Neuroglobin and cytoglobin: two new members of globin family

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The globin family has long been defined by myoglobin and hemoglobin, proteins with the functions of oxygen storage and transportation, respectively. Recently, two new members of this family were discovered: neuroglobin present in neurons and retinal cells and cytoglobin found in various types of tissue. The increased expression of these proteins in hypoxic conditions first suggested a role in oxygen supply. However structural and functional differences, such as the hexacoordinated heme, a high autoxidation rate and different concentrations between different cellular types, have dismissed this hypothesis. The protective role of these globins has already been established. In vitro and in vivo studies have demonstrated increased survival of neurons under stress in the presence of neuroglobin and increased resistance to neurodegenerative diseases. However the mechanism remains unknown. Functions, including detoxification of nitric oxide, free radical scavenging and as an antioxidant and signaling of apoptosis, have also been suggested for neuroglobin and an antifibrotic function for cytoglobin.

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Introduction

Globins are a family of proteins that present the "globin fold": alpha-helical segments arranged symmetrically and a prosthetic heme group. A heme group is a protoporphyrin with an iron atom in the center, where one molecule of oxygen binds reversibly. Two members in the globin family, myoglobin (Mb) and hemoglobin (Hb), have been known for a long time. The main function of Mb, which is mainly found in muscle cells, is oxygen storage. Hb, generally found in the red blood cells of vertebrates transports oxygen from the lungs to tissues. Mb is a monomeric protein, that is, formed by only one protein chain, and Hb is tetrameric (four chains) composed of two identical alpha chains and two identical beta chains. Thus, while Mb binds one oxygen molecule, each molecule of Hb can carry four oxygen molecules.[1,2]

Oxygen binding to Hb and Mb is well described in the literature. The iron atom in the heme group of these proteins is pentacoordinated. When in its ferrous state (Fe^{+2}), iron chelates with the four nitrogens of porphyrin, covalently binds the proximal histidine (F8) and chelates with oxygen, if present. When oxygen is not present this binding site remains unoccupied. The binding of oxygen to iron is stabilized by a hydrogen bond between the oxygen and histidine E7, the distal histidine.[3]

The binding of oxygen to these proteins is an allosteric process. For both Mb and Hb, the binding of oxygen is regulated by the oxygen itself and by the presence of heterotropic effectors such as anions, protons, water, etc. For Hb, oxygen binding is cooperative, i.e. the first oxygen molecule facilitates the binding of successive molecules.[3] In addition to the functions of storing and transporting oxygen, both Hb and Mb perform different roles under specific conditions. Hb undergoes redox reactions forming ferric (Fe^{+3}) and ferryl (Fe^{+4}) Hb, thereby losing the ability to bind oxygen. Mb helps detoxification of nitric oxide (NO), among other functions.[4,5]

In vivo, the methemoglobin reductase system keeps iron in the ferrous state and consequently maintains the functional role of globins. This reduction process can happen by two pathways. One consists of the cytochrome b-5-mediated redox cycle involving methemoglobin reductase enzyme with nicotinamide adenine dinucleotide (NADH) as an electron donor. The other pathway uses flavin for the reduction of methemoglobin, operates in the presence of the flavin-reductase enzyme and also depends on NADH.[6,7]

There are other members of the globin family including globin E, found in bird's eyes, globin X, found in some amphibians and fishes, and the recently discovered cytoglobin (Cgb) and neuroglobin (Ngb).[8-10]

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Ngb is expressed in neurons and retinal cells (although it can be present at very low concentrations in other cells) and Cgb is expressed in several different types of tissue. Both were identified in 2000 through genomic sequence homologies.[11] Additionally, both proteins were first found in human tissue and later in other animal species, suggesting a wide distribution among vertebrates. Phylogenetic analyses indicate that Ngb may have a common evolutionary origin to invertebrate globins and that Cgb has a more recent origin, closer to that of Mb.[10,12]

Ngb is a monomeric protein with 150 amino acid residues and is mainly expressed under hypoxic conditions due to ischemia in both the central and peripheral nervous systems. However, the molecular concentration varies depending on the tissue. In retinal cells, where there is a high demand for oxygen, Ngb concentrations can be up to 100 times greater than in neurons. The effect of hypoxia on the *in vivo* production of Ngb varies from species to species as detailed in the publications of Burmester et al.[13] and Schmidt et al.[14]

Ngb has also been identified in tumor cells (such as induced glioblastomas) with the expression increasing when tumor cell lines are submitted to hypoxic conditions *in vitro*.[15] Cgb, the last globin to be discovered, has 190 amino acids distributed in two chains in a dimeric arrangement. It is found in several organs including the heart, lungs, liver and stomach, yet no specific function has been described.[16-18]

Despite having the same globin folding and similar sizes as Mb, both these new proteins have one important difference compared to the other globins: in the absence of an external ligand the heme is hexacoordinated. In this case, in addition to the five previously described ligands, the distal histidine (E7) becomes the sixth ligand of the iron, a condition known as bis-histidine. Consequentially, binding to oxygen occurs in two steps. First the bond between the iron and the distal histidine has to be broken and only then it is possible for the oxygen to bind to the protein. This unusual behavior raises interest in the role of these new proteins, as this equilibrium between the penta and hexacoordinated forms does not characterize efficient oxygen binding.[5]

Hexacoordinated Hb, previously identified in plants (leg-hemoglobins), facilitates oxygen diffusion in roots.[19,20] Human Hb can be converted into the hexacoordinated species hemochrome (Fe^{+2}) and hemichrome (Fe^{+3}) in situations of stress, such as low pH, low hydration and in the presence of high concentrations of organic solvents.[21] Physiologically, hexacoordinated Hb is associated with disease.

Cytochromes are hexacoordinated hemoproteins and have an important biological role. These widely found proteins possess a heme group but are not globins (they have beta sheets in their structural arrangement, among other features). Cytochromes, which may be in ferrous and ferric states, are involved in redox reactions and in electron transport chain in mitochondria, among other essential cell processes.[22]

Another difference of Ngb and Cgb compared to Mb and Hb is the accessibility to internal disulfide bridges formed by cysteine residues. When oxidized, disulfide bridges can modify the configuration of heme, increasing oxygen affinity and thus facilitating their path by the disruption of distal histidine. Cgb and Ngb have cysteines in different positions of the chains; the effect of disulfide bridge stabilization causes greater disturbance in Ngb than in Cgb. However, during *in vivo* oxygenation, the disulfide bridges are reduced and do not interfere in the affinity for oxygen.[23-25]

The biochemical and biophysical characterizations of Ngb and Cgb have been studied by several authors since their discovery. Ngb and Cgb have some similarities with Hb and Mb which include the acid and alkaline Bohr Effect, but the oxygen affinity of Ngb seems to be more dependent on the pH (the Bohr effect) than with Cgb. The affinity for oxygen, measured by the value of p50, i.e. the pressure of oxygen required to saturate 50% of the protein binding sites, is similar to that of Mb.[5,12,26]

Cgb also presents oxygen binding cooperativity, i.e. the binding of one molecule of oxygen facilitates the binding of subsequent molecules. This same behavior is observed in Hb, but due to the unique structural arrangement found in Cgb compared to Hb, the mechanism of its cooperativity has not yet been elucidated.[19]

Although the overall oxygen affinity of Ngb and Cgb is similar to that of Mb, functionally the proteins differ from Mb and Hb as the structures are stable at low pHs (it has been reported that apo-Ngb maintains its structure at pH 2.0 unlike Hb and Mb), and at high temperatures; the melting temperatures of Ngb and Cgb are 100ºC and 95ºC, respectively. [27,28]

Another peculiar feature of Ngb is the autoxidation rate. At room temperature and in the presence of oxygen, unlike the other globins, the equilibrium of Ngb shifts to the ferric state.[29]

All the properties presented by the two new globins have led researchers to try to discover their functions. Because Cgb was described after Ngb and as it is not so specific, its function has not been explored as much. For Ngb, research has been conducted to try to link intrinsic characteristics with conditions presented by the cell in situations of stress, such as acidosis, presence of reactive species and low oxygenation which has led to identifying a probable role of Ngb as a neural protector.

But what are the possible mechanisms of action? What are the roles of these proteins in cells? From biological and structural information, some functions of Cgb and Ngb have been suggested.

Possible roles for neuroglobin

At first, it was suggested that Ngb had a similar role to that of Mgb, but, for neurons. In this case, Ngb would act as an oxygen reservoir releasing oxygen in stressful situations, such as hypoxia and maintaining the concentration of oxygen in the electron transfer chain and

thus the normal function of the mitochondria. Since the p50 value measured for Ngb is similar to that of Mb, this hypothesis seemed plausible. However, the low concentration of protein in the brain (~ 1 μM) along with the high autoxidation rate discarded this idea. Studies on Ngb autoxidation have shown that oxygen affinity is much lower than that found for Mb.[5,27]

Other functions were then suggested for these proteins, such as hypoxia signaling, enzymatic activity, free radical scavenger and anti-apoptosis.

The suggestion of a NADH oxidant role emerged from the fact that in some strains of *Escherichia coli* Ngb was expressed in the ferrous and not in ferric state, as expected due to the high rate of autoxidation of these proteins as was mentioned earlier. Giufre et al. found that there was an in-cell reductase system, similar to that found in vertebrate cells that prevented oxidation of Ngb by reducing the protein with the oxidation of nicotinamide adenine.[30,31]

Several studies are attempting to identify how Ngb would act as a scavenger of the free radicals produced in redox reactions. It is known that Ngb can react with hydrogen peroxide, superoxide and nitrite peroxide, similar to Hb. However, because of the hexacoordination, the iron in Ngb is protected against electronic changes and consequently, unlike Hb, Ngb does not enter the ferryl state. Thus, the mechanism by which Ngb plays an antioxidant role remains unclear, though decreased formation of free radicals in the presence of Ngb has been observed.[32-34]

The hexacoordination structure provides further protection to the heme group against strong oxidizing agents. Studies on point mutations of the distal histidine which resulted in the elimination of the hexacoordination showed that in the presence of oxidizing agents, such as hydrogen peroxide, Ngb forms free radicals resulting from heme group degradation. When in its native form, the structure of Ngb is maintained even in the presence of oxidizing agents and their subproducts.[35]

To further investigate the possible role of free radical detoxification, several studies have been conducted with Ngb in relation to reactive species derived from oxygen and nitrogen, including NO. It was found that in the presence of NO, Ngb changes by oxidizing from the ferrous (Fe^{+2}) to ferric state (Fe^{+3}) thereby reducing NO. Nevertheless, these results should be analyzed very carefully as this behavior is common to all globin proteins and does not necessarily represent a function *in vivo*.[36] It is true that experiments with cell lines where the expression of Ngb was induced were much more resistant to the presence of reactive species such as hydrogen peroxide, but there is no *in vivo* increase in the expression of Ngb when other scavenger proteins are expressed. Another point to be considered lies in the fact that any globin protein potentially reacts with reactive species, so perhaps, this function *in vivo* is not the primary function of Ngb.[37,38]

In the signaling regulation for cell death inhibition due to hypoxia, Khan et al. suggested that the interaction of Ngb with membrane proteins would inhibit the neuronal death cascade through reorganization of cytoskeletal and mitochondrial aggregation. In this case, they act as inhibitory signals not only for hypoxia but for other conditions.[39]

An interaction of these proteins with cytochromes was suggested from the possibility that Ngb acts in redox reactions. *In vitro*, redox reactions between the ferrous and ferric forms of proteins are possible (mediated by the cytochromes c and b5) and very quick (in the order of 2000 reactions per second). Several suggestions were made with anti-apoptosis of Ngb being the most commented. When neurons are under stress due to increased reactive species or even lack of oxygen, there is an increase in the permeability of the mitochondria membrane and therefore leakage of cytochrome c to the cytosol. In this case, Ngb would react with cytochrome c inhibiting programmed death signaling. A later recovery of the neuron would depend on the type of stress, but there would be an attempt at survival.[40-42] Studies which focused on this hypothesis of an interaction between cytochromes and Ngb are further supported by the fact that Ngb is found near to the mitochondria in cells. However more studies are necessary to confirm this premise.

Possible roles for cytoglobin

Cgb is present in several tissues, but in mammals, it is mainly found in fibroblasts and fibroblast-like cells. For this reason, it was suggested that Cgb plays a role in the fibrosis process, acting as an oxygen donator during synthesis and cross-linking of collagen or acting as a protector of the free radicals formed in the fibrosis process. Recently this hypothesis gained strength with evidence that Cgb-rich liver cells did not enter the fibrosis process when attacked by chemicals. Other experiments have shown a role for Cgb in protection against fibrosis in kidney cells.[43-45] Other studies have reported Cgb associated with protection against fibrosis in cells.[17] Nevertheless, other functions have been linked to Cgb, such as tumor suppression.[46]

Cgb is also present in neurons albeit at a lower concentration than that of Ngb and is mostly located in or near to the nucleus. Studies with neurons that contain Cgb showed that these cells become more resistant to attack by free radicals and reactive oxygen and nitrogen species in a mechanism similar to that described for Ngb.[47]

Final considerations

Since their identification, many studies have been conducted to characterize the functions of Cgb and Ngb. Cgb seems to have a role in cellular fibrosis. Ngb is mostly found in neurons and in the retina and seems to be expressed mainly in hypoxic conditions. It is undeniable that this protein is present to protect cells. *In vivo* and *in vitro* studies show that, in the presence of Ngb, neurons can survive oxidative stress for much longer.

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