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The Globin Superfamily: Functions in Nitric Oxide Formation and Decay

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Abstract

Globin proteins are ubiquitous in living organisms and carry out a variety of functions related to the ability of their prosthetic heme group to bind gaseous ligands such as O₂, NO and CO. Moreover, they catalyze important reactions with nitrogen oxide species, such as NO dioxygenation and nitrite reduction. The formation of NO from nitrite is a reaction catalyzed by globins that has received increasing attention due to its potential as an hypoxic NO signaling mechanism. In this review we revisit the current knowledge about the role of globins in NO formation and its physiological implications.

Keywords

Globin; Nitric Oxide; Nitrite; NO dioxygenation; Cytoglobin; Neuroglobin

Introduction

Nitric oxide (NO) is a critical signaling molecule in eukaryotic systems (Hill et al., 2010). NO formation *in vivo* is tightly regulated; mammalian systems rely mainly on NO synthase proteins (NOS) to produce NO. These are a group of enzymes that catalyze the synthesis of NO from L-arginine and molecular oxygen (O₂) (Daff, 2010). Nevertheless, increasing evidence indicates that other pathways, both protein-dependent and protein-independent, can also produce NO in biological systems. These routes often become important in hypoxic conditions where NOS proteins, due to their O₂ requirement, are rendered ineffective. The production of NO in these pathways largely depends on the reduction of nitrite to NO. The physiological relevance of these pathways has been reviewed elsewhere (Lundberg et al., 2008) and herein we review mechanisms for bioactivation. Focusing on the protein-related pathways, a variety of proteins have been shown to catalyze the reduction of nitrite to NO. These proteins are very different in their reaction mechanisms and electron sources, but in all cases they possess a metal-containing active site, such as molybdenum, iron or zinc. The molybdenum-containing enzymes xanthine oxidase or aldehyde oxidase catalyze the

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reduction of nitrite to NO at the molybdopterin site and can receive electrons from their flavin-containing domains, constituting a self-sufficient catalytic unit. Iron containing proteins that catalyze this reaction are in all cases heme-containing proteins (for example hemoglobin and myoglobin) and usually need a reducing system to maintain turnover, with the exception of flavohemoglobin that has a flavin reductase domain and is also a self-sufficient nitrite reductase. The reaction of the zinc-containing carbonic anhydrase with nitrite may be very different as the Zn does not undergo oxidation or reduction and could be linked to the dismutation of nitrite, but to date the mechanism remains unclear.

In this minireview we will focus on the role of the globins in NO formation and its physiological implications.

NO scavenging by globins

When considering the impact of globins in NO metabolism, an important part of the heme reactivity is related to processes that effectively destroy or dampen NO bioactivity:

The NO dioxygenation (reaction 1) is by far the most common reaction of NO with the heme group of globins in nature, and sometimes constitutes the main function of the protein, as in the case of flavohemoglobins (Gardner, 2005). This reaction is often very fast and close to the diffusion controlled limit, with rate constants up to $2.9 \times 10^9 \text{ M}^{-1}\text{V}^{-1}$ (Eich et al., 1996; Gardner, 2005). The reaction involves the oxyglobin ($\text{Fe}^{\text{II}}\text{O}_2$) form of the protein. It must be noted that globins often have very high oxygen affinities (red blood cell Hemoglobin $\text{P}_{50}\text{O}_2 = 26$ torr; Neuroglobin $\text{P}_{50}\text{O}_2 = 7.5$ torr; Myoglobin $\text{P}_{50}\text{O}_2 = 2-3$ torr; Cytoglobin $\text{P}_{50}\text{O}_2 = 0.7-1.8$; (Emara et al., 2010) and references therein). This implies that moderate to low O_2 levels will be enough to bind to most of the existing deoxyglobin (Fe^{II}) species forming the oxyglobin ($\text{Fe}^{\text{II}}\text{O}_2$) species. However, at very low oxygen concentrations the oxygen binding equilibrium will shift towards the deoxyglobin form (Fe^{II}), which usually binds NO with high affinity (reaction 2). This binding does not chemically modify NO, but due to the very slow NO release rate, can remove NO bioactivity in a biological system. The last NO scavenging reaction (reaction 3) is the binding of NO to the metglobin (Fe^{III}) form; this reaction is an equilibrium that generally favors NO dissociation from the $\text{Fe}^{\text{III}}\text{NO}$ complex, but particular conditions can lead to the formation of enough $\text{Fe}^{\text{III}}\text{NO}$ as to carry out reductive nitrosylation reactions, generating deoxyglobin and nitrite (reaction 4) (Hoshino et al., 1996; Tejero et al., 2012).

NO formation by globins

The formation of NO by globins is due to the reduction of the nitrite anion to NO according to the general expression (Doyle et al., 1981; Huang et al., 2005b):

This reaction with nitrite has been reported for a variety of globins and other heme systems. The equation describes the overall reaction for heme groups in most cases studied, even in non-globin systems (Ascenzi et al., 2013). The $\text{Fe}^{\text{III}}\text{NO}$ species is very probably formed as an intermediate that dissociates into Fe^{III} and NO at a rate faster than its formation rate (Sturms et al., 2011) and therefore the reaction is formally the reverse of the reductive nitrosylation (reaction 4). As shown in the equation, the reaction needs one proton to proceed and thus is favored by acidic conditions (Doyle et al., 1981; Huang et al., 2005b). When oxygen is present, the reaction is complicated by the presence of the oxyglobin species that will catalyze NO dioxygenation (reaction 1) and also react with nitrite (reaction 7):

Reaction 7 is a complex, multi-step process where intermediates as H_2O_2 and NO_2 are formed (Keszler et al., 2008). The cross-talk between these reactions is important from the physiological perspective and has been particularly investigated in the case of red blood cell hemoglobin (Grubina et al., 2007; Helms and Kim-Shapiro, 2013; Huang et al., 2005b). NO scavenging and NO formation reactions require an electron supply and depend critically on oxygen concentration (Figure 1). After NO dioxygenation or nitrite reduction reactions, the final heme species is the oxidized, Fe^{III} form. This species needs to be reduced to sustain the turnover (Smaghe et al., 2008). In addition, the oxygen binding affinity establishes a set point from where the globin will shift from the oxyglobin $\text{Fe}^{\text{II}}\text{O}_2$ and NO dioxygenation reactions to processes mediated by the deoxyglobin species. Accordingly, the intrinsic oxygen affinity and the presence of an electron source are important factors to assess the reactivity of a globin in a living system. We will now summarize the current knowledge about the reactions involving NO formation and decay for different globins.

Hemoglobin

Red blood cell hemoglobin (Hb) is a tetramer made up of four globin monomers, usually two Hb alpha subunits and two Hb beta subunits and a molecular weight of 64 kDa. Each monomer binds a heme prosthetic group through coordination with a histidine side chain (proximal histidine, F8 in standard globin fold nomenclature), whereas another histidine side chain (distal histidine, E7) stays on the other side of the heme plane without binding directly the heme iron, thus resulting in a five-coordinated heme iron with four nitrogen ligands from

the porphyrin and another one from the protein. Hb functions as an oxygen transport and delivery protein in most animals.

The functional role of Hb as oxygen carrier requires a suitable oxygen affinity and high stability of the reduced, oxygen-bound form (oxyglobin, $\text{Fe}^{\text{II}}\text{O}_2$). Consistent with its function, oxyHb levels in arterial and venous blood are usually between 95-100% and 70-75%, respectively. Thus Hb is expected to scavenge NO very efficiently according to the reaction 1 (Doyle and Hoekstra, 1981; Eich et al., 1996). However, in the arteriolar circulation and in many organs, such as the heart, kidney and exercising skeletal muscle, Hb oxygen saturation can reach much lower levels (Tsai et al., 2003). These circumstances require the consideration of other Hb forms apart from oxyHb when assessing Hb reactivity *in vivo*.

The great efficiency of reaction 1 has driven questions about how NO can cause relaxation of the vascular endothelium in the presence of vast amounts of circulating Hb. The theoretical and experimental approach resolving this paradox is beyond the scope of the present review, but involves important diffusional barriers around intact erythrocytes and along the endothelium in laminar flowing blood. For further discussion, the reader is referred to more specialized works on this topic (see (Helms and Kim-Shapiro, 2013) and references therein).

Given the efficiency of Hb as an NO scavenger, its role as a source of NO has been largely ignored. However, deoxyHb can also react with nitrite to form NO according to the reaction 5. The reaction shows a linear dependency with $[\text{H}^+]$ as expected from the equation (reaction 5) but the slope is ~ 0.88 , slightly different from the anticipated value of 1. This deviation could be due to pH-induced allosteric changes that in turn modify the reaction rates (Doyle et al., 1981; Huang et al., 2005b). In any case, the dependence appears to indicate that the reacting species is nitrous acid (HNO_2) and not NO_2^- . Nitrous acid has a pK around 3.15-3.5, and therefore the equilibrium is displaced towards the unprotonated form (NO_2^-) in the pH range studied (5.5-8.0). Conversely, the amount of HNO_2 in solution will vary proportionally to H^+ in this pH range, in agreement with the change in the observed rates (Doyle et al., 1981). The products of the reaction are Fe^{III} and $\text{Fe}^{\text{II}}\text{NO}$; although earlier studies have observed a ratio favoring Fe^{III} formation and thus proposed the formation of other species such as N_2O_4 , further studies have observed ratios closer to the expected 1 Fe^{III} : 1 $\text{Fe}^{\text{II}}\text{NO}$, consistent with the proposed reaction scheme (Huang et al., 2005a; Huang et al., 2005b). Taking into account the possible loss of NO due to traces of O_2 or other side reactions we consider that the reaction 5 largely describes the process for the deoxyHb and nitrite reaction.

Closer examination of the nitrite reduction by Hb performed under conditions of excess nitrite indicates that the single exponential fit is unable to properly fit the decay of the deoxyglobin species, which appears to correspond to a sigmoidal rather than exponential pattern (see figure 1 in (Huang et al., 2005b). Calculation of the instantaneous rate constant ($d[\text{deoxyHb}]/dt \times 1/([\text{deoxyHb}][\text{NO}_2^-])$) during the reaction indicates that the reaction rate constant increases as the amount of deoxyHb in the solution decreases (see figure 2 in (Huang et al., 2005b)). This observation seems to call for a more complex mechanism than

reaction 5; however, further examination shows that the reaction is similar to that described above considering that T and It state Hb have different nitrite reductase rate constants and the binding of NO causes an allosteric change from the initial T-state to the R-state along the reaction. Essentially the nitrite reaction with the T state Hb tetramer is approximately 50-times slower than the R state (Table 1). As the reaction progresses anaerobically, the products of the reaction $\text{Fe}^{\text{II}}\text{NO}$ and Fe^{III} accumulate and their R-state stabilizing effects catalyze a faster reaction. This process is called R-state autocatalysis and explains the sigmoid reaction character of nitrite and deoxyHb under strict anaerobic conditions. Studies with allosteric effectors such as NEM (inducing R-state) or IHP (inducing T-state) demonstrate that the sigmoidal kinetics disappear when Hb cannot transition between allosteric states (Huang et al., 2005b). The reaction of the separated side chains is faster than the reaction of HbA in the R-state, indicating that the tetramer assembly causes significant modulation of the rate constants (JT and MG, unpublished data (Table 1)).

The physiological implications of this reaction are interesting as *in vivo* the R and T distribution is determined by oxygen ligation. The fully deoxygenated Hb has more available sites for the reaction but a slower rate constant as it is in the T-state. Partially oxygenated Hb has less available sites but a faster (R-state) rate constant. Several works have used kinetic models to mathematically address the interplay of these reactions as a function of the oxygen concentration (Gladwin et al., 2006; Gladwin and Kim-Shapiro, 2008; Rong et al., 2013). In summary, calculations show that the maximum NO production appears to happen when the hemoglobin is oxygenated around 50%, with important consequences for hypoxic vasodilation. The reader is referred to other recent reviews for a deeper treatment of this and other features of the reactions involving Hb and NO (Helms and Kim-Shapiro, 2013).

Myoglobin

Myoglobin (Mb) is a globin very similar in sequence and structure to Hb, although it is found in a monomelic form. Human Mb is a 153 amino acid protein in its mature form, with a molecular weight of 17 kDa. It shows a greater oxygen affinity than Hb, with a $P_{50\text{O}_2}$ around 2-3 mmHg. Like Hb, it binds the heme group in a five-coordinated fashion and functions as an oxygen delivery protein in tissues.

Mb can catalyze NO dioxygenation at a very high rate ($k = 3.4 \times 10^7 \text{ M}^{-1}\text{S}^{-1}$), as observed for Hb (Eich et al., 1996; Gardner, 2005). The effect of mutations on this rate constant has been studied for several Mb mutations. The rate constants are sensitive to steric hindrance effects, and can be reduced by 4- to 10-fold by the insertion of bulky side chains in the positions BIO or Ell. Replacement of the distal histidine (E7) yields faster rate constants (2- to 8-fold) regardless of the polarity of the new residue (Eich et al., 1996).

Mb catalyzes the reduction of nitrite to NO with rate constants similar to those of the R-state Hb, with some differences between myoglobins from different species characterized by rate constants of $3\text{-}6 \text{ M}^{-1}\text{S}^{-1}$ at pH 7.4 and 25°C (Huang et al., 2005b; Shiva et al., 2007; Tiso et al., 2011) (Table 1). The reaction is pH dependent, and speeds up as pH decreases with a tenfold increase in the observed rate per unit of pH (Shiva et al., 2007). The reaction

products are Fe^{III} and $\text{Fe}^{\text{II}}\text{NO}$, as expected from reactions 2 and 5. The mutational analyses available are limited to the replacement of the distal histidine (E7) by leucine and alanine (Tiso et al., 2011). The resulting proteins show decreased nitrite reductase rate constants, suggesting that the distal histidine does not decrease the rate constant by steric hindrance, but rather contributes stabilizing interactions with the nitrite ligand.

Neuroglobin

Human neuroglobin (Ngb) is a small globin with 151 amino acids and a molecular weight of 16.9 kDa. The protein is a monomer (Dewilde et al., 2001). Ngb is expressed in several tissues, most notably in some nervous system cells and retina (Hankeln et al., 2004). Unlike Hb or Mb, in the reduced state the heme group is coordinated by two histidine side chains, from the residues E7 and F8 (His64 and His96 in human Ngb). This results in a six-coordinated heme iron. The function of the protein is unknown although several hypotheses have been proposed (Burmester and Hankeln, 2009). Due to the low stability of the oxy $\text{Fe}^{\text{II}}\text{O}_2$ form, which autoxidizes rapidly to Fe^{III} (Dewilde et al., 2001), its function is most probably not related to oxygen storage/transport with the possible exception of the retina (Hankeln et al., 2004; Schmidt et al., 2003). Ngb catalyzes the dioxygenation of NO at a fast rate. After mixing Ngb- $\text{Fe}^{\text{II}}\text{O}_2$ and NO there is rapid formation of an intermediate species ($k > 7 \times 10^7 \text{ M}^{-1}\text{V}^{-1}$), most probably a Fe^{III} -peroxynitrite complex, that decays to yield Fe^{III} and nitrate at a rate independent of NO concentration ($k \approx 300 \text{ s}^{-1}$ at 20°C) (Brunori et al., 2005). Given the fast decay of the $\text{Fe}^{\text{II}}\text{O}_2$ species and the absence -to date- of a functional reducing system, the relevance of this reaction *in vivo* is uncertain. The nitrite reductase activity of Ngb has been well studied (Jayaraman et al., 2011; Petersen et al., 2008; Tejero et al., 2011; Tiso et al., 2011). We have studied the reaction for wild-type human Ngb and several mutants, and found in all cases a 10-fold increase in the observed rate per pH unit (within experimental error) as expected from reaction 5 (Tejero et al., 2011; Tiso et al., 2011). As seen in the case of Hb and Mb, this dependency indicates that the reacting species is nitrous acid (HNO_2). Studies of mouse Ngb have reported much faster rate constants of nitrite reduction (Table 1), an excess of Fe^{III} formation and weak NO binding (Petersen et al., 2008). The causes of these large differences between human and mouse Ngbs are not yet known. Human Ngb has two cysteines (CD7 and D5, corresponding to positions 46 and 55 in human Ngb) that are located in the CD-loop and D helix (Pesce et al., 2003). In the available x-ray structures of Ngb both cysteines have been mutated to prevent protein aggregation (Pesce et al., 2003) but the location of these side chains inside or close to a flexible loop at reasonable distance suggest the possible formation of a disulfide bridge, which has been corroborated by mass spectrometry studies with the wild-type protein. Interestingly, the cysteine in position CD7 is replaced by glycine in mouse Ngb and therefore this disulfide bond cannot be formed (Hamdane et al., 2003). Our lab has investigated the effect of thiol reducing agents on the nitrite reduction rates, and we observed that oxidation of the disulfide bridge causes a 2-fold increase in nitrite reduction observed rates (Tiso et al., 2011). Another modification that has been shown to alter nitrite reductase activity is the phosphorylation of serine side chains. Ngb has at least 6 serine residues that have been identified as possible phosphorylation sites. We observed that Ngb phosphorylation regulates the binding of 14-3-3 proteins (Jayaraman et al., 2011). Upon

protein phosphorylation by ERK2 in vitro, a 3-fold increase in nitrite reductase rates was measured (Jayaraman et al., 2011).

We have also studied the effect of mutations of the distal histidine (His64 in human Ngb) on the nitrite reduction rates. Mutants His64Leu and His64Gln show much faster reaction rate constants (Table 1) (Tiso et al., 2011). There are two possible reasons, not mutually exclusive, for this behavior; firstly, the removal of the histidine abolishes the His binding/dissociation equilibrium and allows the heme to be accessible at all times. Secondly, the new side chains can provide stabilization of the transient heme-nitrite complex and/or steric hindrance for ligand binding. The different chemical characters of the Leu and Gln side chains, but similar rates, seem to indicate that the first part is the most important. However, other His64 mutations (Tejero et al., 2011) indicate that the factors at play may be more complex and the understanding of the regulation of this reaction needs further investigation.

Cytoglobin

Cytoglobin (Cgb) is a globin showing six-coordinate heme binding. The human protein is 190 aminoacids long with a molecular weight of 21.4 kDa. Compared to other globins, Cgb has extra N- and C-terminal fragments about 20 aminoacids long of unknown function, that may be involved in protein-protein interactions or membrane binding (Blank and Burmester, 2012). Cgb can form dimers in a disulfide-independent form (Hamdane et al., 2003) but apparently the monomeric state is predominant (Lechauve et al., 2010). Like Ngb, Cgb binds heme in a six-coordinated fashion. Cgb is expressed ubiquitously in mammalian tissues, probably due to its presence in fibroblasts (Burmester et al., 2002; Hankeln et al., 2004; Trent and Hargrove, 2002) and located in the cell cytoplasm (Schmidt et al., 2004). The physiological function of Cgb is yet unknown, but several reports indicate that Cgb can be involved in cellular NO metabolism (Gardner et al., 2010; Li et al., 2012; Liu et al., 2012). NO dioxygenation by the Cgb $\text{Fe}^{\text{II}}\text{O}_2$ species is similar to the reaction of Ngb. The formation of an intermediate species ($k \approx 10^8 \text{ M}^{-1} \text{ s}^{-1}$) and subsequent decay ($k \approx 370 \text{ s}^{-1}$ at 20 °C) is observed (Gabba et al., 2013). Studies by Gardner and colleagues showed that Cgb can be efficiently reduced by ascorbate, cytochrome *b*₅, CYPOR and NCB50R (Gardner et al., 2010). These reductants can be combined with Cgb to regenerate the reduced form of the protein catalyze the NO dioxygenation reaction in steady-state. Although the observed rates are not as fast as other specialized NO dioxygenases such as flavohemoglobin, they can be adequate to the regulation of the low NO levels produced in mammalian tissues, whereas bacterial flavohemoglobins require a higher pace to cope with large NO bursts from macrophages or other sources (Gardner et al., 2010). In studies using ascorbate and CYPOR as reductants, Cgb has been shown to consume NO at oxygen dependent rates as expected from reactions 1 and 6 (Liu et al., 2012). Using ascorbate as a reductant, Cgb is a much more efficient NO dioxygenase than Mb (Liu et al., 2013). This activity has been related as a possible role of Cgb regulating NO concentrations in smooth muscle cells (Liu et al., 2012; Liu et al., 2013). Cgb can reduce nitrite to NO with rate constants similar to these of human Ngb (table 1) (Li et al., 2012). The reaction shows a rate increase as the pH is varied from 8.0 to 5.5 as expected from the scheme (reaction 5) but the magnitude of the increase does not follow exactly the expected relationship: observed rates increase by only ~ 2 between pH 8.0 and 7.0 and by ~ 15 between pH 7.0 and pH 5.5 (Li et al., 2012). The authors indicate

that part of the shift can be also due to the change of the distal histidine binding and dissociation at different pH (Li et al., 2012). The protonation of the distal histidine probably occurs within the pH range studied and this should have some impact on the rate of the reaction. However, similar effects would be expected in Ngb and plant hemoglobins which are not observed. It is possible that Ngb and Cgb show different patterns in the changes of K_H vs pH; this is an issue that may impact the physiological function of Ngb/Cgb and deserves further examination.

Other globins

The rates of NO formation or dioxygenation have been studied for a number of globins. Flavohemoglobins are globin proteins with a two-domain architecture, which includes an N-terminal, globin domain that binds heme in a five-coordinated manner and a C-terminal, ferredoxin reductase-like domain that binds FAD as a prosthetic group that can accept electrons from NAD(P)H. The reductase domain catalyzes electron transfer from NAD(P)H to the heme domain to keep the heme in a reduced state. The oxygen affinity of flavohemoglobins is very low ($K_{D}O_2 = 4\text{--}36$ nM (Gardner, 2005)) making $Fe^{II}O_2$ the favored state. The reaction of *E. coli* flavohemoglobin with nitrite has been studied at 1 mM nitrite (Gardner and Gardner, 2002). Although the exact relationship between nitrite concentration and the reaction rate is unknown, assuming the linear relationship observed in the other globins studied, we can estimate a rate constant of $40\text{ M}^{-1}\text{S}^{-1}$ (Table 1). Nitrite reduction rates have been determined for several non-symbiotic hemoglobins from plant and algae (Sturms et al., 2011; Tiso et al., 2012). These hemoglobins form a separated clade from the symbiotic plant hemoglobins (leghemoglobins), and their function is unknown but probably related to nitrogen metabolism (Sturms et al., 2011). Non-symbiotic hemoglobins are six-coordinate globins, but the affinity of the distal histidine for the heme iron is variable and in some cases an equilibrium between five and six-coordinate species is observed in the resting deoxy species (Bruno et al., 2007). Plant hemoglobins show higher rates of nitrite reduction compared to their mammalian counterparts (Table 1). In the case of *Arabidopsis*, the observed rates are also $[H^+]$ dependent with a linear correlation between rates and proton concentration and a slope of 1 within experimental error, in a trend similar to Hb, Mb and Ngb (Tiso et al., 2012).

Molecular basis of nitrite reductase rates

As can be expected given the variability in sequences and the slight changes in structure, each globin shows an intrinsic rate constant of nitrite reduction (Table 1). The observed rates for wild-type proteins range from $\sim 0.1\text{ M}^{-1}\text{ s}^{-1}$ in Ngb to over $80\text{ M}^{-1}\text{ s}^{-1}$ in plant hemoglobins. The molecular basis for this variability is not completely understood yet. In the case of five-coordinate globins, a reasonable correlation between redox potential and rate constant has been observed (Huang et al., 2005b). For six-coordinated globins, the relationship appears to be more complex with an important role of ligand access to the heme pocket. Our studies suggest that the more negative redox potential of six-coordinated globins does confer a faster rate constant, but this is limited by the active site accessibility through the intrinsic distal histidine affinity for the heme iron, as this residue blocks nitrite binding.

The relative impact of these and other factors are still under investigation. There a number of globins for which the nitrite reduction rates have not been assessed such as

Globin E and Globin Y, and the rate constant for flavohemoglobin remains to be determined at varying nitrite concentrations. Even when this activity may have little physiological significance in some of these proteins, the intrinsic rate constants can be of interest to assess the factors regulating nitrite reductase activity in different globins.

Biological significance of NO formation by globins

In recent years, an important body of work has demonstrated the relevance of globins in NO formation through nitrite reduction. Thus, our view of globins has changed from being just a sink of NO bioactivity to a more profound, dual role as NO providers in low oxygen conditions and NO scavengers in normoxic conditions (Figure 1). This scenario clearly applies to Hb and Mb. These two proteins seem particularly suited for the role, as they both have notable advantages: first, the concentration of the proteins in cells is very high, in the mM range, which compensate for the slow rates of nitrite reduction, and second, they have a dedicated and effective reduction system that can regenerate the reduced form. There is currently compelling evidence of the role of both Hb and Mb as nitrite reductases *in vivo* (Lundberg et al., 2008). Early studies showing the vasodilatory effects of nitrite indicated the involvement of Hb in the reaction (Cosby et al., 2003). As Mb is more reactive than Hb towards nitrite (Huang et al., 2005b; Shiva et al., 2007), it became apparent that Mb could fulfill the same role. The involvement of nitrite reduction on hypoxic NO signaling through Mb was shown by studies on isolated mitochondria (Shiva et al., 2007) and nicely demonstrated in studies with Mb-knockout mice (Rassaf et al., 2007). Recent studies have shown the activation of sGC in platelets by red blood cell derived NO (Velmurugan et al., 2013), further demonstrating the ability of Hb to catalyze the reaction.

In the case of Ngb, Cgb and other globins, the possible physiological relevance is more complex as they are usually found in low concentration in cells and they may lack appropriate reduction systems. However, this perspective is also evolving: it has been shown recently that Cgb can be reduced by ascorbate, cytochrome *b*₅ and CYPOR (Gardner et al., 2010; Liu et al., 2012; Liu et al., 2013). This can lead to efficient NO dioxygenation but also provides a route to make NO in hypoxic conditions. Furthermore, the influence of external factors on nitrite reductase activities is poorly understood. We have identified two physiological processes that can alter nitrite reductase rates: i) the redox state of the cell can modify the nitrite reduction rate of Ngb through the redox sensitive disulfide bond between cysteines CD5 and D7 (Tiso et al., 2011) and ii) Ngb phosphorylation can also modify the nitrite reduction rates (Jayaraman et al., 2011). As we learn more about the reactivity of globins towards nitrogen species, more new details about their complex role in NO formation and detoxification emerge.

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Abbreviations

Hb	red blood cell hemoglobin
Mb	myoglobin
Cgb	cytochrome b ₅
Ngb	neuroglobin
NO	nitric oxide
CYPOR	cytochrome P450 oxidoreductase
NCB50R	novel cytochrome b ₅ oxidoreductase (cytochrome b ₅ reductase 4)
Cb₅	cytochrome b ₅
NEM	N-ethyl maleimide
IHP	inositol hexaphosphate
P₅₀O₂	partial pressure of oxygen for 50% heme binding

Figure 1.

NO generation and NO denitrosylation pathways are regulated by environmental oxygen and globin oxygen binding affinity. The ferric heme can be reduced back to the initial ferrous form by other proteins or reducing agents, allowing for catalytic NO production or scavenging. In six coordinate globins, availability of the ferrous heme form is determined by the binding of the distal histidine side-chain.

Table 1

Rate constants for the reaction of nitrite with deoxy globins.

	K (M ⁻¹ s ⁻¹)	Reference
<i>Human red blood cell Hemoglobin</i>		
T-state ^a	0.12	(Huang et al., 2005b)
R-state ^a	6	(Huang et al., 2005b)
Isolated α -chains ^a	9.2	(Tejero et al., 2011)
Isolated β -chains ^a	10.3	(Tejero et al., 2011)
<i>Myoglobin</i>		
Horse heart ^b	2.9	(Tiso et al., 2011)
Sperm whale ^b	5.6	(Tiso et al., 2011)
Sperm whale H64A ^b	1.8	(Tiso et al., 2011)
Sperm whale H64L ^b	<0.2	(Tiso et al., 2011)
<i>Neuroglobin</i>		
Mouse ^c	5.1	(Petersen et al., 2008)
Human S-S ^b	0.12	(Tiso et al., 2011)
Human SH ^b	0.062	(Tiso et al., 2011)
Human H64L ^b	259	(Tiso et al., 2011)
Human H64Q ^b	267	(Tiso et al., 2011)
<i>Cytoglobin</i>		
Human ^d	0.14	(Li et al., 2012)
<i>Plant & bacterial Hemoglobins Flavohemoglobin^g</i>		
Rice nsHb1 ^e	83	(Sturms et al., 2011)
<i>Synechocystis</i> ^e	68	(Sturms et al., 2011)
<i>A. thaliana</i> Hb1 ^f	19.8	(Tiso et al., 2012)
<i>A. thaliana</i> Hb2 ^f	4.9	(Tiso et al., 2012)

^a 100 mM sodium phosphate, pH 7.4, 37°C;^b 100 mM sodium phosphate, pH 7.4, 25°C;^c 50 mM sodium phosphate, 0.5mM EDTA, pH 7.4, 25°C;^d 100 mM phosphate, pH 7.0, 25°C;^e 100 mM potassium phosphate, pH 7.0, unknown temperature;^f 100 mM sodium phosphate, pH 7.5, 25°C;^g 100 mM sodium phosphate, pH 7.0, 37°C; estimated from reactions with 1 mM nitrite.