The redox state of the cell regulates the ligand binding affinity of human neuroglobin and cytoglobin

Djemel Hamdane¹, Laurent Kiger¹, Sylvia Dewilde², Brian N. Green³, Alessandra Pesce⁴, Julian Uzan¹, Thorsten Burmester⁵, Thomas Hankeln⁶, Martino Bolognesi⁴, Luc Moens² and Michael C. Marden¹

¹ Inserm U473, 94276 Le Kremlin-Bicêtre, France
² Department of Biomedical Sciences, University of Antwerp, B-2610 Antwerp, Belgium
³ Waters Corporation, Micromass UK Ltd, Atlas Park, Manchester, M22 5PP, UK
⁴ Department of Physics, INFM Advanced Biotechnology Centre, University of Genova, I-16146 Genova, Italy
⁵ Institute of Zoology, Johannes Gutenberg University of Mainz, D-55099 Mainz, Germany
⁶ Institute of Molecular Genetics, Biosafety Research and Consulting, Johannes Gutenberg University of Mainz, D-55099 Mainz, Germany

Correspondence: Michael C. Marden
Inserm U473, 84 rue du General Leclerc
94276 Le Kremlin-Bicetre Cedex, France
E-mail address: marden@kb.inserm.fr

Key words: neuroglobin, cytoglobin, oxygen binding, ligand kinetics, disulfide bond, mass spectrometry

Abbreviations: Hb: hemoglobin; Mb: myoglobin; Ngb: neuroglobin; NGB human neuroglobin; Cygb: cytoglobin; CYGB: human cytoglobin; TNB: 5-thio-2-nitrobenzoic acid; DTT: dithiothreitol; ESI-MS: electrospray ionization mass spectrometry; m/z: mass-to-charge ratio; hx: hexacoordinated; T1, T2, etc: tryptic peptides.
ABSTRACT

Neuroglobin and cytoglobin reversibly bind oxygen in competition with the distal histidine, and the observed oxygen affinity therefore depends on the properties of both ligands. In the absence of an external ligand, the iron atom of these globins is hexacoordinated. There are three cysteine residues in human neuroglobin; those at positions CD7 and D5 are sufficiently close to form an internal disulfide bond. Both cysteine residues in cytoglobin, although localized in other positions than in human neuroglobin, may form a disulfide bond as well. The existence and position of these disulfide bonds was demonstrated by mass spectrometry and thiol accessibility studies. Mutation of the cysteines involved, or the use of reducing agents to break the S-S bond, led to a decrease in the observed oxygen affinity of human neuroglobin by an order of magnitude. The critical parameter is the histidine dissociation rate which changes by about a factor of 10. The same effect is observed with human cytoglobin, although to a much lesser extent (less than a factor of 2). These results suggest a novel mechanism for the regulation of oxygen binding; contact with an appropriate electron donor would provoke the release of oxygen. Hence the oxygen affinity would be directly linked to the redox state of the cell.
INTRODUCTION

Two new globins, neuroglobin (Ngb) and cytoglobin (Cygb), were recently added to the vertebrate globin family (1). Ngb is predominantly expressed in the brain, the retina and other nerve tissues, whereas Cygb is expressed in all organs studied so far. While Ngb is clearly localized in the cytosolic compartment of the cell (2-4), the cellular and subcellular distribution of Cygb is still being investigated (2).

Human NGB and CYGB are composed of 151 and 190 amino acids, respectively, with the presence in CYGB of amino- and carboxy-terminal extensions of ~20 residues each. Although both globins display the structural determinants of the globin fold (5), they share little sequence identity with vertebrate hemoglobin (Hb) and myoglobin (Mb) (6-11).

Ngb and Cygb are hexacoordinated (hx), either in their ferrous or ferric forms, having the distal HisE7 as the internal ligand. Flash photolysis studies of Ngb, at normal temperature, show high recombination (k_{on}) and low dissociation (k_{off}) rates for O2 and CO, suggesting a high intrinsic affinity for both ligands. However, since the rate-limiting step in ligand binding to the ferrous deoxy-hx form involves dissociation of the distal HisE7 residue, ligand binding in vivo is suggested to be low (P_{50} = 1 Torr) (6, 12), although a higher oxygen affinity (due to a lower affinity for the distal histidine) was reported (10-11).

Cytosolic hx-Hbs are also observed in bacteria, unicellular eukaryotes (13), plants (14) and some invertebrates (15-16). It can be hypothesized that hx-Hbs are universally distributed over the living world and thus may have essential function(s) in cell metabolism.

The physiological role of hx-Hbs, mostly expressed at low level, is not well understood. Several roles have been suggested. First these proteins may scavenge O2 under hypoxic conditions and supply it for aerobic respiration (6-7, 17-18). Second, they may function as terminal oxidases by oxidizing NADH under hypoxic conditions and hence enhance ATP production by glycolysis (19). Third, they could be O2 sensor proteins activating other proteins with regulatory function (20-21). Fourth, they may be involved in NO metabolism.

Sun and collaborators (17-18) demonstrated that Ngb is upregulated under hypoxic conditions, in vivo and in vitro, and that it protects the neuron against the deleterious effects of the hypoxia and ischemia. How Ngb protects neurons from hypoxia or ischemia is unclear.

Initial ligand binding studies indicated a heterogeneity in the kinetics (12) or possible relaxation phenomena (10), and measurements over a wide range of temperature suggested the presence of multiple conformations in Ngb-CO (21). Distal heme pocket heterogeneity in NGB was also observed by Raman spectroscopy and NMR (22-24). Unlike most other
globins, NGB and CYGB contain 3 and 2 cysteine residues respectively. The bonding of these cysteines may play a major role in the observed heterogeneity. In this study we focus on the structural and functional role of these residues.

MATERIALS AND METHODS

Expression cloning and purification of recombinant neuroglobin and cytoglobin.

Expression cloning and purification of wild type and mutant mouse and human neuroglobin was performed as described previously (12). Human CYGB cDNA in the expression plasmid pET3a (7) was expressed under the same conditions except δ-amino-laevulinic acid was omitted. Expressed CYGB was purified from inclusion bodies using the procedure described by Geuens et al (2). Reconstruction of the native CYGB from the apoprotein was undertaken by adding a 1.4 fold excess of hemin hydrochloride (stock solution in 0.1 M NaOH and diluted in 50 mM Tris-HCl at pH 7.5 immediately before use) followed by dialysis against 50 mM Tris-HCl at pH 8.5. After reconstruction, insoluble material was eliminated by centrifugation (10,000 rpm; 30 min). Final purification of CYGB was performed by gel filtration using a Sephacryl S200 column equilibrated in 50 mM Tris-HCl at pH 8.5. Mutation of both cysteines (at positions B2 and E9) in the CYGB sequence to serines was performed as described for NGB (12).

A mini CYGB, missing the amino-terminal residues 1-17 and the carboxy-terminal residues 165-190 was constructed. The 5' and 3' ends of the 570 bp coding regions of the human CYGB were deleted by PCR by using an oligonucleotide primer (GAGAGGCATATGGAGCTGTCCGAGGCGGAG) that incorporates an NdeI site at bp 46, and a primer (ACCCAGAGATCTTACTTGTAGGCTGCGGTC) that introduces a stop codon at bp 502 plus a BglII site at bp 505. The Pfu-polymerase was used to minimize amplification errors. The PCR-products were cloned in the pET3a vector using the NdeI and BamHI restriction sites. The clones were verified by sequencing before expression.

Analyses and separation of different neuro- and cytoglobin subforms. Samples of NGB and CYGB were further purified with an Akta purifier system on a Hitrap DEAE Sepharose column from Amersham Pharmacia Biotech (Biosciences). This was done with or without the prior incubation in 1 mM dithiothreitol (DTT) for one hour at 37°C. Because of the low pI of the NGB and CYGB the samples were loaded onto the column equilibrated with
20 mM phosphate buffer at pH 8.0. After a pH decrease to 6.5 (20 mM bis-tris HCl) the protein elution was initiated at 100 mM NaCl from a selective salt gradient. Protein elution was monitored at 280 nm and 410 nm. The fractions of the protein were dialyzed against 100 mM phosphate buffer at pH 7 and its purity was assessed by spectroscopic analysis and by gel filtration on a Superose\textsuperscript{R} 12 HR10/30.

Reaction and binding kinetics of neuroglobin and cytoglobin with thiol reagents. 5,5\textsuperscript{'}-Dithiobis(2-nitrobenzoic acid) (DTNB) was used to examine the reactivity of the sulfhydryl groups in the native NGB as described by Jocelyn (25). The reaction was performed at pH 7 in 100 mM potassium phosphate buffer and 100 µM DTNB, with a protein concentration of 5-6 µM protein, determined from the Soret band for the carboxylated species using the extinction coefficient $\varepsilon_{420} = 190$ mM$^{-1}$ cm$^{-1}$. The absorption at 412 nm, corresponding to the TNB anion ($\varepsilon_{412} = 13.6$ mM$^{-1}$ cm$^{-1}$), was continuously monitored over 4 hours and corrected against a blank to which no protein was added. The reaction is $\text{TNBS-}S\text{TNB} + \text{Cys} \rightarrow \text{TNB} + \text{TNBS-}S\text{Cys}$, where TNB is the species detected by visible spectroscopy.

Human HbA, as well as the alpha and beta globin chains, were used as controls. Under denaturing conditions (8 M guanidinium chloride) these samples showed the same signal amplitude per cysteine; this value was taken as 100% reactivity. The beta chains show about half (44%) reactivity under native conditions: 20 mM phosphate buffer at pH 7.

Ligand binding kinetics. Spectral measurements were carried out with a HP8453 or Varian Cary 400 spectrophotometer. Laser flash photolysis and stopped-flow rapid mixing, the methods used to assess hexacoordination and bimolecular CO and O\textsubscript{2} rate constants, have been described previously (12). Photolysis was performed with 10 ns pulses at 532 nm. Detection of the sample absorption was in the Soret band, typically at 436 nm, using a 50 W lamp and interference filters. Samples from 1 to 10 µM were in 4 x 10 mm quartz cuvettes.

The ligand binding kinetics of proteins with reduced disulfide bond was obtained after incubation of the mouse Ngb and human NGB with 10 mM DTT during 24 hours. The transition from the cysteine oxidized form (capable of forming the disulfide bond) to the reduced form could be followed by measuring the CO binding kinetics at various times after addition of DTT.

Sample preparation and electrospray ionization mass spectrometry. Native samples were infused (5 µL/min) into the mass spectrometer (Quattro Ultima, Micromass Ltd, Wythenshawe, UK) at ~5 µM concentration in 1:1 acetonitrile:water containing 0.2% formic
acid. Data were acquired over the mass-to-charge ratio (m/z) range 600-2000 (5 min) and deconvoluted in order to present the spectra on a molecular weight (mass) scale using the maximum entropy (MaxEnt) based software supplied with the spectrometer. Mass scale calibration employed the series of ions with multiple charges from separate introductions of Mb (sequence mass 16951.5 Da).

Reduced samples were prepared by reacting the native samples (20 µM concentration) with 5 mM DTT in 10 mM ammonium bicarbonate (pH 8-9) for 10 min at 37°C. The resulting solution was then diluted 5-fold in 1:1 acetonitrile:water containing 0.2% formic acid and infused directly into the mass spectrometer as described above.

Reduced and S-carboxyamidomethylated (Cam) samples were prepared by reacting with 5 mM DTT and 5 mM iodoacetamide in 10 mM ammonium bicarbonate/8 M urea and incubated for 10 min at 37°C. Excess reagents were removed from the resulting solutions by washing each of them twice with water as follows. First each solution was centrifuged to dryness in a 10 kDa cut-off centrifugal filter device (Centricon YM10, Millipore Corp., Bedford, MA). Then the proteins retained on the filter were dissolved in 200 µL of water and the solution centrifuged to dryness again. This last procedure was repeated once and the washed proteins finally dissolved in water. Aliquots of the washed reduced and Cam solutions were diluted to ~5 µM concentration in 1:1 acetonitrile:water containing 0.2% formic acid and analyzed as described above or digested with trypsin.

Cam samples were prepared and washed as described for the reduced and Cam samples except that the DTT was omitted from the reaction mixture.

Samples were digested with trypsin for 30-80 min using procedures that were developed for identifying variants in human Hb (26) and adapted to suit the smaller quantities available in this work. Digest mixtures were analyzed by ESI-MS without prior separation of the tryptic peptides. Mass scale calibration used the tryptic peptide peaks from a separate introduction of a normal human Hb digest.
RESULTS AND DISCUSSION

Occurrence of cysteine residues in globins

In globin sequences, cysteine residues are rather scarce and occur at well-defined positions suggesting specific functions. Our globin database, an updated version of that reported in Kapp et al. (27), consists of 811 sequences: 260 alpha, 272 beta, 58 Mb, 12 Ngb, 5 Cygb, and 204 invertebrate sequences. There is a dominant occurrence of Cys at positions G11 (228/260) and G18 (51/260) for the alpha chains, and at positions F9 (234/272), G14 (65/272) and G15 (10/272) for the beta chains (Fig 1A, B). No specific clustering of Cys residues occurs in the globin sequences of invertebrates, except in nematodes (A8 and E15) and in annelids (NA2 and H11).

The role of cysteine residues in globins is most likely the same as in proteins in general, namely, structural by the formation of intra- or inter-molecular disulfide bonds, or catalytic by being part of an active site or a place of direct ligand binding.

Intra-molecular disulfide bonds are well documented in many proteins but so far have never been observed in vertebrate globins. Inter-molecular disulfide bonds between globin chains are unknown in vertebrate Hbs and Mbs but are essential in some invertebrate extracellular Hbs in order to form giant aggregates (~3.5 x 10^6 Mr; Annelids: hexagonal bilayer Hbs), which are necessary to avoid elimination of the Hb molecule from the hemolymph by excretory processes (28-29). The specific S-nitrosylation of vertebrate HbO2 at Cys93 of the β chain is proposed to be part of a dynamic transport cycle of NO leading to control of blood pressure and facilitating efficient delivery of oxygen to tissues (30).

The available Ngb sequences display conserved Cys at positions CD7, D5 and G18/19. There are two exceptions: the Rodentia Ngb are missing the CD7 Cys and the zebrafish Ngb the G18/19 Cys. Based on the crystal structure of the NGB (31), sites CD7 and G19 host solvent accessible residues, while the D5 residue is virtually solvent inaccessible.

The cysteine residues in CYGB occur at positions B2 and E9, different from those in Ngb, and are less conserved. Although the number of Ngb and Cygb sequences available is rather small, the relative conservation of the Cys residues may suggest a functional significance.

Chromatographic analysis of human neuro- and cytoglobin.

Considering the potential effect of disulfide bonds in both molecules, the separation of “crude” NGB and CYGB preparations into fractions by ion-exchange and gel filtration chromatography was performed. Crude recombinant NGB was separated into three fractions, F1, F2 and F3 by DEAE ion exchange chromatography with gradient elution, with F1 being the major fraction. If the
protein solution was first incubated in 1 mM of DTT for one hour at 37°C, there was only one (monomeric) fraction, corresponding to F1, in the ion-exchange chromatography. This suggests the presence of a dimeric fraction in the crude extract, as confirmed by HPLC gel filtration analysis.

The same preparation for CYGB, with or without DTT, gave mainly a band corresponding to a dimeric form; dilution experiments did not show a transition to the monomeric form; these results indicate a stable dimer not based on disulfide bonds. However, the mini Cygb, corresponding to the central globular fragment, was eluted as a monomer.

**Accessibility studies.** The reactivity of the cysteines can be probed by reaction with thiol reagents such as DTNB and iodoacetamide. DTNB binding experiments, under native or denaturing (8M guanidinium chloride) conditions, show that about 35% of the cysteines are reactive for WT NGB (Fig. 3), indicating the presence of a disulfide bond.

The NGB mutant C(G19)ÆS with only two cysteines (CD7 and D5) showed essentially no reaction with DTNB (Table I), under normal or denaturing conditions, indicating that cysteines CD7 and D5 were involved in a disulfide bond. The presence of a CD7/D5 disulfide bond was also recently suggested (32). Overall, these binding studies indicated the presence of disulfide bonds, but more information was necessary to fully determine all the possible bonding patterns, including dimers via intermolecular bonds.

WT mouse Ngb, or mutants with either the D5 or G19 cysteine changed to serine, show nearly 100% reactivity with DTNB. Note that cysteine D5 reacted more slowly than that at position G19 (Fig. 3), consistent with results from the X-ray structure indicating that the D5 site is not directly solvent accessible (31).

Iodoacetamide accessibility for the cysteines in NGB and CYGB was studied using ESI-MS and will be discussed in the next section.

**Mass spectrometry assessment of disulfide bonds and accessibility of cysteines to iodoacetamide**

**Human neuroglobin.** In order to rigorously confirm the presence of disulfide bonds, the fractions obtained by DEAE Sepharose chromatography were analyzed by ESI-MS with the following results. Fraction F1 (Fig. 4a) gave the mass of the dominant component as 16930.9 Da, agreeing, within experimental error, with the sequence mass of NGB with one disulfide bond (16931.4 Da). Also present was a minor component (~10%: 33891.4 Da) that was 30.3 Da higher in mass than expected for a disulfide linked dimer of NGB (33860.9 Da).
Upon reduction with DTT, the mass of the monomer increased to 16933.2 Da (Fig. 4b), agreeing, within experimental error, with the sequence mass of NGB with all Cys free (16933.5 Da). Concomitantly, the minor dimer fraction disappeared.

Fractions F2 and F3 comprised ~50% of NGB (16931.3 and 16931.2 Da respectively) and ~50% of a component of 33862.8 and 33861.9 Da, respectively, in agreement with the sequence mass of a disulfide linked dimer of NGB (data not shown).

The mass spectrum obtained from native NGB, after digestion with trypsin, exhibited all the tryptic peptides (Fig. 5) expected from the sequence with free Cys, except T5, T6 and T13, which contain CD7-Cys, D5-Cys and G19-Cys respectively (Fig. 6). However, two abundant peptides were present in several charge states, whose masses correspond to (T5 + T6 – 2H) and a T13 dimer (T13 + T13 – 2H), inferring that CD7-Cys is coupled to D5-Cys by a disulfide bond and G19-Cys is free in the native protein (Fig. 6a). Presumably, the T13 dimer was formed during the digest procedure, since peptides containing free Cys generally couple with one another under these conditions. However, there was neither evidence for homodimers of T5 or T6 nor for hetero-dimers of T13 with T5 or T6, giving further support for the absence of free Cys in T5 and T6. The disulfide bond between CD7-Cys and D5-Cys was verified by reducing an aliquot of the digest solution with DTT, when the (T5 + T6 – 2H) peptide disappeared with the concomitant appearance of the T5 and T6 peptides (Fig. 6b). Furthermore, the T13 dimer disappeared concomitantly with the appearance of T13 monomer.

The intra-molecular disulfide bond apparently perturbs the three-dimensional structure of the CD-D region in the crystallized protein. The WT NGB was difficult to crystallize; the initial structure was obtained for the mutant without cysteines (CCC\rightarrow GSS) bearing the Cys46\rightarrow Gly, Cys55\rightarrow Ser, Cys120\rightarrow Ser mutations (31). Analysis of the mutated protein structure (i.e. in the absence of any intramolecular disulfide bonds) shows that two of the four crystallographically independent NGB molecules have disordered CD-D regions (residues 44 - 54), whereas the remaining two display a rather extended conformation for the CD-D region (31), deviating remarkably from that of homologous heme proteins. Although the crystal structure observed for the latter two mutated NGB molecules does not suggest the formation of a CD7–D5 intra-molecular disulfide, based on simple geometrical considerations, it should be considered that the local structure of the protein may have been altered by the introduction of a Gly residue at site CD7. Under this hypothesis, it appears structurally plausible that local conformational rearrangements may lead the CD7 and D5 residues to a closer mutual location, allowing formation of the disulfide bond.
If a conformational transition in NGB between an oxidized (i.e. hosting an intramolecular disulfide bond) form and a reduced (i.e. disulfide free) NGB form can be envisaged, such a transition could affect the location of the neighboring E-helix, thus allowing fine modulation of the availability of the endogenous HisE7 ligand for heme hexacoordination. It can thus be proposed that the redox state of the cell acts on the rupture/formation of the intra-molecular CD7-D5 disulfide bond, so initiating a conformation change that affects the overall oxygen affinity of NGB and/or other functional properties as well.

Formation of other intra-molecular disulfide bonds involving Cys CD7 or D5 with Cys G19 is sterically impossible. Moreover, since no intermolecular disulfide bridges can be formed in the NGB mutant without cysteines (CCC→GSS), little can be extrapolated from the analysis of crystal packing contacts concerning intermolecular aggregation of the WT NGB.

Which cysteine residues are involved in the formation of disulfide bonds can also be inferred from their accessibility and potential for derivatization by iodoacetamide. Derivatization of NGB with iodoacetamide to S-carboxyamidomethylate (Cam) the free Cys, even in the presence of 8 M urea, was unsuccessful. Derivatization of DTT reduced NGB was partly successful without denaturation, showing mainly NGB + Cam and NGB + 2Cam (~50% as intense as NGB + Cam), but no NGB + 3Cam.

When the procedure was undertaken in 8 M urea, derivatization was more successful, but all three cysteines were never completely derivatized. Fig. 4c shows ESI-MS spectra from a second preparation of NGB after reduction and S-carboxyamidomethylation in 8 M urea. Four species are present at masses 16931.4, 16990.4, 17047.5 and 17104.4 Da corresponding to oxidized NGB (4%), and NGB with 1Cam (45%), 2Cam (39%) and 3Cam (12%) respectively. A tryptic digest of the same NGB solution showed that T5, T6 and T13 were approximately 94%, 54% and 38% S-carboxyamidomethylated respectively.

From these experiments we conclude that derivatization of all cysteines by iodoacetamide is only possible after reduction of the intra-molecular disulfide bond, when CD7-Cys is easily derivatized, D5-Cys is less easy to derivatize than CD7-Cys and G19-Cys is derivatized with difficulty. A similar ESI-MS analysis of NGB with CD7-Cys replaced by serine did not show any evidence for a disulfide bond between D5-Cys and G19-Cys, and no derivatization of G19-Cys with Cam, as for the WT NGB (data not shown).

**Human cytoglobin.** Analysis of native reconstructed CYGB by ESI-MS demonstrated that both Cys residues were disulfide linked to 2-mercaptoethanol [derivatized CYGB (21556.9 Da) = underivatized CYGB (21404.7 Da) + 2 x 76.1 Da)] (Fig 7). This derivatization
with 2-mercaptoethanol results from the reconstruction procedure used. Upon reduction with DTT, the mass decreased to 21404.7 Da in agreement with the sequence having two Cys free (Fig 7). These observations also underscore the importance of using mass spectrometry to check the authenticity of recombinant protein samples and stress the disadvantage of using 2-mercaptoethanol as a reducing agent. After removal of the DTT, air oxidation of the reduced CYGB (pH 8, 37°C) resulted in a progressive decrease of the mass, over 7 hours, from 21404.7 Da to 21402.6 Da, implying the formation of a disulfide bond. No evidence was found for CYGB dimers that would indicate the formation of inter-chain disulfide bonds. The existence of an intra-chain disulfide bond was substantiated by the analysis of a tryptic digest of oxidized CYGB (Fig. 8). In the spectrum ions were present corresponding to the peptides expected from the sequence with free Cys except T14, which contains E9-Cys (Fig. 5). Peptide T8, containing B2-Cys, was present, but at a low level. In addition, there were triply charged ions at m/z 706.72 and 749.41 corresponding to \([\text{T8} + \text{T14} – 2\text{H}]\) and \([\text{T8} + \text{T(13-14)} – 2\text{H}]\) of calculated m/z 706.69 and 749.39 respectively (Fig. 8a). Upon reduction of the digest with DTT, these triply charged ions disappeared concomitantly with a 10-fold increase in the intensity of doubly charged ions corresponding to the T8 peptide (Fig. 8b) together with the appearance of singly charged ions corresponding to the T14 peptide (Fig 8b inset) and T(13-14) peptide (not shown).

In contrast to NGB, both Cys in reduced CYGB reacted readily with iodoacetamide to form S-carboxyamidomethylated CYGB without denaturing in 8M urea.

From these data we conclude that both Cys of CYGB are involved in an intra-molecular disulfide bond and that there is no evidence for the existence of inter-molecular disulfide bonds resulting in the formation of dimers. Furthermore, a separate series of analyses of CYGB by ESI-MS under non-covalent conditions showed essentially only the monomer with ~5% dimer, whereas classical biochemical techniques showed CYGB to be dimeric. This would indicate that the dimers are not based on disulfide bonds, unlike NGB, where the small fraction dimers were observed with both techniques. CYGB dimers that do not involve the cysteine are compatible with the biochemical data showing that truncated CYGB is monomeric.

**Ligand binding of human neuro- and cytoglobin.** The kinetics of rebinding of NGB and CYGB to CO show the form expected for hexacoordinated globins. The rapid bimolecular phase corresponds to a competitive binding of CO and the internal histidine residue. For the fraction binding histidine, the return to the final (CO bound) state involves
the slow dissociation of histidine. By analyzing the data from experiments performed at different CO concentrations, one can extract the rates for the CO and histidine association, as well as that for histidine dissociation; the CO dissociation rate is independently measured (10-12). Results from experiments with O₂ or a mixed CO/O₂ atmosphere allow a determination of the O₂ binding rates. As previously reported the intrinsic O₂ or histidine dissociation is quite slow, requiring about 1 s.

A major change in kinetics is observed with NGB after addition of DTT, known to reduce the cysteines and therefore break the S-S bonds (Fig. 9). The dominant change is in the histidine dissociation rate which decreases by nearly an order of magnitude (Table II). Since the overall O₂ affinity depends on the histidine binding, this leads to an effective decrease in O₂ affinity. A similar shift was observed for NGB with mutated cysteines. Thus breaking the S-S bond by DTT, or elimination of the bond by genetic engineering, results in a low O₂ affinity conformation of NGB.

For WT NGB this transition is reversible; however, we have never obtained 100% of the kinetics corresponding to the S-S state NGB. Attempts to purify or oxidize the protein resulted in samples that show at most 90% of the high O₂ affinity form; perhaps even with the disulfide bond, the protein is not completely in a single conformation. This means that the NGB studied so far was always a variable mixture of several forms: with and without the disulfide bond. This would explain the heterogeneity reported in our earlier study (12) and would correspond to some of the multiple conformations reported by Kriegl et al (21).

Reduction of the protein leads to a single species. The kinetic curve shows a clean biphasic form, as expected for the competition of two ligands (Fig 9A). While we have demonstrated two distinct sets of ligand binding rates for NGB, we did not observe any additional reactions during the time course of the (ms-s) kinetics. The slow phase of the flash photolysis experiments had essentially the same rate as the kinetics observed in stopped flow. Thus the overall kinetics of a given sample can be described by the ligand competition, without requiring a dynamic conversion between the forms with and without the disulfide bond. Switching to the form without the S-S bond by addition of DDT is slow, requiring about 30 minutes at 37°C; for the analysis of the flash photolysis data, the NGB sample can be considered as a static mixture of the two forms.

As for NGB, CYGB shows the characteristic absorption spectra and ligand binding for the hexacoordinated globins. In general, the ligand association reactions are slower for CYGB relative to NGB, however, since this occurs for both oxygen and histidine, the overall oxygen affinity remains on the order of 1 torr. The ligand binding rates for CYGB (Table II) are in
agreement with those of Trent and Hargrove (10), although they reported no slow phase for the flash photolysis experiments; we differ by a factor of 1000 in the values for $K_{\text{His}}$ of NGB, where they apparently analyzed the data in a different manner.

For CYGB, there was a similar effect of the disulfide bond on the ligand binding kinetics, but much smaller in magnitude. The shift in oxygen affinity is at most a factor of 2 (data not shown), indicating a weaker coupling of the disulfide bond and ligand binding. The possible disulfide bond for CYGB (B2-E9) is not in the same region as that proposed for NGB (CD7-D5); however both bonds may influence the final position of the E-helix which in turn may determine the affinity for the distal histidine with the heme iron.

A hypothetical model for the heme-cysteine coupling in neuroglobin

Structural analysis by mass spectrometry clearly shows the presence in NGB of a disulfide bond between CD7-Cys and D5-Cys. Substitution of CD7-Cys and D5-Cys by Ser and eliminating as such the disulfide bond, or its reduction by DTT, lowers the distal histidine dissociation rate by a factor 10 with a consequent lowering of the O$_2$ affinity the same factor. This suggests the existence of two major conformations for the protein: one with the disulfide bond present, resulting in a slightly stressed protein, and one without the disulfide bond. Breaking the disulfide bond allows a shift in the orientation of the E-helix resulting in a lowered affinity and a release of O$_2$.

This phenomenon might explain the involvement of NGB in hypoxia (17-18). Indeed, hypoxia will result in the accumulation of reduction equivalents (NADH + H$^+$) in the cell. It can be expected that, under these conditions, the disulfide bond in NGB will be reduced with a subsequent release of O$_2$, counteracting the hypoxia. The existence of a reductase, similar to myoglobin reductase (33), catalyzing this reaction can be hypothesized. When the O$_2$ concentration increases, the free cysteines will be oxidized mainly into an intra-molecular disulfide bond with the concomitant increase of the O$_2$ affinity and O$_2$ storage. A small percentage of inter-molecular disulfide bonds are formed as well. Concomitant with an O$_2$ release, related to the redox state of the cell, the biosynthesis of NGB is induced in order to be able to bind more O$_2$ during a temporary increase in the O$_2$ partial pressure for release in the forthcoming period of hypoxia.

The scheme for this hypothetical model for NGB function is presented in Fig 10. In this model, formation and cleavage of a disulfide bond influences the functional characteristics
of the protein. The same may be true for CYGB, although much less is currently known about the physiological role of this globin. This mechanism would explain how different globins could co-exist; specific partner molecules would provoke release of the oxygen, as opposed to a simple Hb-O₂ equilibrium with the local environment.

Until recently, the disulfide bonds present in mature proteins were thought to be inert and remain unchanged during the life of the protein. However, it now appears that this is not necessarily the case: disulfide bonds can be cleaved and reformed and this event may have significant consequences for protein function (34). Examples of disulfide bond cleavage / formation that have an effect on the protein function, and the factors facilitating it, are: thrombospondin-1/protein disulfide isomerase (35), plasmin/annexin II (36) and CD4/thioredoxin (37). Our model for the regulation of the oxygen binding affinity in NGB is thus another example of the potential dynamic role of disulfide bonds in proteins, affecting their structure and function.

The cellular function of NGB is still an open question, and recently it has been suggested that the iron-oxidized form of NGB interacts with the α subunit of G proteins to influence the signal transduction cascade in the hypoxic brain (32). However, our present results show a coupling between the oxygen affinity of NGB and the redox state of the cell via disulfide bond formation, in agreement with a role of NGB in cellular oxygen homeostasis.
Reference List


Table I: TNB binding to Cysteines.

<table>
<thead>
<tr>
<th>globin</th>
<th>cysteines</th>
<th>State</th>
<th>% reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ngb</td>
<td>WT</td>
<td>D5, G19</td>
<td>F</td>
</tr>
<tr>
<td>Ngb</td>
<td>CC→SC</td>
<td>D5</td>
<td>F</td>
</tr>
<tr>
<td>Ngb</td>
<td>CC→CS</td>
<td>G19</td>
<td>F</td>
</tr>
<tr>
<td>human</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NGB</td>
<td>WT</td>
<td>CD7, D5, G19</td>
<td>F</td>
</tr>
<tr>
<td>NGB</td>
<td>WT</td>
<td>CD7, D5, G19</td>
<td>U</td>
</tr>
<tr>
<td>NGB</td>
<td>CCC→CCS</td>
<td>CD7, D5</td>
<td>F</td>
</tr>
<tr>
<td>CYGB</td>
<td>WT</td>
<td>B2, E9</td>
<td>F</td>
</tr>
<tr>
<td>CYGB</td>
<td>WT</td>
<td>B2, E9</td>
<td>U</td>
</tr>
</tbody>
</table>

The experimental conditions were 20 mM phosphate buffer at pH 7 for the folded (F) state, or 8 M guanidinium chloride for the unfolded (U) form. Typical errors were 4%. Total reactivity of 100% was based on the average signal for unfolded Hb alpha and beta chains.
Table II. Rates of ligand binding to NGB and CYGB

<table>
<thead>
<tr>
<th></th>
<th>CO</th>
<th>Oxygen</th>
<th>Histidine</th>
<th>Oxygen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>k\textsubscript{on}</td>
<td>k\textsubscript{on}</td>
<td>k\textsubscript{off}</td>
<td>1/ K\textsubscript{5}</td>
</tr>
<tr>
<td>NGB</td>
<td>40</td>
<td>140</td>
<td>0.8</td>
<td>5.7</td>
</tr>
<tr>
<td>NGB +DTT</td>
<td>50</td>
<td>170</td>
<td>0.8</td>
<td>4.7</td>
</tr>
<tr>
<td>NGB CCCÆGSS</td>
<td>50</td>
<td>170</td>
<td>0.7</td>
<td>4.1</td>
</tr>
<tr>
<td>mNgb E7 HÆV</td>
<td>230</td>
<td>900</td>
<td>230 255</td>
<td></td>
</tr>
<tr>
<td>mNgb E7 HÆQ</td>
<td>290</td>
<td>800</td>
<td>11 15</td>
<td></td>
</tr>
<tr>
<td>CYGB +DTT</td>
<td>5</td>
<td>27</td>
<td>0.9 33</td>
<td></td>
</tr>
<tr>
<td>SW Mb</td>
<td>0.5</td>
<td>14</td>
<td>12 857</td>
<td></td>
</tr>
</tbody>
</table>

The on and off rates were measured at pH 7, 25°C, using the flash photolysis or stopped-flow technique. K\textsubscript{His} = k\textsubscript{on}/k\textsubscript{off} is dimensionless. The ratio of oxygen binding rates provides the intrinsic ligand affinity of the pentacoordinated form: K\textsubscript{5} = k\textsubscript{on}/k\textsubscript{off} in units of 1/M, with a conversion factor of 1850 for torr to nM. For hexacoordinated Hbs, the observed oxygen affinity depends on the competition with the protein ligand (histidine in this case) K\textsubscript{obs} = K\textsubscript{5}/(1+K\textsubscript{His}). Note that the P\textsubscript{50} values determined from oxygen equilibrium measurements are 1.2 torr for mouse neuroglobin and 0.5 torr for SW Mb; the CO off rate for NGB is 0.05/s.
Figure legends

Fig 1: Occurrence of cysteine residues in globins.  1A) Number of cysteines, or alanines, per sequence. The % is relative to the entire database of 811 sequences; for example, the peak frequency of 1 cysteine per sequence corresponds to 297/811 or 36.6%.  1B) Location of cysteine residues in globins: probability (%) versus sequence position.  Notable positions are F9 (essentially beta chains), G11 (alpha chains), CD7 and D5 for Ngb (12 sequences), B2 and E9 for Cygb (with one exception: A7 and GH4 for Danio rerio (zebrafish).  The % is relative to the number of sequences within the same group: there are 12 Ngb sequences, and 7 have Cys at position CD7 (58.3%); of the 260 alpha globin chain sequences, 226 have Cys at position G11 (86.9%).  The database and the “SeqView” software are available (MCM).

Fig. 2: Location of critical residues, based on the crystal structure of sperm whale Mb.  The sites selected correspond to cysteine pairs in NGB (blue: CD7 and D5), CYGB (yellow: B2 and E9), and Danio rerio (zebrafish) Cygb (green: A7 and GH4).  Note that the third cysteine residue in NGB at position G19 is close to that at GH4.

Fig. 3. Accessibility of cysteines to DTNB binding.  NGB shows only a partial reaction with DTNB, even under denaturing conditions, indicating the presence of a disulfide bond. Mutation of one cysteine in mouse Ngb resulted in nearly 100% interaction of DTNB with the remaining cysteine residue.  For human NGB, mutation of the third cysteine (G19) to serine showed essentially no reaction.  Control experiments were made with human HbA (not shown) and its component alpha (not shown) and beta chains.

Fig. 4: Electrospray ionization mass spectra of NGB fractions. (a) Fraction F1 without DTT; (b) Fraction F1 with DTT. (c) after reduction with DTT followed by derivatization with iodoacetamide (Cam).

Figure 5: Amino acid sequence of NGB and CYGB with indications of the tryptic fragments.

Fig. 6: Partial electrospray ionization mass spectra from an 80-minute tryptic digest of NGB (a) before and (b) after reduction with DTT.
Fig. 7 Electrospray ionization mass spectra of CYGB (a) before and (b) after reduction with DTT, and (c) after oxidation of the reduced sample. MCE: mercaptoethanol. Myoglobin was introduced with the samples to internally calibrate the mass scale.

Fig. 8: Partial electrospray ionization mass spectra from an 80 min tryptic digest of CYGB (a) before and (b) after reduction with DTT.

Fig. 9: A) The kinetics of ligand rebinding after photodissociation of NGB-CO, for samples equilibrated under 0.1 atm CO; the detection wavelength was 436 nm. The biphasic shape is characteristic of the hx globins; data is presented on a log-log scale with time in s. The rapid phase reflects competitive binding of CO and the distal (E7) histidine; the slow phase corresponds to the replacement reaction of the distal histidine by CO. Reduction of the disulfide bonds with DTT leads to a change in the kinetics; there is less slow phase and the replacement reaction is much slower. The solid lines are simulations using the model for competitive ligand binding. B) Kinetics of the slow phase (involving histidine dissociation) showing the large decrease in rate upon addition of DTT; data was normalized to the amplitude for the slow phase.

Fig. 10: Overall scheme showing the coupling between the oxidation state of the cysteines and the oxygen binding to the iron atom. Reduction of the disulfide bond (between CD7 and D5 for NGB; A7 and GH4 for CYGB) leads to a decrease in oxygen affinity; thus an external electron donor could provoke the release of oxygen.
Figure 1 B
Figure 3
Figure 4

(a) Rel. Int. (%) Molecular Mass (Da)

(b) Rel. Int. (%) Molecular Mass (Da)

(c) Rel. Int. (%) Molecular Mass (Da)

NGB
16930.9
16913.2
16949.5

Reduced NGB
16933.2
16915.4
16951.2

NGB+Cam
16990.4
NGB+2Cam
17047.5
NGB+3Cam
17104.4
Figure 7

(a) CYGB+MCE

(b) Mb

(c) CYGB
Figure 8

(a) Relative Intensity (%)

(b) Relative Intensity (%)

Mass-to-Charge Ratio (m/z)
Figure 9 A

NGB CO rebinding kinetics

$\Delta A_N$

$\log (t)$

+ DTT
Figure 9 B

![Graph showing time (ms) vs. ΔA_N with three lines: NGB, NGB+DTT, and NGB-CO at 55°C, slow phase.](image-url)
Figure 10

[Diagram showing the process of oxidation and reduction involving iron (Fe), histidine (His-E7 and His-F8), oxygen (O₂), and sulfur (S).]
The redox state of the cell regulates the ligand binding affinity of human neuroglobin and cytoglobin

Djemel Hamdane, Laurent Kiger, Sylvia Dewilde, Brian N. Green, Alessandra Pesce, Julien Uzan, Thorsten Burmester, Thomas Hankeln, Martino Bolognesi, Luc Moens and Michael C. Marden

J. Biol. Chem. published online October 6, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M309396200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2003/10/06/jbc.M309396200.citation.full.html#ref-list-1