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Review

Neuroglobin, seven years after

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Abstract. Neuroglobin is expressed in vertebrates brain and belongs to a branch of the globin family that diverged early in evolution. Sequence conservation suggests a relevant role in the nervous system, with tight structural restraints. Experiments *in vivo* and *in vitro* showed increased hypoxic stress damage upon repressing neuroglobin biosynthesis and improved recovery following overexpression. Neuroglobin shows internal heme hexacoordination, which controls oxygen affinity and kinetics. Neuroglobin concentration, oxygen affinity and enhanced autooxidation question a role in oxygen delivery; thus it was proposed that the neuroprotective effect might be due to radical scavenging or activation of protection mechanisms. Neuroglobin's structure shows a peculiar internal cavity of very large size. Binding of heme ligands is associated to a conformational change involving the heme that "slides" into the pre-existing cavity and makes the sixth coordination position available. These features may pave the way to an understanding of neuroprotection by neuroglobin.

Keywords. Globins, ligand binding, crystallography, evolution, neuroprotection, radical scavenging.

Introduction

Hemoglobin (Hb) and myoglobin (Mb) make blood and muscles red, carry O_2 and are among the most extensively characterized proteins, yet they are evolutionarily young in the globin family. In vertebrates there are at least two older and more discreet members of the globin family, neuroglobin (Ngb) and cytoglobin (Cygb), that are expressed in much smaller amounts and were discovered at the beginning of the new millennium [1, 2]. The precise function and mechanism of action of Ngb and Cygb are still elusive [3] but since their discovery, they have been enthusiastically investigated using the wide array of methodologies that have been tuned by decades of biochemical studies on their more outgoing relatives, *i.e.*, Hb and Mb.

In the case of Ngb, its localization in the neurons [1] elicited particular interest, and several hypotheses were advanced for its patho-physiological role in the brain [3]. Among others, very interesting experiments *in vitro* and *in vivo* published by Greenberg and colleagues [4, 5] showed that Ngb has a role in neuroprotection; the recovery from stroke in experimental animals was ameliorated by overexpression of Ngb, a very exciting discovery.

It seems unlikely (although not excluded) that Ngb is an O_2 reservoir or transporter, because of its fairly low average concentration in the neurons (~1 μM). Some of the alternative functions that were proposed group into the following categories: (i) signaling of hypoxia; (ii) radical scavenging (constitutive or under hypoxic

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stress); (iii) NADH oxidase to sustain anaerobic glycolysis [3].

Evolutionary considerations on the environment experienced in the early stages of life on earth have suggested that O_2 binding and delivery was a function acquired by globins when aerobic metabolism became predominant. Ngb might have retained some enzymatic function endowed by the ancestral globin; an alternative may be the participation in a novel signaling function to trigger protective responses to hypoxia.

Since the discovery of Ngb by Burmester and colleagues [1], its three-dimensional structure has been determined in different oxidation states of the heme iron, its kinetics and reactivity towards O_2, CO and NO analyzed, extensive spectroscopic investigations carried out, and both *in vivo* and *in vitro* studies performed to shed light on its mechanism of action in the nervous tissue. Examination of the full body of experimental data available could contribute to single out the most likely hypothesis on Ngb's function as well as to exclude some less likely possibilities. This paper highlights some aspects of the biochemistry of Ngb without pretending to be exhaustive; we apologize with our colleagues for the papers that we may have inadvertently missed quoting.

Gene evolution and structure

The ancestor of globins is thought to be present in organisms as far back as the Last Universal Common Ancestor (LUCA) [6] since members of the globin gene family are spread among virtually all kingdoms of organisms [7–9]. The function of the ancestral globin might have been different from O_2 uptake and delivery if it arose before oxygenic photosynthesis, as suggested by the discovery of ”protoglobins” in two *Archea* [9]. Enzymatic functions are observed in several non-vertebrate systems where globins can carry out reactions towards O_2, NO and H_2S [10–13]. A likely scenario envisages the recruitment of an ancestral globin (with an original redox function) for O_2 binding and scavenging, which finally evolved in vertebrates into the canonical dioxygen carriers.

Delivery and facilitated diffusion of O_2 is the commonly acknowledged function of the most abundant globins in vertebrates, Hb and Mb. Nevertheless, enzymatic properties of these and other globins have been reported and generally accepted [14–18], other members of the globin family have also been identified in vertebrates, although their functions await being unambiguously established. The presence in vertebrates of these additional tissue globins remained elusive, given their low expression levels in tissues, until they were discovered by genomic database analysis that searched for globin-like sequences [1, 2, 19]. The discovery of Ngb by Burmester et al. [1] came as a surprise and triggered the interest of several groups, given its prevalent expression in the brain.

The family of globins in vertebrates, so far, is formed by Hb, Mb, Ngb, Cygb, globin E (GbE, eye-specific in chicken) [20] and globin X (GbX) recently discovered in fish and amphibians [21]. Phylogenetic analysis of vertebrate globins indicates that they can be associated with two distinct branches of metazoa. Ngb and GbX belong to a branch that diverged before the split of Protostomia and Deuterostomia (700 million years ago) (Fig. 1) and include Annelid (Polychaeta, *Glycera dibranchiata*) nerve Mb and Chordata (Tunicata, *Ciona Intestinalis*) Hbs [21].

The position and number of introns within globin genes led to considerable debates [7, 22, 23] dealing with their antiquity and their possible “sliding”. Two introns, positioned at B12.2 and G7.0 are present in most vertebrate and invertebrate globin genes and are considered ancestral; a third intron is present in different positions within helix E in symbiotic and nonsymbiotic plant Hbs as well as in nematodes and arthropods [22, 24]. Moreover, the presence of an intron within helix E has been reported also in globin genes from algae, protozoa and crustacea [21].

Analysis of the gene structure showed, for the first time in vertebrate globins, an intron in helix E of Ngb and GbX; the presence of this intron, absent in all other vertebrate globins, reinforces the assignment of Ngb and GbX to a gene family separate from that including Mb, Hb and Cygb [21]. In Ngb, the position of the intron within helix E is shifted 1 bp (E10.2) with respect to that found in GbX and in *Ciona intestinalis* Hbs (E11.0); given the phylogenetic relationship between these proteins, this could indicate a rare case of intron sliding rather than its independent occurrence within this gene group [25]. Mammalian Cygb contains a third intron at the 3’ end, before ten additional amino acids that do not pertain to the canonical globin fold; GbX also contains an unprecedented intron in helix H (H10.0). Both are considered recent and seem to be limited to these two groups of globins.

Reliable sequences of Ngb from 11 mammals, 1 bird and 4 teleost fish species have been determined [26]; in all species apart from the trout, Ngb seems to be present as a single-copy gene. Structural analysis showed that Ngb sequences are consistent with the globin fold template, given the conservation of amino acids involved in heme binding and ligand interactions, *i.e.*, PheB10, PheCD1, TyrCD3, ValE11, LeuF4, ValFg1, ValFG3 and PheG5 [27, 28]. Comparison of Ngb with Hb and Mb shows less than 25% of

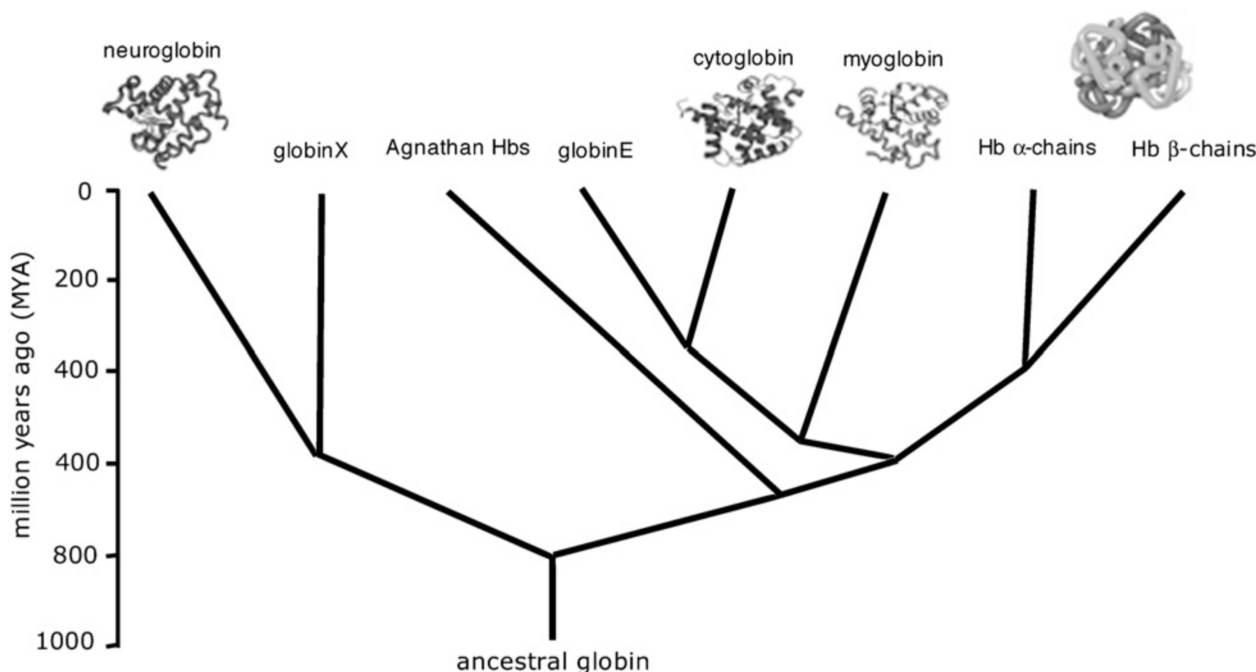


Figure 1. A simplified phylogenetic tree of vertebrate globins.

conserved residues; on the other hand ~0.4×10^{-9} amino acid changes/year are observed within mammalian sequences of Ngb, a frequency which corresponds to one third of that observed for Hb [21]. In fact 46.4% of all residues (corresponding to 70 positions) are conserved within Ngbs from vertebrates, suggesting that tight functional requirements induce a strong purifying selection. Insertions are observed at the C and N termini of Ngb sequences and, limited to one amino acid, at the flexible CD corner. In Ngbs from different organisms, up to three cysteines are observed but only one, in topological position D5, is conserved in all sequences.

Finally, the fact that Ngb seems to be allowed little evolutionary divergence, and the very similar ligand binding parameters observed in man, mouse and zebrafish Ngb, suggest that its function has tight requirements in terms of protein sequence and structure.

Functional parameters

The canonical role of globins in vertebrates is delivering O_2 for aerobic metabolism, from Hb to Mb and finally to cytochrome *c* oxidase. The transfer is achieved by proper modulation of the affinity for this ligand. The discovery that Ngb binds O_2 reversibly led initially to the assumption that this novel protein was also involved primarily in O_2 transport in the neurons.

Determination of the O_2 affinity of Ngb is particularly difficult due to its tendency to autooxidize with a half time, in air, that varies from ~20 to ~3 min depending on pH [29–31]. Nevertheless, the O_2 half saturation pressure (P_{50}) was estimated from kinetics [29, 30] and also by direct measurements [1] to be somewhat similar to that of Mb (about 2.0 torr for Ngb and 1.0 torr for Mb at 20°C). O_2 equilibria of human Ngb, as a function of temperature and pH under conditions that would minimize autooxidation, indicate that at 37°C and physiological pH, $P_{50} = 7.5$ torr, *i.e.*, almost ~5 times greater than that observed by previous measurements [31]. The low average concentration of Ngb in the brain (~1 μM), its tendency to autooxidize and its relatively low O_2 affinity under physiological conditions seem to suggest a primary role other than O_2 storage and/or facilitated diffusion.

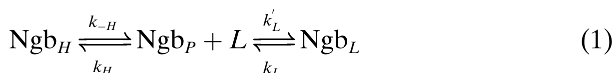
Several investigations on the kinetics of reduced Ngb with O_2, NO and CO have been carried out to derive information useful to single out its physiological role. Contrary to Mb, the reaction of Ngb is complex because this vertebrate globin is characterized by endogenous heme iron hexacoordination in the absence of external ligands. The heme iron in both the ferrous and the ferric forms is directly bound not only to the proximal His(F8) (as canonical in all globins), but also to the distal His(E7). This peculiarity was first inferred from spectroscopy [1, 2, 30, 32] and later proven, at least for the MetNgb state, by the three-dimensional structure of human and murine Ngb [27, 28]. This internal coordination on the distal side

Table 1. Rate constants for the reaction with gases of reduced neuroglobin (Ngb), sperm whale myoglobin (swMb) and human hemoglobin (Hb) chains. The on-rate constants for Ngb (k') are relative to the pentacoordinate derivative, *i.e.*, the species which is found (scarcely) in the unbound state after spontaneous rupture of the bond between the heme iron and the distal (E7) histidine. To directly probe the reactivity of this species, the ligand rebinding after flash photolysis, before hexacoordination is reestablished, is measured. Conditions: neutral pH and 20°C.

Ligand	k′ ($\mu M^{-1} s^{-1}$)	k (s^{-1})	Species	Ref
O_2	140	0.8	Human Ngb	[33]
CO	40	N.D.	Human Ngb	[33]
NO	150	2×10^{-4}	Mouse Ngb	[36]
O_2	19	10	swMb	[53]
CO	0.51	0.019	swMb	[54]
NO	170	$1x10^{-4}$	swMb	[55]
O_2	28, 110	12, 22	Hb A (α,β)	[54]
CO	2.9, 7.1	0.0046, 0.0072	Hb A (α,β)	[54]
NO	260	1×10^{-3}, 1×10^{-5}	Hb A (α,β)	[55–57]

implies that only upon rupture of the bond with His(E7) can an externally added ligand (e.g. O_2) bind; and therefore competition with the internal ligand is an additional component involved in determining the overall affinity for the gaseous ligands.

A minimum two-step mechanism has been proposed to describe such a reaction:



$$\mathrm{Ngb}_H \underset{k_H}{\overset{k_{-H}}{\rightleftharpoons}} \mathrm{Ngb}_P + L \underset{k_L}{\overset{k'_L}{\rightleftharpoons}} \mathrm{Ngb}_L \quad (1)$$

where the pentacoordinate species (Ngb_P) is the only state competent in binding the exogenous ligand (L). Upon mixing ferrous Ngb with several ligands (O_2, CO, NO and methyl isocyanide), the formation of the adduct was found to be a slow process ($t_{1/2}$ ~1 s) and the relevant rate constant(s) was ligand concentration independent [2, 30, 33–35]. These experiments showed that binding of O_2 (and other ligands) is rate-limited by the rupture of the internal coordination with His(E7); the relevant rate constant (k_{-H}) was determined by several groups to be from ~1 s^{-1} to 4 s^{-1}. The reactive pentacoordinate species is not significantly populated because its ligand affinity is high. This was shown by extensive flash photolysis experiments [2, 30, 33, 36, 37], which showed that the equilibrium between the six- and the five-coordinate states favors the former species by ~1000-fold for both murine and human Ngb. Analysis of the transient kinetics after flash photolysis (which instantly populates Ngb_P) led to the determination of the intrinsic rate constants for the binding of L to the pentacoordinate state; a summary of the consensus values is reported in Table 1 for O_2, CO and NO. Perhaps the most surprising result was to find that k'_L for CO is very large (*e.g.*, compared to deoxy Mb and even R-state Hb). In addition, the flash photolysis experiments led to estimation of the rate constant for rebinding of His(E7) to the ferrous iron in competition with binding of L; the consensus value for k_H is ~2000 s^{-1} [2, 30, 33, 35, 38]. This allowed the equilibrium constant for the hexa-to-pentacoordinate states $K_H=(k_{-H}/k_H)$ to be estimated, ~10^{-3}, in complete agreement with the rapid mixing experiments (see above) and with spectroscopic data.

Rapid mixing experiments of ferrous Ngb with CO and methyl isocyanide yielded a biphasic ligand binding time course [35]; the two rate constants (k_{app} ~ 2 s^{-1} and 0.2 s^{-1}) and the relative amplitude of the two phases (~7:3) suggest that the kinetics of rupture of the bond with distal His(E7) is heterogeneous. Spectroscopic and mutational data seem to indicate that differences in the heme pocket structure and accessibility may be invoked to explain the kinetic heterogeneity [32, 37, 39]. In this respect, it is relevant to recall that solid evidence exists [27, 28, 40] to indicate heme orientational disorder in metNgb (see below). It is not clear, however, if the heme orientational disorder (with a ratio 7:3) is an artifact of heterologous Ngb synthesis in *E. coli* or an intrinsic property of the protein, as expressed in the neurons.

In Ngb, the overall affinity constant for O_2 or other exogenous ligands results from a competition with His(E7) rebinding, as illustrated in Eq. (1). Therefore, ligand affinity is not modulated exclusively by the on and off rate constants to the pentacoordinate state as in the case of Mb. This was clearly shown by mutational studies of Ngb [30, 31, 33]. Upon changing the distal His(E7) to Leu, Val or Gln, the affinity for O_2 of mNgb and hNgb is increased with respect to the wild type (by a factor that ranges from 3 to 100 in different mutants), in spite of the variations of k_L and of k'_L that

would imply a decrease in affinity [30, 31]. The absence of competition for the endogenous His(E7) ligand is most likely the cause of the increase in the apparent affinity for O_2. Another remarkable effect of the mutations His(E7)Gln and His(E7)Leu/Lys(E10)-Leu is the drastic reduction of the autooxidation rate in air [31], indicating that His(E7) enhances the reactivity of oxygenated Ngb.

In the light of a possible enzymatic activity of Ngb directed towards NO or other radicals, it may be useful to recapitulate some information available in the literature. Assuming that Ngb is involved in redox reactions in the cell, a suitable intracellular reductase is expected to exist to allow cycling from the ferric to the ferrous state, and therefore (for example) radical scavenging. So far, identification of a specific metNgb reductase is lacking. The photo-activated NADH/FMN mixture, which can efficiently reduce Ngb [34], may perhaps mimic a putative physiological reduction system; however, it is likely that other physiological reductants, or even some canonical redox-active metalloproteins, may efficiently donate electrons to metNgb. It is finally relevant to recall that characterization of the reduction kinetics of hexacoordinated Hbs (hxHbs), including human Ngb [41], showed that in these proteins electron transfer to the heme iron is much faster than in the case of pentacoordinated hemeproteins. This behavior has been explained on the basis of the more favorable reorganization energy term involved in the reduction of the hexacoordinated hemeproteins, which supports the hypothesis that hxHbs may be tuned for redox cycling rather than binding functions.

If confronted with O_2 or NO in the presence of a photo-activated NADH/FMN electron donor system, murine Ngb seems not to function as a reductase for either one of these two ligands [34]; it cannot be excluded, however, that more complex experiments may shed light on possible redox reactions catalyzed by Ngb in the cellular environment. As an example, it was shown that oxygenated Ngb reacts with NO very rapidly (complete within the dead time of stopped-flow, ~3 ms) to yield a peroxynitrite intermediate that decays to nitrate and metNgb with a first order rate constant of 90 s^{-1} (at 5°C) and 200 s^{-1} (at 20°C). This behavior seems not inconsistent with NO scavenging under partially hypoxic conditions in the nervous system where, at the same time, NO synthesis would be enhanced by induced expression of NOS [42]. In such a scenario, binding of NO to ferrous Ngb would yield essentially a dead-end adduct, given that its thermal dissociation is very slow ($t_{1/2}$>10 min [36]). Interestingly, it was shown [43] that Ngb(Fe^{2+})NO reacts rapidly with harmful peroxynitrite (second-order rate constant ~1.3×10^5 M^{-1} s^{-1}, almost 100 times faster than Hb) to yield NO and metNgb. This reaction was proposed to play a role in the neuroprotective effect of Ngb [44].

For human Ngb, Hamdane et al. [33] reported a ten-fold decrease in O_2 affinity (P_{50} from 0.9 to 8.4 torr) upon reduction of an intramolecular disulfide bridge between Cys(CD7) and Cys(D5). Although the general physiological relevance of this finding is questionable because Cys(CD7) is absent in murine Ngb, it is interesting that a consistent effect of this disulfide bond was observed also by kinetics [36]. Finally, it should not be overlooked that the presence in Ngb of reactive cysteines might suggest an involvement in *S*-nitrosation, a very important phenomenon for NO metabolism and functional role [15, 18], although no evidence for the formation of SNO-Ngb has been reported so far.

Structural features

The structures of human Ngb in the met (Fe^{3+}) state, and of mouse Ngb in the met (Fe^{3+}) and CO (Fe^{2+}) bound states have been determined by crystallography [27, 28, 45]. In spite of low sequence homology (less than 25%, [27]), Ngb displays the same fold as sperm whale Mb with an average root mean square deviation (RMSD) for C_α atoms of 2.0 and 1.85 Å for met and NgbCO, respectively (Fig. 2a). Structural superposition with hexacoordinated rice nonsymbiotic hemoglobin (nsHb, 1D8U, [46]) yielded a comparable result (average RMSD=2.24 Å) upon exclusion of the short N-terminal extension of nsHb (Fig. 2b).

As expected from spectroscopic data, metNgb (in the absence of an exogenous ligand) is hexacoordinated with His96(F8) bound to the iron on the proximal heme side and His64(E7) on the distal one. To bind the iron, the position of the distal His64(E7) in mNgb is closer to the heme by 2.38 Å with respect to sperm whale Mb, and the whole helix E is somewhat repositioned, with a rotation around Leu70(E13) leading to a displacement of the N and C ends of the helix by 3.79 and 2.92 Å, respectively. Also, in hNgb, the position of helix E is shifted by about 3 Å towards the heme. Inspection of metNgb and comparison with the structure of nsHb suggests that the region joining helices C and D (CD turn) may play a role in the structural rearrangement that follows exogenous ligand binding, due to its high mobility and the position it occupies in the globin fold, where it acts like a hinge to allow helix E displacement (Fig. 2).

Significant heme rotational isomerism was observed [27, 28] in both human and murine metNgb structures,

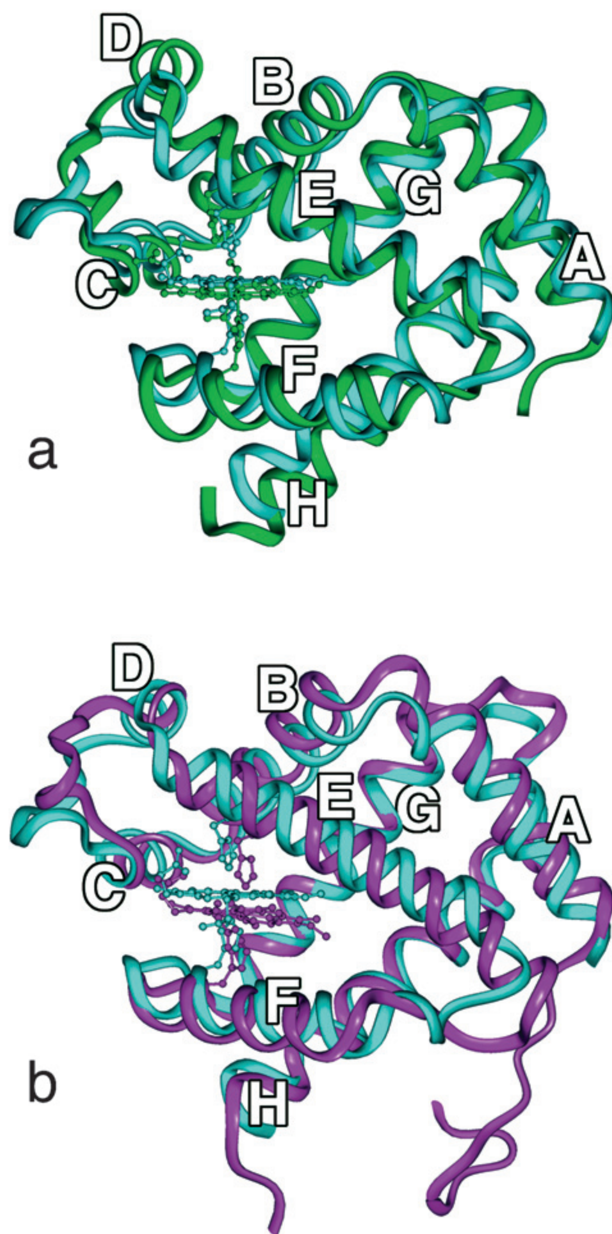


Figure 2. Structural superposition of murine neuroglobin (Ngb, light blue), sperm whale myoglobin (Mb, green) and nonsymbiotic hemoglobin (Hb, purple), based on the C_α atoms. The letters indicate globin helices A–H, according to standard nomenclature. (*a*) Best-fit superposition of murine Ngb and sperm whale Mb. (*b*) Best-fit superposition of murine Ngb and nonsymbiotic Hb.

with a ratio of 7:3 in mNgb (Fig. 3), in complete agreement with earlier NMR data [40]. The two conformers differ by a 180° rotation of the heme moiety along the α–γ meso axis. In the main (A) conformer the metal lies within the heme plane, whereas in the minor (B) conformer it is 0.2 Å out of the heme plane; the propionate groups have identical conformations in both isomers. This difference, which is also reflected in the positions of the distal and proximal His, may or may not account for the heterogeneity observed in the dynamics of ligand binding (as mentioned above).

Both hNgb and mNgb in the met hexacoordinate state possess a large cavity (120 and 290 $Å^3$, respectively) that is localized between the heme distal side and helix E; in mNgb this extends towards the heme proximal side, including the so-called Xe1 and Xe2 binding sites identified in sperm whale Mb [47]. Heme orientational disorder may or may not be related to the presence of this huge internal packing defect that leaves some empty space between the heme rim and the protein moiety, thus allowing alternate positions for the heme vinyl and methyl groups; however, this seems unlikely to us given that heme isomers were first identified by La Mar and coworkers [48] in sperm whale Mb, which contains several internal packing defects [47] but lacks the huge tunnel typical of Ngb.

To allow binding to the sixth heme coordination site, not only must the bond with the distal His64(E7) break, but a conformational transition has to take place to create space to accommodate the external ligand. In analogy with the gating role played by His64(E7) in Mb and Hb, it was initially expected that, in Ngb, a swinging motion of the distal His might allow, at the same time, availability of the sixth coordination position and formation of an empty niche for an external ligand to bind. Unexpectedly, determination of the structure of mNgbCO [45] showed that this protein adopts a "heme-sliding mechanism" to remove the blockage of the binding site. In fact, CO binding is associated to a major heme displacement within the frame of the globin fold (Fig. 4a), which may underlie a new mechanism for ligand affinity modulation in the globin family. Repositioning of the heme upon CO binding leaves the position of the distal His64(E7) almost unchanged (0.5–0.7 Å), while being associated to a displacement of the proximal His96(F8). The heme in this new position seems to reside in a pre-formed docking site and, consistently, only Phe106(G3) is drastically repositioned (by 3.8 Å). The regions that experience the largest movement upon binding are the EF-F-FG module and the CD loop that moves to a slightly more open conformation, but altogether the total RMSDs displacement of the C_α is comparable to that observed in sperm whale Mb upon ligation (0.49 Å *versus* 0.54 Å). The ^1H NMR data from met mNgb in the absence and in presence of cyanide presented by Du et al. [40] were analyzed by Walker [49], leading to the indication that the solution structure, notably the orientation of the proximal and distal histidines, could be different from the one determined in the crystal state. On the other hand, EPR spectroscopy seems to indicate good

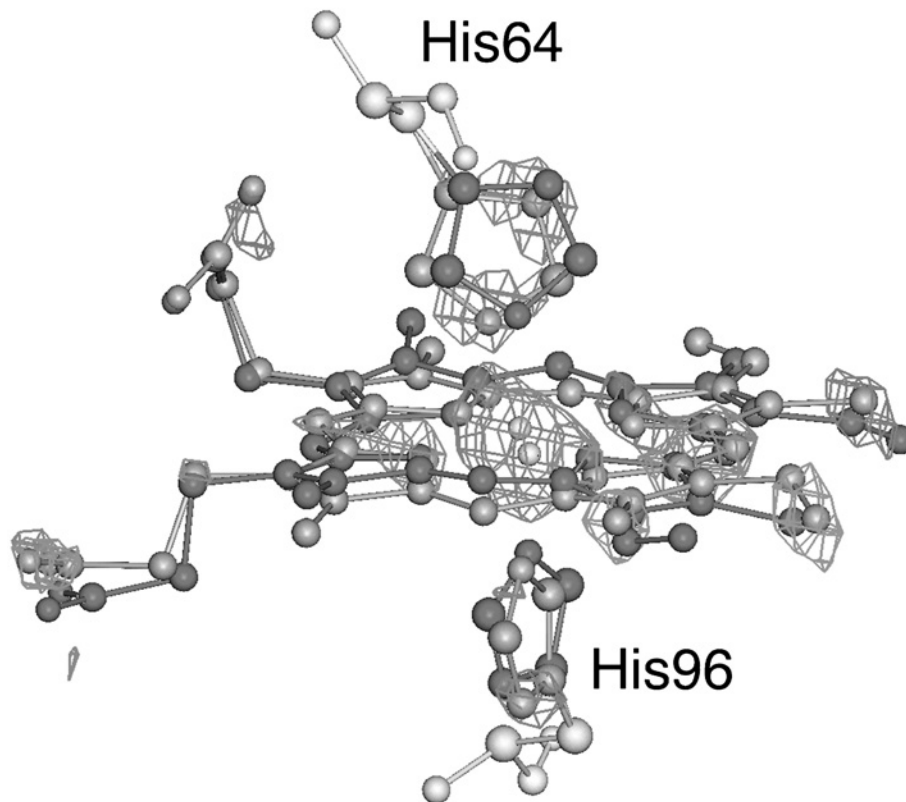


Figure 3. The two conformers of the heme (called A and B) in metNgb from mice are shown. The A conformer is drawn in dark gray and the B conformer in light gray (population ratio 70:30). The two configurations correspond to a heme rotation of 180° along the α-γ meso axis. Fo-Fc positive electron density map was calculated omitting the heme B conformer and the distal and proximal His of the B conformer; the density was contoured at 3 σ.

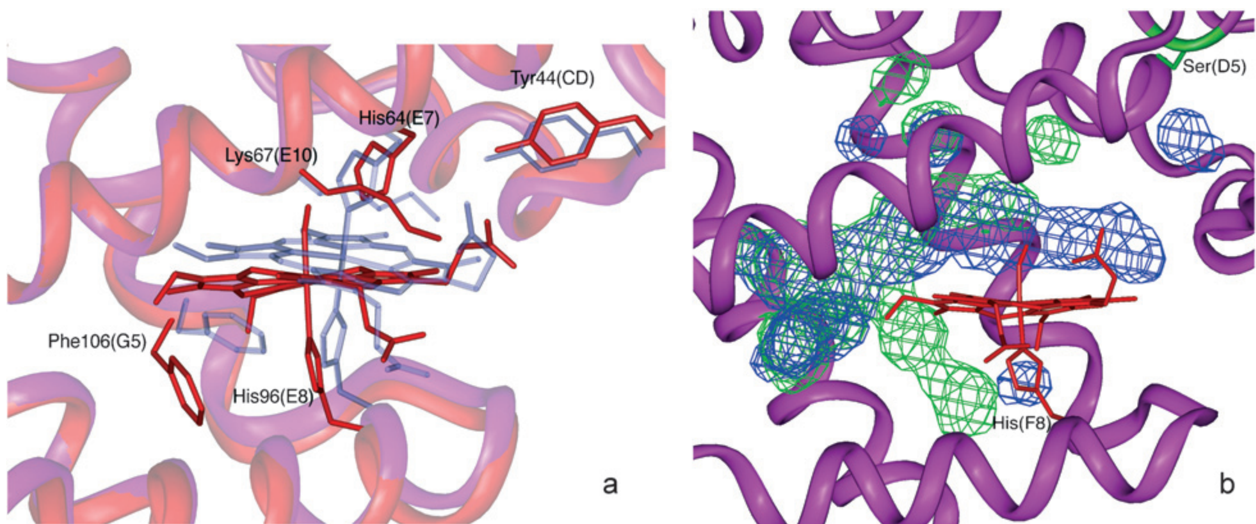


Figure 4. (*a*) Close-up view of the active site of murine NgbCO (red) and metNgb (violet-blue). The sliding motion of heme, as well as the proximal and distal histidines and the amino acids [notably Phe106(G5) and Tyr44 in the CD turn] that undergo the largest displacements are highlighted. (*b*) The major cavity observed in NgbCO (blue) and metNgb (green) are shown. The heme displacement associated with CO binding induces a reshaping of this cavity, with the disappearance of its branch in the region proximal to the heme.

agreement of solution and crystallographic parameters in met mNgb [50].

A reshaping of the large internal cavity is also observed upon CO binding, with the disappearance of its proximal branch (Fig. 4b) and expansion of the distal region; moreover, a new, small (13.3 Å^3) open space is created in the proximity of topological position D5, which is a Cys in the wild type and a Ser in the mutant used for crystallization. Another relevant feature of mNgbCO with respect to the internally hexacoordinate structure, is the decrease of the thermal B-factors of the EF region, that contrib-

utes the residues surrounding the access of the large tunnel to the bulk [Leu70(E13), Val71(E14), Ala74(E17), Leu85(EF), Tyr88(EF)]. The increased rigidity of the EF region might restrict external accessibility and trap small molecules inside the protein tunnel.

In the absence of an external ligand, access to the heme distal binding side is restricted by the presence of a salt bridge between one of the heme propionates (distance of 2.7 Å) and Lys67(E10), which is reinforced by a close contact with the hydroxyl group of Tyr44(CD3) and a water molecule. This network is weakened in mNgbCO, where the minor displacement of His64(E7) induces a movement of Tyr44(CD3) that is communicated to the CD loop (Fig. 4b). This is consistent with data obtained from spectroscopy and mutagenesis experiments indicating that, upon ligation and mutagenesis of Lys67(E10), the accessibility of the distal site is increased [38].

In summary, the structure of NgbCO reveals the likely mechanism whereby the iron hexa-coordination (expected to lower considerably ligand affinity) is made labile enough to allow binding of exogenous ligands. The factor that counterbalances the presence of internal coordination (which *a priori* should be fairly strong) seems to be the docking of the heme in a new position, leading to a different protein conformation and improving iron coordination geometry with O_2 and other external ligands.

What can we infer on the mechanism of action of Ngb, in the light of the structures of its liganded and unliganded derivatives? Ngb is most likely reduced and (partly or largely) ligated by O_2 under physiological conditions (normoxia); upon deoxygenation, Ngb is expected to adopt the hexacoordinate structure. The thermal B-factors indicate that exogenous ligand release will induce a marked change in the mobility of the EF loop and a reorganization of the CD loop. It was suggested [34, 45] that this conformational change may be the signal to transmit the information "low oxygen", to allow interaction of metNgb with GDP-bound G_α to liberate $G_{\beta\gamma}$ and thus protect the cell against neuronal death [51]. Enhanced Ngb expression, as observed under conditions of hypoxia [4], would enhance this signaling pathway. Moreover, hypoxia is known to reduce the pH of a neuronal cell [52]; acidity decreases the rate of exogenous ligand binding and favors hexacoordination [38] and the coupled conformational changes involving the EF loop.

Concluding remarks

Ngb seems to belong to a globin gene family separate from the canonical one of Hb, Mb and Cygb, that diverged early in animal evolution and shows little sequence variation, suggesting conserved structure and function and an important physiological role in the brain.

Functional parameters cast some doubt on the primary role of Ngb as an O_2 buffer or in facilitating diffusion in the nervous tissue; nevertheless, *in vivo* and *in vitro* studies have shown a neuroprotective role of Ngb during hypoxic stress. An analysis of available functional and structural data seems to indicate that the mechanisms involved may be either enzymatic by scavenging radicals, or signaling by activating cellular responses through a conformational transition controlling binding of Ngb to a G-protein component.

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