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EPR analysis of cyanide complexes of wild-type human neuroglobin and mutants in comparison to horse heart myoglobin

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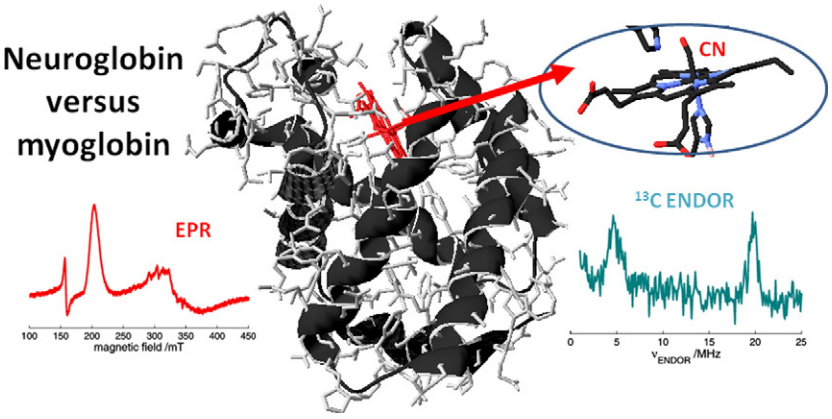
HIGHLIGHTS

- First reported EPR study of cyanide-ligated neuroglobins.
- First determination of the full ^{13}C hyperfine tensor of the cyanide carbon in a cyanide-ligated globin.
- Disagreement of ^{13}C hyperfine data of cyanide-ligated myoglobin with predictions from NMR reveal flaws in theortical model.
- The present ^{13}C hyperfine data challenge the current interpretation of ^{13}C NMR shifts in cyanide-ligated haem proteins.

GRAPHICAL ABSTRACT



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ABSTRACT

Electron paramagnetic resonance (EPR) data reveal large differences between the ferric (^{13}C-)cyanide complexes of wild-type human neuroglobin (NGB) and its H64Q and F28L point mutants and the cyanide complexes of mammalian myo- and haemoglobin. The point mutations, which involve residues comprising the distal haem pocket in NGB, induce smaller, but still significant changes, related to changes in the stabilization of the cyanide ligand. Furthermore, for the first time, the full ^{13}C hyperfine tensor of the cyanide carbon of cyanide-ligated horse heart myoglobin (hhMb) was determined using Davies ENDOR (electron nuclear double resonance). Disagreement of these experimental data with earlier predictions based on ^{13}C NMR data and a theoretical model reveal significant flaws in the model assumptions. The same ENDOR procedure allowed also partial determination of the corresponding ^{13}C hyperfine tensor of cyanide-ligated NGB and H64QNGB. These ^{13}C parameters differ significantly from those of cyanide-ligated hhMb and challenge our current theoretical understanding of how the haem environment influences the magnetic parameters obtained by EPR and NMR in cyanide-ligated haem proteins.

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1. Introduction

Since its discovery in 2000 [1], neuroglobin (Ngb) has been intensively studied. This haem-containing globular protein has the typical globin fold consisting of 8 α-helices (A to H) and it is expressed in the brain of a variety of vertebrates, such as mammals, fish, amphibians, birds and reptiles [1–4]. It has about 25% sequence identity with vertebrate haemoglobin (Hb) and myoglobin (Mb) [5]. Interestingly, it is retained in the Antarctic icefish *Chaenocephalus aceratus* that lacks Hb and Mb [6]. The physiological role of Ngb is still unclear [7]. Possible involvement in protection against apoptosis, removal of reactive oxygen or nitrogen species and a role in ligand sensing have been proposed [7].

In the absence of exogenous ligands, the haem iron in the ferric and ferrous forms of Ngb is ligated to two histidines, namely His96 at position 8 of the F-helix, which is highly conserved in all globins, and His64 at position E7 on the distal side of the haem [8–11] (supplementary information, Fig. S1). This hexa-coordination of the haem-iron contrasts to ferrous mammalian Hb and Mb, in which the haem is only ligated to F8His leaving the distal side open for coordination of exogenous ligands. Due to competition with the endogenous distal HisE7, Ngb shows a slower binding of exogenous ligands, although the intrinsic affinity of the penta-coordinated form to bind O_2 and CO is quite high [12–14]. Moreover, in ferrous human neuroglobin (NGB), the His-binding affinity is found to decrease by a factor of ten due to a disulfide-bridge formation between Cys55 (position 5 of the D helix) and Cys46 (at position 7 of the loop connecting helices C and D) [13]. This leads to a concomitant increase in the O_2 affinity, suggesting that the release of O_2 in NGB is regulated by the redox state of the disulfide bridge. Formation of the disulfide bridge is found to alter the haem-pocket structure [15], a process in which Phe28 (position B10) plays an important role [16].

A crystallographic study revealed that an unusual sliding motion of the haem occurs when CO ligates to the ferrous haem of murine Ngb (mNgb) [17]. Although molecular dynamics (MD) simulations corroborate the haem-sliding ability [18,19], MD simulations comparing crystal and solution conditions also revealed that the experimentally observed dynamics of the haem group may be affected by the crystal packing [18]. Since the Fe(III)–CN^- complex in ferric cyanide-ligated globins is believed to be isostructural and isoelectronic to the Fe(II)–CO complex, it comes as no surprise that cyanide binding to Ngb and related mutants has been investigated [14,20–23]. Except for the cyanide-affinity determination of NGB [14], all currently reported information on cyanide-ligated Ngb variants is based on NMR studies [20–23]. NMR data revealed haem orientational disorder in ferric wild-type (wt) mNgb [20] and NGB [24] and in cyanide-ligated forms of ferric wild-type NGB [23], mNgb [20] and related mutants [22,23]. The disorder consists of a 180° haem rotation about the α–γ-meso axis (see Fig. S2, supplementary material). The cyanide ligation rate is faster for one of the two haem orientations ([20,23], Table S1). In NGB, this is attributed to the lower stability of the distal His-iron coordination bond and a reduced steric hindrance at the bottom of the haem cavity in this conformer, allowing for haem sliding [23]. The haem orientational disorder is found to persist in the H64Q/V68F mNgb double mutant [22]. Formation of the earlier mentioned disulfide bridge in NGB also influences the cyanide binding rate [23].

Although electron paramagnetic resonance (EPR) has often been used to characterize ferric cyanide-ligated globins [25–31], no EPR data of cyanide-ligated Ngbs have been reported so far. In the present work, continuous-wave (CW) and pulsed EPR techniques are used to investigate the cyanide-ligated ferric form of wild-type NGB and its H64Q and F28L mutants (mutations on positions E7 and B10, respectively). The mutants were chosen to test the influence of two key amino acids in the distal haem-pocket region. All findings are compared in detail with reported EPR data on other cyanide-ligated ferric globin forms. Furthermore, although ^{13}C NMR of the iron-bound cyanide in ferric cyanide complexes of haem proteins has been shown to reveal important information about the haem reactivity [32–35], only a few studies have attempted to determine the full ^{13}C hyperfine tensor of the cyanide carbon of such haem complexes [26,36]. Here, Davies ^{13}C-ENDOR is applied to extract information on this tensor for cyanide-ligated NGB variants in comparison to those of the cyanide complex of horse heart myoglobin (hhMb-CN).

2. Materials and methods

2.1. Cloning, expression and purification of NGB and its H64Q and F29L mutants

The QuickChangeTM site-directed mutagenesis method (Stratogene) was used to make the F28LNGB and H64QNGB mutants as described earlier [16,37]. Expression of wt NGB and its mutants was done as reported earlier [8]. In short, the plasmids (cDNA cloned in pET3a) were transformed into *E. Coli* strain BL21(DE3)pLysS. Cells were grown at 25 °C in a TB medium containing 1.2% bactotryptone, 2.4% yeast extract, 0.4% glycerol, 72 mM potassium phosphate buffer, pH 7.5, 200 μg/ml ampicillin, 30 μg/ml chloramphenicol and 1 mM δ-amino-levulinic acid. After induction, the cells were grown overnight, and subsequently harvested, resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 0.1 mM EDTA, 0.6 mM dithiothreitol) and exposed to three freeze–thaw steps. Sonication was used until the cells completely lysed. The extract was clarified by low (10 min at 10,000 *g*) and hi% gh (60 min at 105,000 × *g*) speed centrifugation. The obtained supernatant was collected and fractionated by 60 ammonium sulfate precipitation. The pellets were dialyzed against 5 mM Tris–HCl, pH 8.5 and loaded onto DEAE-Sepharose Fast Flow column for ion exchange chromatography. The wt NGB or NGB mutant (F28L or H64Q) was eluted with 200 mM NaCl from the sepharose. The NGB fractions (wt, F28L or H64Q) were concentrated by Amicon filtration (PM10) and passed through a sephacryl S200 column for gel filtration. They were then stored at −20 °C. SDS-PAGE screening was done for testing the protein purity.

2.2. Preparation of the ferric cyanide-ligated forms

Ferric horse heart myoglobin (hhMb) was puchased from Sigma-Aldrich as lyophilized powder. This powder was then dissolved in a 50 mM Tris–HCl buffer at pH 8.0. KCN (all nuclei in natural abundance) and $K^{13}CN$ were bought from Sigma-Aldrich. The ferric cyanide-ligated forms of the NGB variants and of hhMb were prepared by adding a 40 times excess of KCN or $K^{13}CN$ to the ferric proteins. Formation of the cyanide-ligated form was checked by optical absorption spectroscopy. For EPR measurements, 20 wt.% of the cryoprotectant glycerol was added. Final concentrations were ca. 2 mM for hhMb-CN, 0.8–1 mM for NGB-CN and H64QNGB-CN, and ~0.4 mM for F28LNGB-CN (and their analogues with $^{13}CN^-$).

2.3. EPR spectroscopy

X-band CW-EPR measurements were performed on a Bruker ESP 300E spectrometer (microwave frequency 9.44 GHz) equipped with a liquid Helium cryostat (Oxford Inc.). EPR spectra were obtained with a modulation frequency of 100 kHz, a modulation amplitude of 0.5 mT and a microwave power of 1 mW at a temperature of 10 K.

X-band pulsed EPR experiments were performed at 7 K on a Bruker Elexsys instrument equipped with Helium cryostat (Oxford Inc.).

Single matched resonance transfer hyperfine sublevel correlation (SMART-HYSCORE) experiments were performed using the sequence HTA-t_1-π-t_2-HTA-τ-π-τ-*echo* [38] with a high-turning angle (HTA) pulse of length 32 ns, a π pulse of 16 ns and a τ value of 120 ns. A microwave field strength of 15.625 MHz was used. The HYSCORE traces were baseline corrected using a third-order polynomial, apodized with a Hamming window and zero-filled. After Fourier transformation absolute-value spectra were computed.