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Is the heme pocket region modulated by disulfide-bridge formation in fish and amphibian neuroglobins as in humans?☆

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ABSTRACT

Neuroglobin, a globin characterized by a bis-histidine ligation of the heme iron, has been identified in mammalian and non-mammalian vertebrates, including fish, amphibians and reptiles. In human neuroglobin, the presence of an internal disulfide bond in the CD loop (CD7–D5) is found to modulate the ligand binding through a change in the heme pocket structure. Although the neuroglobin sequences mostly display conserved Cys at positions CD7, D5 and G18/19, a number of exceptions are known. In this study, neuroglobins from amphibian (*Xenopus tropicalis*) and fish (*Chaenocephalus aceratus*, *Dissostichus mawsoni* and *Danio rerio*) are investigated using electron paramagnetic resonance and optical absorption spectroscopy. All these neuroglobins differ from human neuroglobin in their Cys-positions. It is demonstrated that if disulfide bonds are formed in fish and amphibian neuroglobins, the reduction of these bonds does not result in alteration of the heme pocket in these globins. Furthermore, it is shown that mutagenesis of the Cys residues of *X. tropicalis* neuroglobin influences the protein structure. The amphibian neuroglobin is also found to be more resistant to H_2O_2-induced denaturation than the other neuroglobins under study, although all show an overall large stability in high concentrations of this oxidant. This article is part of a Special Issue entitled: Oxygen Binding and Sensing Proteins.

1. Introduction

Neuroglobin (Ngb), a vertebrate heme protein belonging to the globin superfamily, was originally identified in mammalian species [1]. However, it is also present in non-mammalian vertebrates including fish (zebrafish, goldfish and Antarctic fish), amphibians (*Xenopus*) and reptiles (freshwater turtle), which suggests a widespread occurrence [2–5]. The finding that icefish retain the Ngb-gene, despite having lost hemoglobin (Hb) and myoglobin (Mb) in most species, is fascinating [6]. The presence of Ngb in the brain of red-blooded Antarctic notothenioid fish and in at least 13 of the 16 icefish species suggests a crucial biological function [3]. Comparison of mammalian and non-mammalian Ngbs may assist in identifying conserved features and will help to reveal its function. Human Ngb (NGB) is composed of 151 amino acids and shares only a small number of amino acids with vertebrate Hb and Mb (<25% identity) [1,7]. Due to additional amino acids at the N- and C-termini, *Danio rerio* Ngb (*D. rerio*Ngb), *Chaenocephalus aceratus* Ngb (*C. ace*Ngb) and *Dissostichus mawsoni* Ngb (*D. maw*Ngb) and *Xenopus tropicalis* Ngb (*X. trop*Ngb) are 8 and 13 residues longer, respectively, than mammalian Ngb proteins (Fig. 1) [2–4]. Mammalian and fish Ngb proteins demonstrate ~55% identity and ~78% similarities. *X. trop*Ngb is on average 67% identical and 85% similar to mammalian Ngbs. These values are higher than the observed similarity scores of the orthologs Mbs and Hbs of these taxa, as the result of a significant substitution decrease in the mammalian Ngb branch. Amphibians occupy a key phylogenetic position between fish and amniotes (mammals, reptiles and birds), and are thus an important target of comparative genomic research. The amino-acid substitution rate for *X. trop*Ngb (0.61×10^{-9} amino acid replacement per site and year) [4] is between the enhanced rate estimated for fish Ngbs ($\sim0.93\times10^{-9}$) and the slowed-down rate of mammalian proteins ($\sim0.39\times10^{-9}$). The low rate of mammalian Ngb substitution might be explained by the particular requirements of Ngb in the nerve system of these taxa. Furthermore the heme-binding helices are remarkably conserved, suggesting similar physicochemical and kinetic protein properties [2].

After more than a decade, the physiological role(s) of Ngb is (are) still unclear and many functions, including involvement in protection against apoptosis, in reactive O_2/nitrogen species removal and in ligand sensing, have been proposed [8]. Ngb is expressed predominantly in neurons [1,9,10]. The ferrous form (Fe^{2+}) of the protein reversibly

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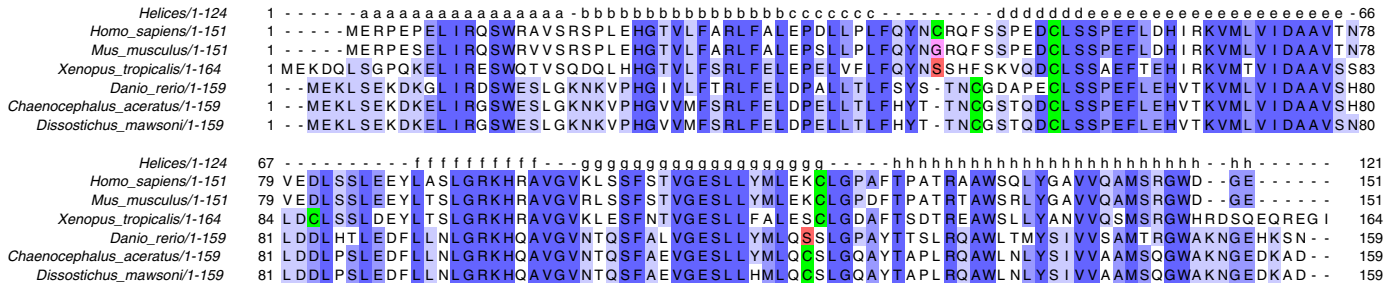

Fig. 1. Amino acid sequence alignment of the Ngbs of man (*Homo sapiens*), mouse (*Mus musculus*), the zebrafish *Danio rerio*, two Antarctic fish *Chaenocephalus aceratus* and *Dissostichus mawsoni* and the amphibian *Xenopus tropicalis*.

binds exogenous ligands, such as O_2, CO, and NO, in competition with the internal E7His ligand [11,12]. Similarly, in the ferric form (Fe^{3+}) of the protein, the heme iron is ligated to the His residues at positions E7 and F8 [7,11]. The intracellular concentration of Ngb is low (~1 μM) and its O_2-binding affinity is comparatively weak [e.g. P_{50} = 2.2 Torr for mouse Ngb (mNgb)] under physiological conditions [13]. Furthermore, the ligand-binding properties of NGB are found to depend on the presence of an internal disulfide bond between the Cys at positions CD7 and D5 [14,15]. The His-binding affinity of ferrous NGB decreases by a factor of ten due to disulfide bridge formation, with corresponding increase of the O_2-affinity, as determined by laser-flash photolysis experiments [14]. This suggests that the release of O_2 from NGB is controlled by a reduction/oxidation mechanism of the disulfide bridge. Electron paramagnetic resonance (EPR) has shown that the heme pocket structure of ferric NGB is influenced by a reduction of the disulfide bridge [16,17]. More specifically, the disulfide bridge formation affects the relative orientations of the E7His and F8His imidazole planes [16]. The B10Phe residue was found to be important in mediating the structural changes in the heme region upon disulfide-bridge formation [17]. All these observations may explain the role of NGB during hypoxia. Under hypoxic conditions, the disulfide bond is reduced, with a subsequent release of O_2 possibly counteracting the hypoxia. When the O_2 concentration increases, the free Cys residues are oxidized to form the intra-molecular disulfide bond with the concomitant increase in O_2-affinity [15]. However, except in the retina, the expression of NGB seems too low for this to be a viable function of the protein. On the other hand, a signaling function may be possible with (i) O_2 acting as signaling molecule or with (ii) the protein conformational change modulating downstream signaling pathways [15].

The Ngb sequences display conserved Cys at positions CD7, D5 and G18/19 with the following exceptions: (i) the rodentia Ngbs, *X. trop*Ngb and *X. laevis*Ngb lack the CD7Cys, (ii) *D. rerio*, and *Carassius auratus* and *X. laevis* Ngbs lack the G18/19, (iii) *D. rerio*, *D. maw* and *C. ace* Ngbs have a Cys in the CD loop at CD9 instead of CD7, and (iv) *X. trop*Ngb has a unique Cys at position EF4 (Fig. 1 and Table 1) [1–5]. The present study focuses on unraveling the effect of Cys positions on the heme pocket in the *D. rerio*, *X. tropicalis* and the Antarctic fish (*D. mawsoni* and *C. aceratus*) Ngbs. Furthermore, the stability of these Ngbs against H_2O_2 is tested.

Table 1
Overview of the position of the Cys residues in different Ngbs relevant for this paper (see also Fig. 1). X indicates the presence of a Cys residue.

Ngb	CD7Cys	CD9Cys	D5Cys	EF4Cys	G18/19Cys
NGB	X		X		X
Rodentia Ngb			X		X
*X. laevis*Ngb			X		
*X. trop*Ngb			X	X	X
*D. rerio*Ngb		X	X		
*C. ace*Ngb		X	X		X
*D. maw*Ngb		X	X		X

2. Materials and methods

2.1. cDNA

The cDNA of *C. ace*Ngb and *D. maw*Ngb was cloned as described [18]. The cDNA from *D. rerio*Ngb, cloned in pET3a-vector, was kindly donated by prof. T. Hankeln and T. Burmester (Institute of Molecular Genetics, Mainz, Germany). *X. tropicalis* embryonic cDNA was kindly donated by prof. M. Dewerchin (Vesalius Research Center, K.U. Leuven, Belgium). *X. trop*Ngb specific primers were made according to the published sequence (gene bank AAI61728.1). The 5′ primer XenoF (cgccatatggagaaagatcagctgctgtcgggacc) contains a *NdeI* restriction site that covers the initiating Met codon of the globin gene, and the 3′ primer Xeno3aR (cgcgcatccttagatgcccctctcgctgttc) contains a *BamHI* restriction site. The amplified product was cleaned and cut with *NdeI* and *BamHI* and subsequently ligated into the equivalently cleaved expression vector pET3a. The resulting plasmid was sequenced to verify that *X. trop*Ngb was successfully cloned in the pET3a vector.

The Cys of *D. rerio*Ngb, *C. ace*Ngb, *D. maw*Ngb and *X. trop*Ngb were replaced by Ser using the QuickChange™ site-directed mutagenesis method (Stratagene). The mutants bearing the Cys→Ser substitution are annotated as *D. rerio*Ngb*, *C. ace*Ngb*, *D. maw*Ngb* and *X. trop*Ngb*, respectively.

*C. ace*Ngb (H80Y117) and *D. maw*Ngb (N80H117) only differ in 2 amino acids located at position 80 and 117. Two mutants (N80Y117Ngb and H80H117Ngb) were created using the QuickChange™ site-directed mutagenesis method (Stratagene).

2.2. Expression and purification of Ngb

Recombinant expression plasmid was successfully transformed in the *Escherichia coli* strain BL21(DE3)pLysS (Invitrogen). The growth of the transformed bacteria and the overexpression of *D. rerio*Ngb, *C. ace*Ngb, *D. maw*Ngb and *X. trop*Ngb were performed as described by and purification was adapted from Dewilde et al. [19]. After expression, the cells were harvested and resuspended in lysis buffer [50 mM Tris–HCl, pH 8.0; 5 mM EDTA; 1 mM PMSF; 0.5 mM dithiothreitol (DTT)]. The resuspended cells were exposed to three freeze-thaw steps and sonicated. The extract was clarified by low (10 min at 10,700×*g*, 4 °C) and high (60 min at 105,000×*g* at 4 °C) speed centrifugation.

Further fractionation was performed by ammonium sulfate precipitation, 40–60% for *C. ace*Ngb and *D. maw*Ngb and 60% for *D. rerio*Ngb, *X. trop*Ngb, N80Y117Ngb and H80H117Ngb. The 60% ammonium sulfate pellet was dissolved in and dialyzed against 5 mM sodium phosphate pH 6.8. A DEAE-Sepharose Fast-Flow column (Amersham Biosciences) was used and the bound Ngb was eluted with 5 mM sodium phosphate pH 6.8 (8.5 for *X. trop*Ngb, 7.7 for *X. trop*Ngb*) 300 mM NaCl. The Ngb fractions were dialyzed against gel filtration buffer (50 mM Tris–HCl, pH 8.5; 150 mM NaCl; 0.5 mM EDTA) and concentrated using a Stirred Cell (Cat nr 5122, Millipore) under 2 bar air pressure. The concentrated material was loaded on a Superdex™

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