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Research Communication

Structure and Dynamics of Antarctic Fish Neuroglobin Assessed by Computer Simulations

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Summary

Neuroglobin (Ngb) is a heme protein, highly conserved along evolution, predominantly found in the nervous system. It is upregulated by hypoxia and ischemia and may have a neuroprotective role under hypoxic stress. Although many other roles have been proposed, the physiological function is still unclear. Antarctic icefishes lack hemoglobin and some species also lack myoglobin, but all have Ngb and thus may help the elucidation of Ngb function. We present the first theoretically derived structure of fish Ngb and describe its behavior using molecular dynamics simulations. Specifically, we sequenced and analyzed Ngbs from a colorless-blooded Antarctic icefish species *Chaenocephalus aceratus* and a related red-blooded species (*Dissostichus mawsoni*). Both fish Ngbs are 6-coordinated but have some peculiarities that differentiate them from mammalian counterparts: they have extensions in the N and C termini that can interact with the EF loop, and a gap in the alignment that changes the CD-region structure/dynamics that has been found to play a key role in human neuroglobin. Our results suggest that a single mutation between both fish Ngbs is responsible for significant difference in the behavior of the proteins. The functional role of these characteristics is discussed.

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INTRODUCTION

Neuroglobin (Ngb) is a heme protein, evolutionarily conserved, predominantly found in the brain and retina of vertebrates (*1*). It is transcriptionally upregulated by hypoxia and ischemia; *in vivo* and *in vitro* evidence suggests a neuroprotective role of Ngb during hypoxic stress (*2, 3*). Although many other roles have been envisaged, including scavenging of reactive nitrogen and oxygen species (*4*), signal transduction (*5*), and regulation of apoptotic pathways (*6*), the physiological function is still unclear. Protein dynamics, ligand binding, and its migration in the protein matrix have been investigated in great detail using time resolved techniques, providing important insights into structural and dynamic properties in the reactivity of mammalian Ngbs (*7, 8*). Nevertheless, the full understanding of the biochemical mechanisms explaining how Ngb may perform its function seems hindered by the (multiple) ligand-binding features.

Despite low-amino acid sequence identity (ca. 20%) between human Ngb and myoglobin (Mb), the 3D structure displays the classical globin fold (*9*), which is endowed with several unique properties. Ngb binds small gaseous ligands (oxygen, CO and NO) at the 6-coordination position of the heme iron. In the absence of an exogenous ligand, the heme iron (in the ferric and ferrous form) is 6-coordinated (6c), with distal HisE7 occupying the sixth coordination site. Distal His can be readily replaced by the external ligands. Another prominent feature of

the Ngb structure is a huge cavity, open toward the exterior that affords potential transit to ligands. Additionally, in human Ngb, two cysteyl residues in the CD region, Cys46-CD7 and Cys55-D5, may form an internal disulfide bond that modulates oxygen affinity (*10*). This regulation was not observed in zebrafish Ngb, where the first cysteine is shifted by two positions (*11*), or in murine Ngb, where Cys46-CD7 is absent (*12*).

Ngb was originally identified in mammals but is widespread in all nonmammalian vertebrates, *e.g*., the zebrafish *Danio rerio* and other teleost fishes (*11*). Mammalian and zebrafish Ngbs share about 50% sequence identity and their oxygen-binding properties are similar (*11*).

Recently, we have characterized the Ngb gene in fishes of the Antarctic ocean (*13, 14*) and cloned and sequenced the Ngb cDNA from the brain of an icefish *Chaenocephalus aceratus* (family Channichthyidae) and from the retina of the closely related, red-blooded *Dissostichus mawsoni* (family Nototheniidae). Both species belong to the suborder Notothenioidei, the predominant teleost group in the Antarctic ichthyofauna. Antarctic icefishes (16 species) lack hemoglobin, and six of the species also lack Mb. Elucidation of the icefish Ngb structure may potentially shed light on the physiological function of Ngb, especially considering apparent Ngb localization in tissues of increased oxidative metabolism and mitochondrial activity (*15*). The highest Ngb level was found in the retina, which has the highest oxygen-consuming rate in the body (*16*). Unique among vertebrates, Antarctic colorless-blooded icefishes of the family Channichthyidae lack erythrocytes and hemoglobin, and thus oxygen supply largely occurs through circulatory and diffusive flux (*17*). Although loss of globin genes in icefishes is due to relaxed selection in the oxygen-rich Antarctic marine environment is a matter of debate, but interestingly they have not lost the Ngb gene or its transcription.

In this study, we analyze the Ngb from the Antarctic icefish *C. aceratus* and the red-blooded notothenid *D. mawsoni* as well as other teleost Ngbs in comparison with mammalian Ngbs. We performed homology modeling and molecular dynamics simulations (MDS) and described the first theoretical model of fish Ngb and its behavior according to MDS. Both Antarctic notothenioid Ngbs are 6-coordinated but show peculiarities that differentiate them from mammalian counterparts.

EXPERIMENTAL PROCEDURES

Ngb Sequence

The amino acid sequences of *C. aceratus* and *D. mawsoni* Ngb were derived from cDNA sequences. The cDNA sequences were obtained by RT-PCR amplification of total RNA from retina of *D. mawsoni* and from whole brain of *C. aceratus* using appropriate primers. Briefly, total RNA was isolated using Ultraspec RNA isolation reagent (Biotecx, TX). Lock-dock oligo-dT30 primed and reverse-transcribed first strand cDNA was PCR-amplified with a notothenioid-specific 5′UTR primer, 5′GTGTGCATCTCTAGCCGAGGAATCC3′ and 5′GGAATCC TGTCTCCAACAGTTGTGTCCC3′ for *C. aceratus* and *D. mawsoni*, respectively, paired with a degenerate teleost 3′ UTR primer 5′GACCYCAYTCAMAGAGCAAATGTACAGCG3′. The cloning and sequencing process leading to the design of notothenioid-specific 5′UTR primers were detailed elsewhere (Girodano et al., 2010, submitted). In *silico* translation of the cDNA sequences provided the Ngb protein sequences used for homology modeling and were submitted to UniProt Knowledgebase under accession numbers P86880 (*C. aceratus*) and P86881 (*D. mawsoni*).

Multiple Sequence Alignment and Structure Modeling

Antarctic fish Ngb sequences together to those of mammalian and other fish species downloaded from Swiss-prot (*18*) were aligned by the 3DCoffee program (*19*) following standard parameters. The model of 6c *D. mawsoni* Ngb was generated with the Modeller9 program (*20*), using the human X-ray structure (PDB entry 1OJ6) as a template. The resulting structure was then used as starting point to generate the complete set of simulations.

Classical Molecular Dynamics

The initial structure explained above was placed in a pre-equilibrated octahedral TIP3 water box. The standard protonation state at physiological pH was assigned to ionisable amino acid residues. Special attention was paid to protonation of His residues, which were assigned on the basis of the hydrogen bond pattern with neighboring residues. For distal HisE7 and proximal HisF8, protonation was chosen to be in the Nd position. Equilibration of the system (~25,000 atoms) and running parameters were performed as described (*21*). All simulations were performed at 300 K and pressures of 1 bar using Berendsen thermostat and barostat. The Amber99 force field (*22*) was used for all residues but not for the heme, whose parameters had been developed and thoroughly tested by our team in previous work (*23–26*). All simulations were performed with the PMEMD module of the AMBER9 package (*27*). Equilibration consisted of energy minimization of the initial structures, followed by slow heating up to 300 K (4 steps of 50 ps at 150, 200, 250, and 300 K). The structure was considered to be stabilized after a 20-ns MD run of the 6c *D. mawsoni* Ngb. From this equilibrated structure, the other structures where generated by deleting the His-Fe bond (5c-state) and/or introducing the two point mutations that differentiate the two Antarctic Ngbs, followed by the same four-step equilibration protocol. For each structure, 80-ns long MD production runs were performed where the backbone root mean square distance does not exceed 1.9 Å with respect to the initial frame. Trajectories were analyzed from frames collected at 2-ps intervals.

Essential Dynamics

Dynamical differences between *D. mawsoni* and *C. aceratus* Ngbs in their 5c state were studied using essential dynamics

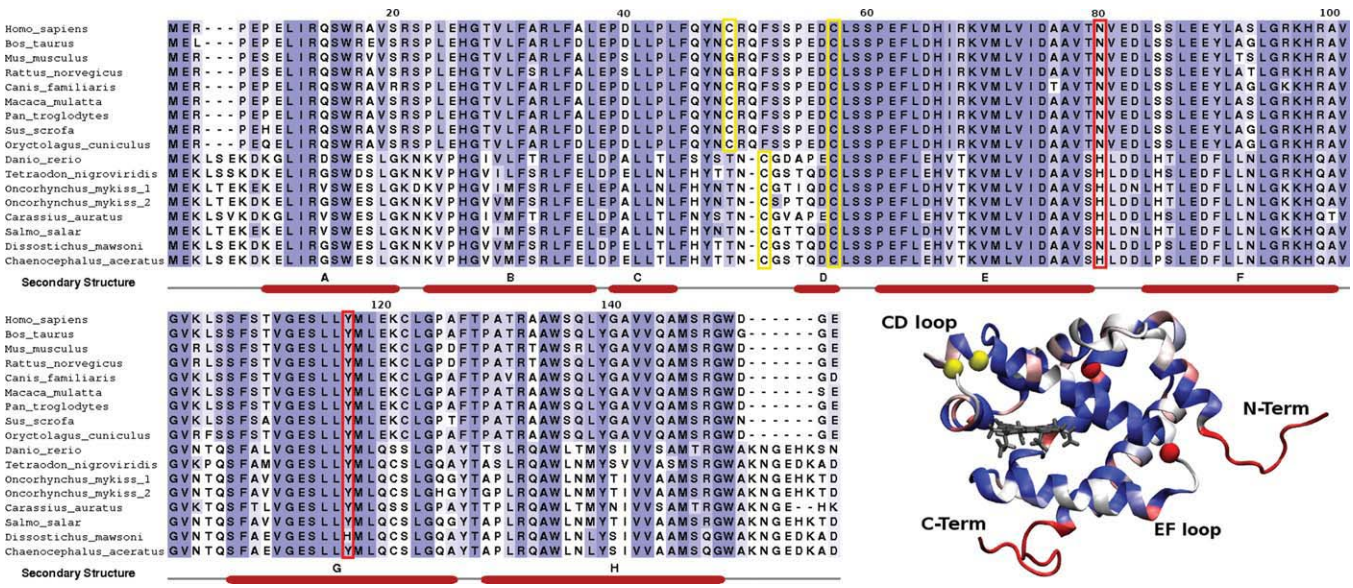

Figure 1. Sequence alignment of mammal and fish Ngbs. Residues are shaded according to sequence conservation. Relevant positions, *e.g.*, mutations between Antarctic fish proteins or conserved Cys residues, are boxed. A scheme of the secondary structure found in human Ngb with helices A-H is below the alignment. Inset: Modeled structure of fish Ngb colored by sequence conservation with human Ngb. N- and C-terminal extensions are in red, residues 80 and 117 in red circles and Cys in yellow circles. Numbering is according to fish Ngb. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(ED) analysis (*28*). ED analysis was performed with the ptraj module of the AMBER suite and consisted in the diagonalization of the covariance matrices of atomic positions along the trajectory. From them we obtained the eigenvectors that define the essential motions of the protein. To analyze the configurational space explored by the proteins, projections of their essential modes onto the last 50 ns of the MD trajectory were performed. Only backbone atoms were considered.

RESULTS

Mammalian vs. *Fish Neuroglobins*

When compared with mammalian counterparts in a multiple sequence alignment (Fig. 1), fish Ngbs display some striking peculiarities. They all show three- and six-residue extensions, composed of charged residues, in their N and C termini, respectively. The extra residues protrude toward the EF loop causing potentially relevant interactions, as discussed below. The alignment also shows a gap (position 51) in the CD region, which may be involved in aiding heme coordination and shows correlated motions with the so-called His-gate (*21, 29*). As shown in Fig. 2A, in fish Ngbs, the average Cys-Cys distance in the CD loop is several-Å shorter than in the human protein, in which Cys46-CD7 and Cys55-D5 are known to form a disulfide bridge and appear to be involved in redox-state sensing (*11, 21, 30*). Thus, although the residues are in the reduced form, they remain very close to each other during the time scale of the simulations (Fig. 2B). This short distance allows the protein to adopt a conformation, which is more suitable to form a disulfide bridge than in human Ngb, where a more important rearrangement is needed.

Apart from these differences, conservation is high in the rest of the Ngb sequences between mammals and fish, with Antarctic fish Ngbs about 55% identical to human, as well as between members of each group. Notably, sequence conservation is significantly higher among mammals than among fish species as previously described (*31*).

Red- vs. *Colorless-Blooded Antarctic Fish Ngb*

Sequence analysis of *D. mawsoni* and *C. aceratus* Ngbs shows that the only differences between these proteins are at positions 80 and 117, the icefish having His and Tyr and red-blooded *D. mawsoni* Asn and His, respectively. Both residues are located away from the heme, exposed to the solvent and distant from the dynamically relevant CD corner.

As shown by MDS, the two proteins differ in loop structure and flexibility (Figs. 2C and 2D and Table 1). Moreover, different interactions are established between the EF loop and its N-terminal region of *D. mawsoni* Ngb and the corresponding regions of *C. aceratus*. This interaction, absent in human Ngb, helps to stabilize an incipient α-helical structure in the N-terminal region. In *D. mawsoni* Ngb, formation of the H bond between Lys9:HD2 and Asn80:O involves disruption of that between Asn80:O and Asp83:H, and *vice versa* (Figs. 2C and 2D). In contrast, in *C. aceratus* Ngb, the EF loop and N-terminal interaction is present but is less specific and fluctuates contacting different residues.

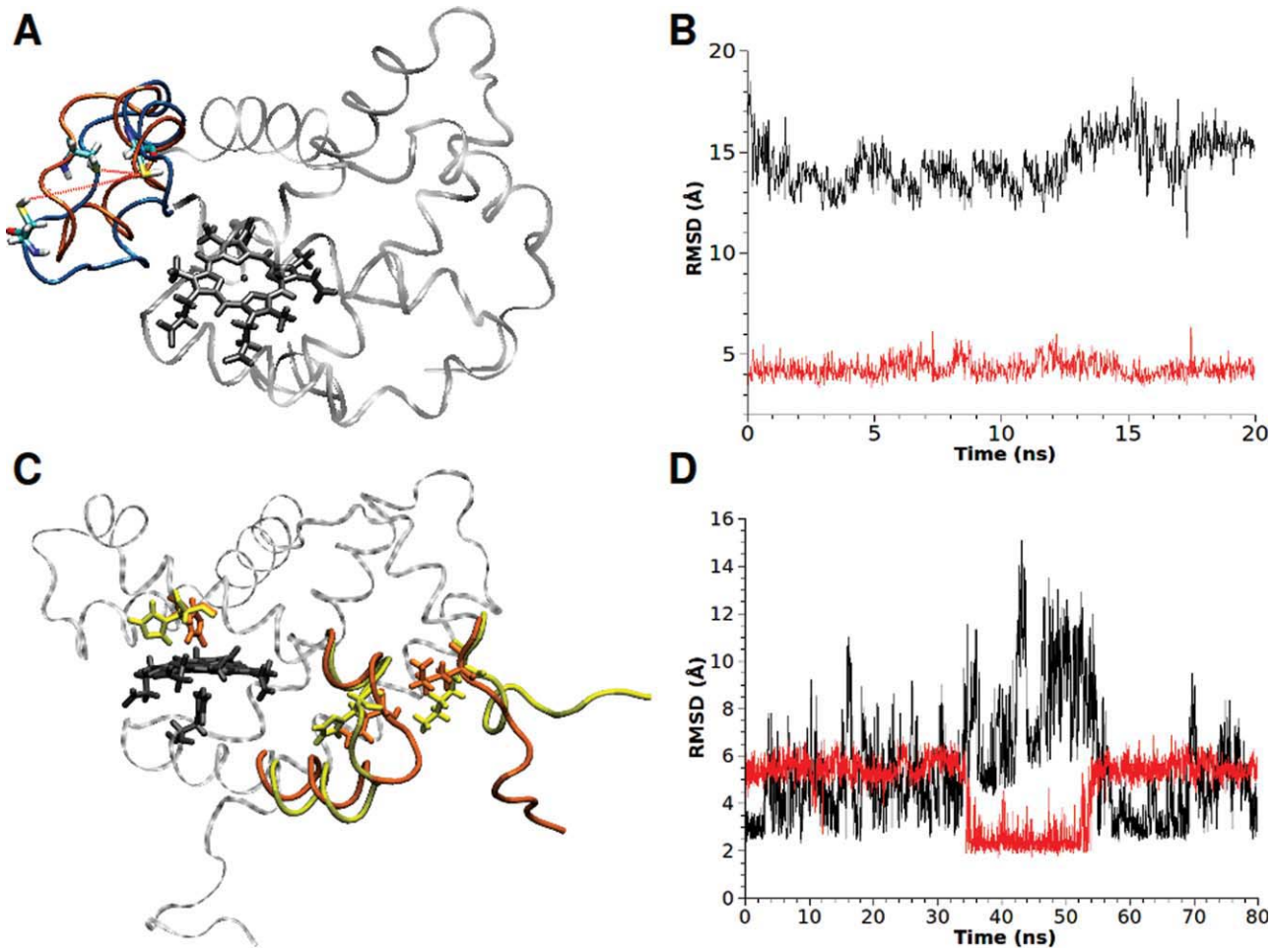

Figure 2. Peculiarities of Antarctic fish Ngbs. A: Comparison between Cys-Cys average distance in *D. mawsoni* Ngb (orange) and human Ngb (blue) at the CD loop. In human Ngb, the disulfide formation requires a relevant rearrangement of the CD region (14.4-Å average distance); in *D. mawsoni* Ngb, the two Cys are close (4.2-Å average distance) and in adequate orientation to form a bond. Similar values were obtained in *C. aceratus* Ngb (not shown). B: Time trace of the Cys-Cys distance along 20 ns for human (black) and *D. mawsoni* (red) Ngb. C: Backbone representation of *D. mawsoni* Ngb (orange) and *C. aceratus* Ngb (yellow), highlighting the changes in EF-loop conformation and its interaction with the N-terminal region in colored sticks. D: Time trace for two selected distances in *D. mawsoni* Ngb (Asn80:O-Asp83:H in red; Lys9:HD2-Asn80:O in black) illustrating the interaction between the EF loop and N termini. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

A static view of the conformation adopted by the proteins can be obtained by comparing average structures (Fig. 2, Table 1). Root mean square deviation (RMSD) values clearly show that the EF loop of *C. aceratus* and *D. mawsoni* Ngbs significantly differ and that this difference is independent of the coordination state (Table 1). Furthermore, this difference is not only static but also affects the dynamics of the protein (Fig. 3), where root mean square fluctuations (RMSF) and ED projections show a remarkably higher flexibility in the *C. aceratus* EF loop, whereas in *D. mawsoni* Ngb, the overall flexibility is spread along different loops (Figs. 3A and 3B).

In the distal site, the main consequence is that His is able to open in a "His-gate"-like movement only in *C. aceratus* Ngb in the time frame under consideration. Notably, the His opening modifies the CD-region conformation and flexibility. RMSD values for this region (Table 1) show that *C. aceratus* Ngb is significantly different in the 5c state after His opening. Furthermore, RMSF values and ED analysis show that CD region becomes less flexible (Figs. 3A and 3B). All these conformational and dynamical differences are dictated by the replacement of Asn 80 by His, as they can be reverted by mutating these residues. This experiment consisted in converting *C. aceratus* Ngb to *D. mawsoni* Ngb at position 80 after the conformational change that the loop suffer was acquired and stabilized. Within 5 ns after reversion, the EF loop readopted the conformation consistent with the corresponding residue at position 80. This

Table 1
RMSD between average structures for both fish Ngbs

			C. aceratus Ngb		*D. mawsoni* Ngb	
			5c	6c	5c	6c
CD region	*C. aceratus* Ngb	5c		**0.64**	**0.75**	**0.77**
		6c	**0.82**		0.82	0.79
	D. mawsoni Ngb	5c	**0.96**	0.79		0.53
		6c	**0.92**	0.74	0.54	
EF region	*C. aceratus* Ngb	5c		0.8	**0.81**	**0.8**
		6c	0.82		**0.52**	**0.45**
	D. mawsoni Ngb	5c	**0.96**	**0.79**		0.54
		6c	**0.92**	**0.74**	0.54	

Average structures for both fish Ngbs either in the 5c or the 6c states for the last 50 ns were compared and RMSD calculated. We focused our analysis in the conformation of the CD region (upper) and the EF region (lower). The values above the diagonal inform on the RMSD excluding the loop regions (residues 40–60 for the CD region and residues 75–95 for the EF region), while those below the diagonal inform on the total RMSD. Highlighted in bold are values where differences between including and excluding CD or EF regions are higher, implying a significantly different loop conformation between protein species.

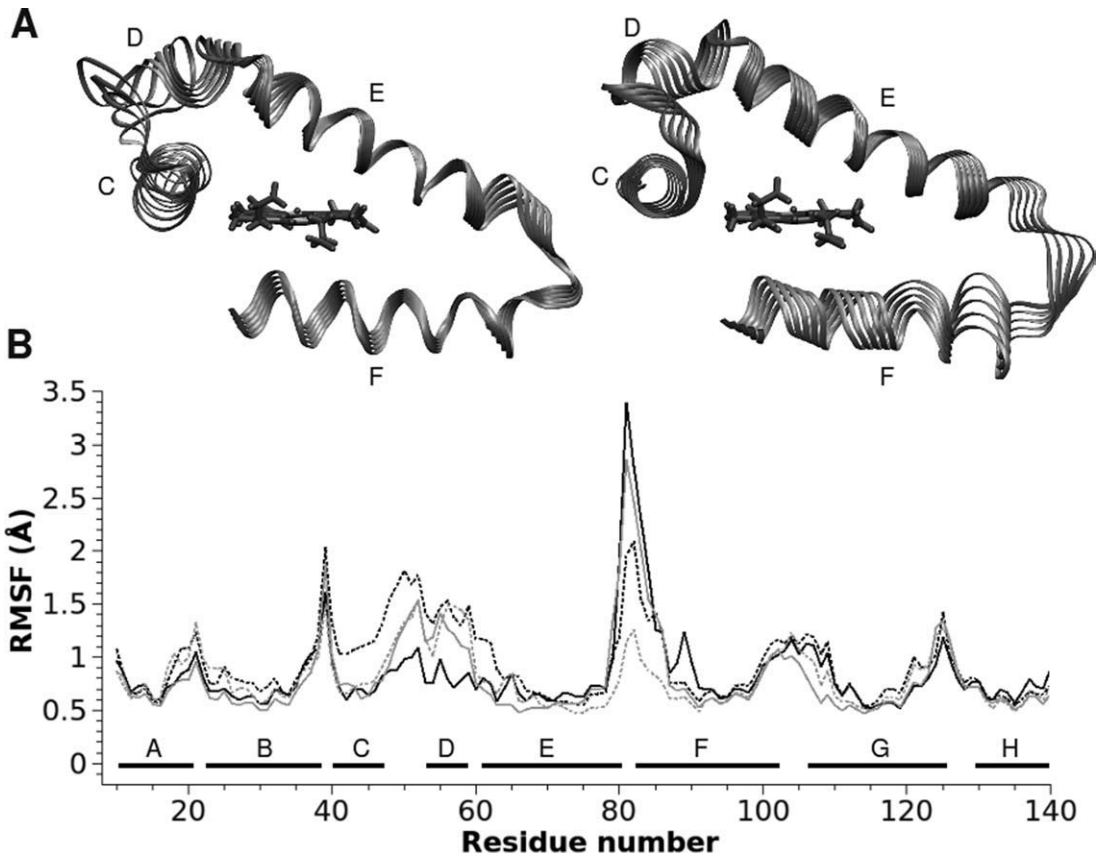

Figure 3. Dynamical differences between fish Ngbs. A: Projection of the normal mode with highest amplitude in *D. mawsoni* (left) and *C. aceratus* (right). B: Root mean square fluctuations along the last 50 ns in *D. mawsoni* (dotted line) and *C. aceratus* (continuous line). 5c-species are in black and 6c-species in gray.

implies that the single mutation modifies EF loop conformation and dynamics and propagates, through the heme-coordinating His, up to the CD region in the opposite part of the protein.

DISCUSSION

Isolation and environmental history have shaped a unique Antarctic marine biota. Many fish groups became extinct because of the constraints of increasingly cold and icy conditions, and the cold-adapted and antifreeze-protected notothenioids emerged as the predominant teleost taxon (*32, 33*). In the modern notothenioid family Channichthyidae, mutational events led to remnant α-globin genes and complete deletion of the genes encoding the β globins of hemoglobin (*34–36*). Six of the 16 icefish species including *C. aceratus* also fail to produce cardiac Mb (*37*). The lack of these globins is closely correlated with compensatory changes in the icefish anatomy, *e.g.*, larger heart and gills and blood volume (*38*).

Because oxygen transport and supply must be achieved without hemoglobin and Mb, icefishes are an excellent system to investigate possible enhancement of other factors to compensate for the loss of these essential hemoprotein functions. Icefishes serve as the natural knock-outs for functional studies of oxygen-binding hemoproteins and the correlated nitrogen-monoxide-oxygenase activity. Current research in mammals suggests that nearly all icefish hallmark traits are linked to high levels of NO (*39*).

To our knowledge, this is the first structural modeling and MD study on such proteins from Antarctic fish. When compared with mammals, fish Ngbs display some striking peculiarities in regions considered relevant for protein function: (*i*) Ngbs of Antarctic and temperate fish have extensions of charged amino-acid residues at the N and C termini. In the zebrafish *D. rerio*, the N-terminal region was demonstrated to be implied in cell-penetrating capability (*40*). These extra residues extend toward the EF loop; (*ii*) Antarctic and temperate fish Ngbs are shorter by one residue in the CD region, involved in heme coordination and in the "His-gate." The shorter loop approaches the two Cys that form the disulfide bridge in human Ngb so that the Cys-Cys distance is several Å shorter. The residues remain very close during the simulations, allowing easier formation of the disulfide bridge than in human Ngb. Disulfide formation in human Ngb has been experimentally shown to decrease protein flexibility (*41*), particularly, in the CD region. This in turn enhances O_2 affinity about 10-fold by stabilizing the 5c state, making the protein adopt a conformation prone to bind exogenous ligands (*10, 21*).

The UV–vis spectra of ferric and ferrous form of both *C. aceratus* and *D. mawsoni* Ngbs proteins are typical of 6c-state (Giordano et al., unpublished) in analogy with other Ngbs (*42*). Preliminary results obtained using multiple steered molecular dynamics and the Jarzynski equality as in Nadra et al. (*21*) show that the 6c-state is preferred in both Ngbs by about 6 kcal mol^{-1}. Unexpectedly, but consistent with the Cys-Cys distance, the overall conformation of the 5c state for both proteins is much more similar to the oxidised state of human Ngb (with the Cys residues forming an intramolecular disulfide bond).

A single amino-acid replacement appears sufficient to induce much higher flexibility in *C. aceratus* Ngb in comparison with red-blooded *D. mawsoni*. MDS analysis suggests that the Asn→His substitution in position 80 produces changes in the conformational and dynamical features in the icefish protein. In the distal domain, the main consequence is the "His-gate"-like movement, causing rearrangement of the CD region that correlates with EF-loop movements. These differences are dictated by this replacement, because they disappear by reversal mutation. No effect is associated to the Tyr→His replacement at position 117.

Adaptive changes appear restricted to regions that influence conformational mobility. This finding has important implications for rates of protein evolutionary adaptation, because a single substitution is sufficient for potential functional shifts. As these sequence differences are far from the active site or previously described relevant regions for protein function, in principle, we did not expect these mutations to be very functionally relevant. To our surprise, this appeared to be true only for the Tyr117→His mutation but not for His80→Asn. The latter is located in the EF loop, which connects the helices, which include heme-coordinated His, and is very close to the N-terminal extension. Although this loop is less variable in sequence than the CD region, it displays flexibility (*21, 29*) and may indeed have a relevant role in heme coordination.

These results support the general hypothesis that alterations in protein conformational mobility can happen through one/few substitutions, providing insights into the evolutionary rates at which adaptive change may occur (*43, 44*). They also indicate that a small change in the primary structure, namely a short-term response, may be very efficient as such for generating an adaptive response to a challenge.

However, prudence should be exercised in assigning a general value to these conclusions. First, one needs to be cautious in interpreting whether the single change in primary structure is due to adaptation or phylogenetic variation (or both, as they are not mutually exclusive). Nonetheless, we must take the icefish exceptional oxygen-transport system into account. In the icefish brain and retina, the delivery of oxygen by diffusion could be highly insufficient. Thus, Ngb may also have the task to fulfil the role of a classical oxygen carrier, and the conformational flexibility of Ngb can be included into the suite of anatomical and physiological compensations that icefish had to engineer as a result of the evolutionary loss of hemoglobin and Mb. To support this hypothesis, Ngb concentration and oxygen affinity should be measured for these species and be high enough to accomplish the proposed function. Second, His 80 is present in all fish sequences except in *D. mawsoni*. However, in these sequences, additional substitutions distributed in key positions in the structure may well mask the effect at position 80 in icefish Ngb.

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