et al., 2007). This protein has no Tyr residues but has 15 phenylalanines (Fig. 5). By substituting Tyr residues for specific Phe residues one is, therefore, able to insert a Tyr residues at selected positions and thereby construct a putative electron transfer pathway where one did not exist before. There are two Phe residues (Phe 41 and 98) close to the heme and when either of these is substituted by a Tyr the rate constant for ferryl heme reduction is greatly enhanced. For example, for the F98Y variant the increase is 1200 fold with ascorbate as reductant and 1600 fold with deferiprone as reductant (Reeder et al., 2012). In Table 1 it is seen that for *Aplysia* Mb where no Tyr residues are present no through protein electron transfer occurs but on insertion of Tyr residues the pathway is evident, and the position of the residue dictates the $k_{\max 1}$ and K_D values.

These results cannot be explained in terms of changes in the donor acceptor distance or redox potential but shows that the Tyr residue must act as a redox co-factor by virtue of the fact that the aromatic phenolic moiety can harbor a radical. The mechanistic details of how Tyr forms a radical by donating an electron to the ferryl heme have been discussed by Reeder et al. and these authors propose that the reason that this electron transfer appears to be so slow in comparison with expectation based on the Marcus theory is because electron transfer can only occur in a small fraction of the protein (10^{-7} of the total), that has both protonated oxyferryl and deprotonated tyrosine (Reeder et al., 2012). Rapid protonation equilibration ensures that all the protein flows through this species and thus all the oxoferryl form is reduced but at a rate 10^7 fold slower than if all the protein could participate in Tyr to heme electron transfer.

The donation of electrons from an exogenous reductant through the Tyr residue to the heme has also been shown to inhibit lipid peroxidation and formation of the heme to protein cross-link. These observations will be further discussed and illustrated below.

From the above several conclusions may be drawn that are useful when considering heme induced oxidative damage *in vivo* and how to develop therapeutic strategies to intervene and limit these processes. These are:

- 1) Mb and Hb, once outside their cells and in spaces unprotected by cellular defense systems, e.g. post glomerular space, CFS, synovial

fluid, are toxic. This stems from the formation of ferryl heme that can oxidize lipids to form molecules with powerful vasoconstrictive properties. A unique biomarker for this activity is the formation of heme to protein covalent bonds.

- 2) The ferric/ferryl cycle, fueled by peroxides may be prevented from oxidizing lipids by the addition of a wide range of reductant, e.g. ascorbate. Many of these are safe to take either orally or by infusion and therefore may offer therapeutic opportunities.
- 3) A wide range of molecules developed to act as iron chelators *in vivo* (e.g. hydroxypyridones, desferrioxamine, etc) also act as ferryl reductants. This is an important point to emphasize as it means that amelioration of the effects of frequent transfusions for patients with thalassemia can be through the mechanism of ferryl reduction of the heme and not solely due to iron chelation following heme degradation by heme oxygenase.
- 4) Reduction, at low reductant concentrations, is essentially through the pathway involving surface Tyr. This is important to recognize as it informs the design of Hb based blood substitutes (Hb based oxygen carriers, HBOCs), see below.

4. Therapeutic intervention in rhabdomyolysis

Acetaminophen, Ac, is a mild painkiller available without prescription in most countries. Depending on the country and the formulation this compound goes under any of over one hundred brand names e.g. Paracetamol, Panadol, Tylenol etc. It has been reported that Ac inhibits prostaglandin H_2 synthases by reducing the oxoferryl porphyrin radical in the enzyme that is essential for the oxygenation of arachidonic acid (Anderson, 2008; Aronoff et al., 2006). As mentioned above it is known that Mb similarly oxidizes arachidonic acid in the presence of H_2O_2 and Boutaud et al. reported that this reaction was also inhibited by Ac and that Ac can donate electrons to the ferryl species through both the through-protein, Tyr route, and the heme edge pathway. These *in vitro* results suggest that Ac may possibly act similarly *in vivo* and offer a simple, safe and cheap way to ameliorate the deleterious effects of rhabdomyolysis. This hypothesis has been tested by Boutaud et al. in a rat model of rhabdomyolysis (Boutaud et al., 2010). The results of this study in which control animals, rhabdomyolytic animals and rhabdomyolytic animals with Ac administered (400 mg/kg) were compared proved very encouraging. All measures of oxidative stress reactions,

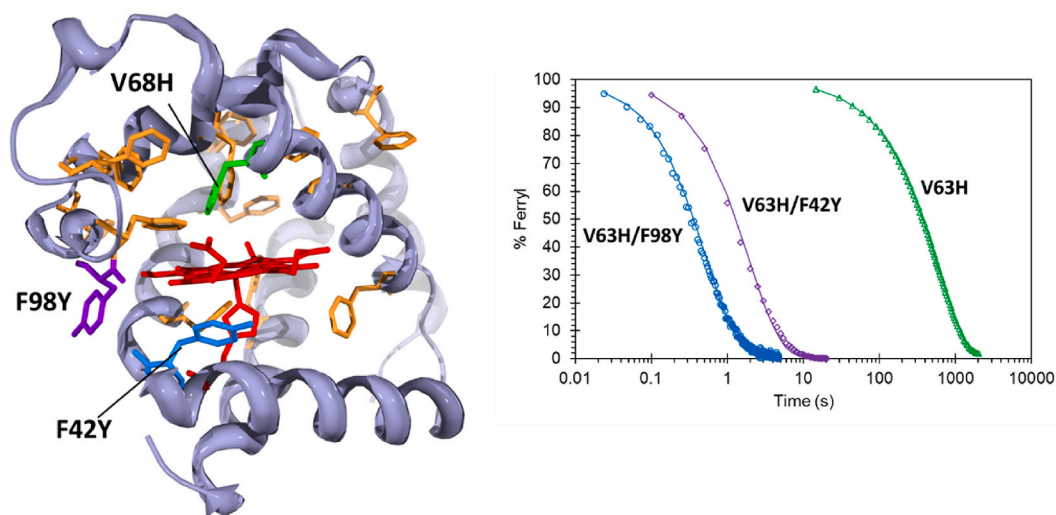


Fig. 5. *Aplysia* myoglobin exhibits extensive enhancement of the ferryl reduction kinetics by reductants upon selected tyrosine mutations. Left: Crystal structure of myoglobin from *Aplysia faciata* (*Aplysia limacina* as a junior synonym). PDB code 1MBA, this wild type protein was mutated (V63H, F42Y and F98Y) *in silico*. This protein has 15 phenylalanine residues close to the heme iron that can be selectively mutated to redox active tyrosine residues. Right: The V63H variant (green) was used to provide a distal histidine present in most human globins. V63H/F42Y (purple) and V63H/F98Y (blue) mutations exhibits significantly enhanced kinetics of ferryl reduction by low micromolar levels of reductants (50 μ M Ascorbate shown). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

much elevated in the rhabdomyolytic animals, were brought closer to values seen in the controls by administration of Ac. Treatment using Ac reduces both urinary and plasma F_2 isoprostanes, reduces creatine plasma levels and enhances creatinine clearance. In addition, the biomarker, cross-linked Mb, in the urine was greatly decreased. All these parameters indicate that Ac administration leads to less kidney damage and improved kidney function. Furthermore, visual inspection of kidneys, removed postmortem, revealed that while the rhabdomyolytic rat kidney was strongly discolored that from the treated rats had a relatively normal aspect.

These results indicate Ac or other compound that can target the oxyferryl species may offer a useful additional treatment for human patients.

5. Strategies for designing hemoglobin based oxygen carriers (HBOC's)

Blood transfusions are lifesaving interventions in many serious medical conditions including trauma, surgery, thalassemia etc. The blood for these transfusions is given, either freely or for a fee, by human donors. Inherent to this process are a number of problems that must be overcome to ensure the safety of the patient receiving the blood. The major one of these is that donated blood may be contaminated with viruses, e.g. HIV, hepatitis etc and new blood borne viruses are being found at the rate of at least two per year. For developed countries, the risk of such contamination can be essentially removed through screening but in poorer countries this is often not possible and indeed even testing the blood for viruses may not be routinely undertaken. Once the blood has been taken, erythrocytes must be stored in a refrigerator and have a life span of some 42 days (FDA regulation). However, biochemical and morphological changes to the stored erythrocytes suggest that older blood has adverse biological characteristics in critically ill patients, although studies thus far are far from conclusive (Bennett-Guerrero et al., 2007). In the United Kingdom, the maximum erythrocyte storage time is set at 35 days. These problems, together with the need for blood-typing and the expense of running an efficient, safe transfusion service, have driven the search for alternatives. The ideal would be an oxygen carrying therapeutic that is virus free, has no requirement for immunity typing, is easily stored and has a long shelf life. One possibility that has been explored is an Hb based -oxygen carrier (HBOC), using human or animal Hb (or its engineered variants). Classically such HBOCs are generated from chemical modifications of Hb purified from out-of-date human or animal blood. However, more recently, recombinant Hbs purified from transformed *E coli* are being developed. Such 'artificial' blood could solve the problems of compatibility, storage, viruses etc. Although having large quantities of virus-free HbA is a necessary requirement for producing a therapeutic oxygen carrier for human use, there is a number of other problems that must be solved before it could fulfil this role. In solution the tetramers of Hb can dissociate into $\alpha\beta$ dimers that can pass through the kidney and thereby lead to organ failure (see above). To prevent this, tetramers can be stabilized either through chemical crosslinking at the dimer-dimer interface or by mutation. Furthermore, the tetramers themselves may be either encapsulated or joined together and decorated with polyethylene glycol (PEG) chains, which ensures that the crosslinked Hb remains within the vasculature. Crosslinking and PEGylation also reduce the osmotic pressure of the preparation and reduce its immunogenicity. Despite these advances and the many years during which satisfactory HBOCs have been sought, clinical trials have not led to any product securing regulatory approval for general human use, although use in specific critical medical situations they have been used for individuals (Nellis et al., 2020). The reasons why HBOCs have not been approved and found wider use are complex and have been extensively discussed by Alayash (Alayash, 2014, 2019; Buehler and Alayash, 2008) and by Olson (Benitez Cardenas et al., 2019; Varnado et al., 2013) and will not be further discussed here, except to draw attention to two important

factors. The first of these is that HBOCs, unlike erythrocytes, may closely approach the epithelial surface lining the capillaries and remove NO, produced by the epithelium, either by binding it or by converting it to nitrate. As NO is a potent relaxant of the vascular muscle tone this results in elevated blood pressure. The second is related to oxidative damage as discussed above. The use of recombinant technology provides an opportunity to address both the issue of NO scavenging and of oxidative damage through selected mutation of the Hb. Recombinant Hb also allows mutations to enhance protein stability (Varnado et al., 2013) or the use of other Hbs such as the more inherently stable fetal Hb (HbF) (Chakane et al., 2017; Ratanasopa et al., 2016; Simons et al., 2018). Olson et al., through specific site mutations of the heme cavity, have successfully produced HBOCs with lower NO reactivities (Benitez Cardenas et al., 2019). The first attempt at a commercial recombinant HBOC, rHb2.0, developed by Somatogen (later Baxter Healthcare) specifically focused on combating the intrinsically rapid NO scavenging capability of Hb (Burhop, 2005; Raat et al., 2005). This entered clinical trials, but was discontinued due to adverse side reactions triggered by extracellular Hb (Varnado et al., 2013). Therefore, NO scavenging seems not to be the sole reason for adverse effects such as elevated blood pressure. The second important factor we suggest is the peroxidatic activity of the heme that, as in the kidney and CFS, generates vasoconstrictive oxidize lipids that act on the arteries and capillary bed. In addition, Alayash and coworkers have shown that the radical formed on HbA, as a consequence of the reaction between ferric Hb and H_2O_2 (Eq (2)), may preferentially migrate to a cysteine residue ($\beta 93$) and irreversibly oxidize this to cysteic acid. This oxidation process accumulate throughout blood storage and has major effects on Hb stability and is a major cause of aging of the red blood cell during storage (Alayash, 2021).

To address the intrinsic oxidative toxicity of HBOCs the group at Essex have engineered an HBOC that suppresses the oxidative activity of Hb in the presence of low concentrations of reductants. In WT human HbA the α chains possess a surface tyrosine (Tyr 42) that can act as a conduit for electron transfer to the heme while the β chains (or the γ chains of HbF) do not possess a suitably located Tyr but have a Phe (Phe 41) close to the heme. If this is substituted by a Tyr a new electron pathway is formed and ferryl heme in the β/γ chains is, as the α chains, reduced more rapidly and at lower reductant concentrations and is also less active as a lipid peroxidase under these conditions. These encouraging results have led to the β Phe41Tyr variant, or other Tyr variants, to be platforms from which to develop a new generation of HBOCs (Cooper et al., 2019; Silkstone et al., 2016a, 2016b, 2018), see Fig. 6. An additional effect of some Tyr variants is an enhancement of the ferric to ferrous reduction, more rapidly returning the protein from its inactive ferric form to the physiologically active oxygen carrying ferrous form (Silkstone et al., 2018).

Besides encapsulation and polymerization, which deserve their own extended review, PEGylation is one of the preferred strategies to keep the HBOC in the vasculature, preventing rapid renal clearance and hence decrease renal damage. PEGylation has been used in a variety of products in clinical trials (Abuchowski, 2017; Vandegriff and Winslow, 2009; Vincent et al., 2015). However, PEGylation using standard reactive agents such as maleimide-PEG typically generates highly heterogeneous mixtures that disrupt oxygen binding affinity and cooperatively. The use of recombinant HBOCs allows specific sites to be engineered that do not modify protein functionality and creates a much more homogeneous product (Cooper et al., 2020). Current research combining the strategies of using recombinant Hb to combine Hb stability, decreased NO scavenging, decreased oxidative damage and homogeneous PEGylation is ongoing at Essex.

The work over the past couple of decades has led to a much better understanding of globin toxicity. It is well established that release of Hb or Mb from the protective environment of the erythrocyte or myocyte leads to pathological consequences for a variety of reasons. An important one is the peroxidatic activity of these proteins. Above we have

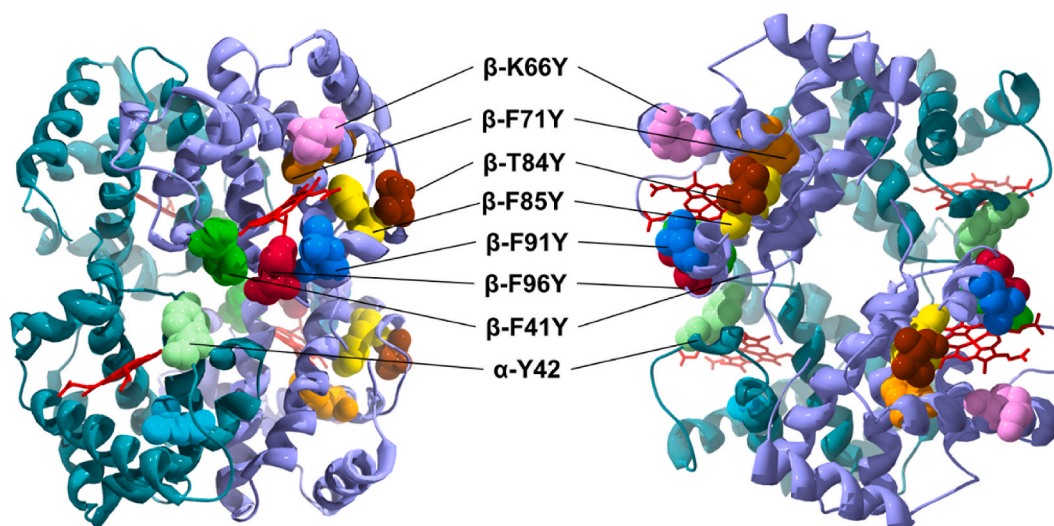


Fig. 6. Sites of mutations introduced on β subunit of hemoglobin to enhance ferryl reduction by reducing agents. Each site on the beta chain (blue ribbon) adds a redox active tyrosine to shuttle electrons from a reductant to the heme iron (β -F41Y, β -K66Y, β -F71Y, β -T84Y, β -F85Y, β -F91Y, β -F96Y). The alpha chain (green ribbon) already has this pathway (α -Y42). Hemes shown in stick form. PDB 1HHO was used to construct this image with the two images rotated by 90° . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

addressed some examples of these such as rhabdomyolysis associated acute kidney injury, delayed vasospasm following subarachnoid hemorrhage and hemoglobin-based blood substitutes. However, this list is far from complete and there are many other conditions in which Mb and Hb, lysed from cells, may be involved in pathological processes. Examples of these include hemolytic anaemias (such as sickle cell) and snake bite (e.g. Russell's viper), the latter causing severe lysis of erythrocytes and is a significant cause of death in Asia. One might seek interventions for these conditions through administration of suitable reductants to remove cytotoxic ferryl and associated radical species and research to this end is ongoing.

A short review, such as this, cannot hope to cover adequately all the medical consequences of Mb and Hb acting as peroxidases. However, this brief survey of some major pathologies may serve to illustrate the wide-ranging repercussions of freeing Mb and Hb from their cellular environments. The fundamental chemical reaction between heme and H_2O_2 , to generate ferryl heme and radicals, we suggest, has wide-ranging ramifications and is the underlying contributing cause of many severe pathological conditions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mam.2021.101045>.

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