HUMAN NEUROGLOBIN FUNCTIONS AS A REDOX REGULATED NITRITE REDUCTASE

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Running Head: Neuroglobin heme-coordination regulates nitrite reduction to nitric oxide

Neuroglobin is a highly conserved hemoprotein of uncertain physiological function that evolved from a common ancestor to hemoglobin and myoglobin. It possesses a six-coordinate heme geometry with proximal and distal histidines directly bound to the heme iron, though coordination of the sixth ligand is reversible. We show that deoxygenated human neuroglobin reacts with nitrite to form nitric oxide (NO). This reaction is regulated by redox sensitive surface thiols, cysteine 55 and 46, which regulate the fraction of five-coordinated heme, nitrite binding and NO formation. Replacement of the distal histidine by leucine or glutamine leads to a stable five-coordinated geometry; these neuroglobin mutants reduce nitrite to NO approximately 2000-times faster than the wild type, while mutation of either C55 or C46 to alanine stabilizes the six-coordinate structure and slows the reaction. Using lentivirus expression systems we show that the nitrite-reductase activity of neuroglobin inhibits cellular respiration via NO binding to cytochrome c oxidase and confirm that the six-to-five coordinate status of neuroglobin regulates intracellular hypoxic NO signaling pathways. These studies suggest that neuroglobin may function as a physiological oxidative stress sensor and a post-translationally redox regulated nitrite reductase, that generates NO under six-to-five coordinate heme pocket control. We hypothesize that the six-coordinate heme globin superfamily may subserve a function as primordial hypoxic and redox regulated NO signaling proteins.

A phylogenic analysis of the heme-globin family indicates that the well-characterized proteins hemoglobin and myoglobin were antedated by neuroglobin, which existed already 800 million years ago (1,2). Neuroglobin (Ngb) sequences remained highly conserved throughout mammalian evolution, suggesting a strongly selected vital functionality (3). This heme containing, monomeric, 16.9 kDa protein shares 21 and 25% sequence similarity with myoglobin and hemoglobin. However, unlike myoglobin and hemoglobin, it possesses a bis-histidine six-coordinate heme geometry, such that the proximal and distal histidines in the heme pocket are directly bonded to the heme iron (both Fe^{2+} or Fe^{3+} oxidation states) (4). Indeed at equilibrium the concentration of the five-coordinate neuroglobin is very low, reported from 0.1 up to 5% (5). Binding of oxygen or other gas ligands, such as nitric oxide (NO) or carbon monoxide, to the heme iron occurs upon displacement of the 6th coordination bond with the distal histidine 64 residue (6,7). Despite this structural difference with myoglobin, neuroglobin displays comparable α-helix globin folding and high oxygen affinity (P_{50} about 1-2
However, the low tissue concentration of neuroglobin and the rapid auto-oxidation of the oxygen bound species suggest neuroglobin has not evolved to store and supply oxygen, leading to a number of different hypotheses about its molecular functionality (2,10).

In vitro and in vivo expression of neuroglobin produces cytoprotective effects, limiting neuronal cell death during glucose deprivation and hypoxia and limiting the volume of brain infarction in stroke models (11-14). Understanding the functionality of neuroglobin could provide a paradigm shift in both biology and therapeutics because several highly conserved heme-globins, ubiquitous in plants and animals, exist in equilibrium between dominant six-coordinate heme geometry and a less frequent five-coordinate state. Examples include cytoglobin, cytochrome c, Drosophila melanogaster hemoglobin, and the non–symbiotic plant hemoglobins (15-17).

Over the last five years, our groups have examined the ability of deoxygenated hemoglobin and myoglobin to react with and reduce nitrite to NO (18,19). We have proposed that this reaction subserves a function similar to the bacterial nitrite reductases, in which a coupled electron and proton transfer to nitrite generates NO.

In the heart, myoglobin can reduce nitrite to NO to regulate hypoxic mitochondrial respiration and enhance the cellular resilience to prolonged ischemia, analogous to the cytoprotective effects of neuroglobin (19). Studies using the myoglobin knockout mouse have now confirmed that myoglobin is necessary for: 1) nitrite-dependent NO and cGMP generation in the heart, 2) nitrite-dependent cytoprotection after ischemia/reperfusion and, 3) nitrite-dependent control of hypoxic cellular respiration (20). It is therefore apparent that both myoglobin and neuroglobin may have roles in limiting cell death after ischemia-reperfusion injury. Of relevance to neuroglobin, we have recently discovered that the mitochondrial protein cytochrome c can reduce nitrite to NO more rapidly than either hemoglobin or myoglobin, but only when it assumes the five-coordinate conformation (21). This conformation only occurs during the interaction with anionic phospholipids or upon oxidation or nitration of protein residues, suggesting a post-translational tertiary structure regulation of nitrite reduction and NO generation.

Interestingly, human neuroglobin contains two surface cysteines (C46 and C55) that form a disulfide bridge upon oxidation (22). Disulfide bond formation is accompanied by a decrease in the distal histidine binding affinity to heme iron \((K_{His, has} \text{ decreased from } \sim 3000 \text{ to } 280, \text{ values are calculated as } k_{on}/k_{off} \text{ and are dimensionless}) (23). This in turn increases the sub-population of five-coordinate neuroglobin and increases the affinity for endogenous ligands such as oxygen \((P_{50} \text{ shift from about } 9 \text{ to } 1 \text{ mmHg}) (22). Nicolis et al. reported that the oxidized disulfide-bridged neuroglobin also exhibits a higher affinity for nitrite than the thiol reduced form (24).

We therefore hypothesized that neuroglobin, and more generally the six-coordinate heme globins, may function as post-translationally redox-regulated nitrite reductases, that generates NO under control of the six-to-five coordinate heme iron transition. Such functionality may underlie hypoxic neuroprotective signaling and the control of hypoxic cellular respiration.

**EXPERIMENTAL PROCEDURES**

Reagents and standards samples preparation – All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. UV-visible spectra and kinetic data were recorded on an HP8453 UV-Vis spectrophotometer (Agilent Technologies, Palo Alto, CA). Superdex S200 gel filtration columns were purchased from GE Healthcare Life Sciences (Piscataway, NJ). Solutions of sodium dithionite and nitrite were prepared with argon degassed 0.1 M phosphate buffer (pH 7.4) and kept at 25°C under inert gas. Purchased horse heart myoglobin was further purified by passing through a Sephadex G-25 gel filtration column and eluting with 0.1 M phosphate buffer (pH 7.4). Neuroglobin was oxidized with excess potassium ferricyanide or reduced by incubation with 500 mM sodium dithionite; excess reagents were removed by passing the mixture through two sequential Sephadex G-25 desalting columns. Met-Ngb concentrations were estimated by measuring the absorbance of the heme Soret band using \(A_{414} =\)
129 mM$^{-1}$ cm$^{-1}$. Standard reference species of recombinant Ngb for spectral deconvolution were prepared following procedures previously described for hemoglobins (19,25). Reference spectra were recorded for deoxy-Ngb, iron-nitrosyl-Ngb, met-Ngb, and oxy-Ngb. When necessary, anaerobic reduced Ngb samples were prepared in glovebox under a 2% - 4% H$_2$ atmosphere of catalyst-deoxygenated nitrogen, collected directly in cuvettes and sealed with rubber septa inside the glovebox. To reduce the intramolecular Ngb disulfide bond, Ngb solutions were dialyzed in PBS containing 10 mM DTT dissolved in degassed 100 mM phosphate buffer and 0.5 mM EDTA as previously described (24).

Cloning, expression and purification of recombinant Ngb – Molecular biology was performed using standard techniques. For the expression of the 151 amino acids polypeptides of human Ngb the cDNA SC122910 was cloned in BL21(DE3)pLysS(pET28a). Cells were grown in LB broth containing 30 µg/ml kanamycin and 25 µg/ml chloramphenicol, expression was induced with 1 mM IPTG and carried out for 4 hours at 37°C including $\alpha$-amino-levulinic acid (0.4 mM) in the media. Purification was carried out as described with minor modifications (26). To increase purification yield, human Ngb cDNA was fused with a 6xHis tag in the N-termini, cloned into E. coli BL21(DE3)pET28a for proteins over-expression and His tagged human Ngb was purified using Ni-NTA-agarose (Qiagen, Valencia, CA) affinity column according to the manufacturer’s instructions. The eluted protein was dialyzed against PBS at 4°C, concentrated with 10 kD cutoff filter and stored in aliquots at -80°C. The additional amino acids at the N-terminus of His tagged Ngb were removed using a thrombin cleavage capture kit (Novagen, Gibbstown, NJ). The purity of each recombinant Ngb batch prepared was assessed by SDS-PAGE and UV-visible spectroscopy. The number of accessible thiol groups per heme was measured by the 4-PDS assay (27).

Mutagenesis of recombinant Ngb – Site directed mutagenesis was performed using QuickChange II kit (Stratagene, Palo Alto, CA). The oligonucleotides for mutation C46A, C55A, H64L and H64Q are reported in Supplementary Table 1. The template used for C46A and C55A was pCMV-1A and for H64L and H64Q was pET28a. Clones were sequenced to confirm the desired mutations. Expression and purification of mutant Ngb were carried out using the same procedures as for wild type Ngb.

Anaerobic reactions of globins with excess nitrite – Reaction kinetics of known amount of Mb or Ngb with nitrite were monitored by absorption spectroscopy for the indicated time in a cuvette in the presence or in the absence of 2-4 mM sodium dithionite. All reactions were run at 25°C or 37°C in 0.1 M phosphate buffer at controlled pH. Previously deoxygenated nitrite was added, using an airtight syringe, to a sealed anaerobic cuvette to initiate the reaction. Oxygen contamination was prevented by application of positive argon pressure without a channel for gas escape. Concentrations of single species during reactions were determined by least squares deconvolution of the visible absorption spectrum into standard reference spectra using Microsoft Excel analysis. OxyNgb was included to confirm successful deoxygenation before the reaction. To vary pH, deoxy-Ngb and nitrite were prepared in phosphate buffer adjusted to the target pH values. Fast kinetic studies were performed using an Applied Photophysics DX-17 stopped-flow instrument equipped with rapid-scanning diode array detection (Applied Photophysics Ltd., Leatherhead, Surrey, UK). Experiments were carried out at 25°C by rapidly mixing a solution of reduced deoxy-Ngb containing 2 mM dithionite with a known solution of nitrite at controlled pH. To determine bimolecular rate constants all reactions were analyzed with Pro-K software (Applied Photophysics Ltd., Leatherhead, Surrey, UK) using singular value decomposition followed by fitting of the reduced data matrix to a pseudo-first order kinetic model.

Model of the wild type human Ngb structure – Crystallization of the wild type human Ngb is hindered by aggregation and precipitation problems. Mutation of the three cysteine residues yielded a protein suitable for crystallization studies (28). The reported structure (PDB 1OJ6) thus includes the mutations Cys46Gly, Cys55Ser and Cys120Ser. To assess the possible structure of the wild type enzyme a homology model was built using the Swiss-Model server (29) with the sequence of the wild type Ngb and the available human structure as template. The coordinates of
the heme molecule were copied from the 1OJ6 structure.

**Determination of the midpoint redox potential of the thiol/disulfide couple in Ngb** – Wild type and C55A mutant Ngb (50-60 µM) were incubated at 37 °C in anaerobic glove box with solutions containing various ratios of reduced (GSH) and oxidized (GSSG) glutathione, with the total GSH and GSSG concentration fixed at 20 mM in 0.1 M phosphate buffer pH 7.0 (30). The GSH/GSSG ratio was varied to establish a gradient of redox potentials between -130 and -240 mV. After 1 or 2 hours incubation, glutathione was removed anaerobically by passage through a G25 column and Ngb was reacted immediately with 10 mM nitrite in 0.1 M phosphate buffer, pH 7.0 as described above. The observed rate constant determined at each glutathione ratio was fitted using the Nernst equation and the midpoint reduction potential of the thiol/disulfide couple of Ngb calculated.

**Determination of nitrite binding constants** – The binding constant of nitrite to met-Ngb was determined by incubation of 10 µM wild type or mutant Ngb with increasing concentrations of nitrite in 200 mM phosphate buffer, pH 7.4, in a cuvette at 25 °C and the UV–visible spectra were recorded after each increase in nitrite concentration. The dissociation constant $K_D$ for each protein was determined by interpolation of the absorbance difference data following procedures in Nicolis et al. (24). The reaction rate of nitrite and met-Ngb was then determined under the same conditions at 100 mM nitrite.

**NMR spectroscopy** – $^1$H NMR spectra in $^1$H$_2$O were collected at 29 °C on a Bruker DRX-600 NMR spectrometer (Bruker, Billerica, MA) operating at 599.79 MHz with a 5 mm triple resonance probe using a water presaturation pulse sequence with 1 s irradiation time. Samples of wild type and mutant 250-300 uM met-Ngb were prepared in 0.1M phosphate buffer pH 7.4. Typically 1024 transients were averaged, using 90 degree pulses, spectral width of 80 ppm and 16K time domain points. Spectra are referenced indirectly through the resonance of the water, which occurs at 4.76 ppm downfield from the methyl resonance of DSS (2,2-dimethyl-2-silapentane-5-sulfonate).

**Electron Paramagnetic Resonance spectroscopy** – Iron nitrosyl species were measured by EPR spectroscopy using a Bruker EMX 10/12 spectrometer (Bruker, Billerica, MA) operating at 9.4 GHz, 5-G modulation, 10.1-milliwatt power, 327.68-ms time constant and 163.84-s scan over 600 G at 110 K as described previously (21,31). The concentrations of Mb and Ngb species were determined by performing the double integral calculation and comparing to standard samples.

**Direct measurement of NO release by chemiluminescence** – Deoxy-Ngb (final concentration 20 µM) was injected in a reaction vessel containing 100 mM phosphate buffer, pH 7.4 (3 ml) pre-equilibrated and purged with helium and connected in line to a Nitric Oxide Analyzer (NOA 280i) (Sievers, GE Analytical Instruments, Boulder, CO). Once a stable baseline was established Ngb was reacted with a known amount of nitrite as previously described (18). Traces were smoothed by a running average spanning 2 s.

**Respiration of isolated mitochondria in the presence of Ngb** – Mitochondria were isolated from the livers of male Sprague Dawley rats and incubated with wild type or mutant Ngb proteins in a sealed, stirred chamber at 37 °C. State 3 respiration was stimulated with succinate (15mM) and ADP (1mM) and oxygen consumption was measured with a Clark-type oxygen electrode. All experiments with respiring mitochondria were performed according to previously reported procedures (19). Similar experiments were performed with SHSY5Y cells suspended in the respirometer and treated with the uncoupler FCCP (5µM) to measure hypoxic inhibition of cellular respiration.

**Immuno-blotting of Ngb expression in SHSY5Y neuronal cells** – Equal amounts of denatured total proteins (25 µg) from the SHSY5Y neuronal cells expressing GFP vector, wild type and H64L mutant Ngb, were subjected to 4-15% SDS-polyacrylamide gradient gels and immunoblotted with anti-GFP monoclonal antibody (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA) then scanned using an Odyssey imaging system (LI-COR Biosciences, Lincoln, NE).

**Statistical Analysis** – Each experiment was performed at least in triplicate and values are representative of two or more independent determinations using different batches of protein.
pumped separately. Data were analyzed using Origin 8.0 (OriginLab Corporation, Northampton, MA) and expressed as mean ± standard deviation of the mean. Analysis for statistically significant differences among mean values was done, when applicable (fig. 2B, 5B and 6B), using the one-way ANOVA analysis.

RESULTS

Nitrite is reduced to NO via reaction with deoxygenated human neuroglobin. In order to examine the reaction of nitrite with neuroglobin we expressed and purified recombinant human neuroglobin. Spectrophotometric analysis of our proteins confirmed the six-coordinate heme structure in both the ferrous and ferric states of Ngb, with visible α and β peaks around the 550 nm wavelength (Supplemental Figure 1A). We prepared ferrous deoxy-Ngb in an anaerobic glove box as detailed in the experimental procedures and recorded the visible spectra of the reaction between 10 µM deoxy-Ngb and 10 mM nitrite at 25 °C at constant intervals in a sealed air tight cuvette under external argon pressure (Figure 1A). The time-dependent changes of deoxy-Ngb, ferric met-Ngb and iron-nitrosyl-Ngb (Fe +2-NO) species (Figure 1B) were calculated by least squares deconvolution of the reaction spectra using standard reference spectra (Supplemental Figure 1A). In an anaerobic environment nitrite is reduced to NO according to equation 1 and the NO generated has very high affinity for the ferrous Ngb heme (Fe +2) (wt Ngb: $k_{on} = 1.5 \times 10^8 \text{M}^{-1}\text{s}^{-1}; k_{off} = 2 \times 10^4 \text{s}^{-1}$; H64L Ngb: $k_{on} = 2.7 \times 10^8 \text{M}^{-1}\text{s}^{-1}; k_{off} = 2 \times 10^4 \text{s}^{-1}$; H64Q Ngb: $k_{on} = 1.9 \times 10^8 \text{M}^{-1}\text{s}^{-1}; k_{off} = 3 \times 10^3 \text{s}^{-1}$) thus yielding ferric Ngb heme (Fe +3) and iron-nitrosyl-heme (Fe +2-NO) as a final reaction product (equation 2).

$$\text{Fe}^{+2} + \text{NO}_2^- + \text{H}^+ \rightarrow \text{Fe}^{+3} + \text{NO}^- + \text{OH}^- \quad (\text{eq. 1})$$

$$\text{NO}^- + \text{Fe}^{+2} \rightarrow \text{Fe}^{+2} - \text{NO} \quad (\text{eq. 2})$$

We observed a reaction stoichiometry consistent with the reaction of nitrite with hemoglobin or myoglobin, with two deoxy-Ngb molecules forming one iron-nitrosyl-Ngb and one ferric Ngb (Figure 1B). Analysis of the bimolecular rate constant indicated that the reaction of nitrite with Ngb proceeds overall at $0.12 \pm 0.02 \text{M}^{-1}\text{s}^{-1}$ at 25°C, pH 7.4 (0.26 ± 0.02 M$^{-1}\text{s}^{-1}$ at 37°C). A recent study (33) reported that the reaction of deoxy mouse neuroglobin with nitrite in the range 7–230 µM generated ferric met-Ngb in excess of ferrous nitrosyl-Ngb at apparent second-order rate constant of $5.1 \pm 0.4 \text{M}^{-1}\text{s}^{-1}$ however our experimental conditions with human neuroglobin differ considerably.

Both Salhany and the Gladwin group have shown (34,35) that the reaction of nitrite with hemoglobin in the presence of dithionite proceeds via equation 1-2, but the ferric heme that is formed is reduced back to the ferrous form to continue the reaction. Thus, iron-nitrosyl-heme forms at the same rate as deoxyheme is consumed and the overall stoichiometry is one deoxy-Ngb forming one iron-nitrosyl-Ngb. Performing the reaction in the presence of dithionite limits the auto-oxidation of the ferrous heme prior to the reaction with nitrite and allows for facile assessments of anaerobic reaction mechanisms and kinetics. By complementary studies using myoglobin we verified that the rate-limiting step of the reaction in the presence of dithionite is the heme iron catalyzed conversion of nitrite to NO (Supplemental Figure 2A and 2B). We then performed the reaction of anaerobic nitrite and deoxy-Ngb (10 mM and 10 µM respectively) as described above in the presence of 3 mM excess dithionite at pH 7.4 in 100 mM phosphate buffer (Figure 1 C and D). The stoichiometry was consistent with one deoxy-Ngb forming one iron-nitrosyl-Ngb and the calculated bimolecular rate constant was $0.11 \pm 0.01 \text{M}^{-1}\text{s}^{-1}$, in accordance with the value obtained in the absence of dithionite. We further investigated the reactivity of deoxy-Ngb with nitrite in the concentration range 0.25 - 20 mM (Figure 1E). The second-order bimolecular rate constant derived from the linear fit of the observed rate constants versus nitrite concentration is $0.12 \pm 0.02 \text{M}^{-1}\text{s}^{-1}$ in agreement with the calculated instantaneous reaction rate.

Proton dependence of the nitrite reductase reaction with neuroglobin. We next explored whether deoxy-Ngb dependent nitrite reduction requires a proton (equation 1). We determined the pH dependence of the bimolecular rate constant of the nitrite reductase reaction near the physiological range (pH 6.5-8.0) (Figure 1F). We found that increasing concentration of protons accelerates the reaction rate by 10-fold for each pH unit decrease. The slope of the linear fit, which represents the
order of rate dependence on $[H^+]$ is 0.96, close to the ideal 1.0, and it extends through the zero point (Figure 1F inset) indicating the requirement for one proton in the reaction. We conclude that the reaction constitutes a concerted electron and proton transfer to nitrite to form NO analogous to bacterial nitrite reductase.

Surface cysteines C46 and C55 regulate the heme pocket coordination and the rate of nitrite reduction to NO. Unlike most other globins, human Ngb displays 3 conserved cysteines (notable exception being mouse Ngb) at positions 46, 55 and 120 located on the protein surface as shown in the wild type thiol reduced human Ngb structure model (Figure 2A). When oxidized, cysteines 46 and 55 form an intra-molecular disulfide bond (36), which influences the position of the E-helix containing the distal histidine (22) and regulates the heme ligand binding equilibrium. Reduction of the disulfide bond allows additional structural freedom in the orientation of the E-helix (Figure 2A), that leads to an increased proportion of molecules in the six-coordinate state and thus reduced oxygen and nitrite binding affinities (22,24). We determined by the 4-PDS assay the number of accessible thiols per heme in our wild type Ngb, as purified, reduced by DTT and in the C55 to alanine mutant Ngb (Figure 2B). The results are consistent with the quantitative formation of a disulfide bond during protein purification and the presence of the single reduced Cys120 in the oxidized thiol form. To determine if the rate of nitrite reduction is influenced by the redox state of cysteines 46 and 55, we first reduced the cysteines by incubation with 10 mM DTT and then measured the rate of nitrite reduction after anaerobic DTT removal. Figure 2C shows that reduction of the disulfide bond slows down the rate by about 2-fold ($0.062 \pm 0.005 \text{ M}^{-1}\text{s}^{-1}$ at 25°C, pH 7.4). To directly test the hypothesis that disulfide bridge reduction affects the nitrite reactivity of neuroglobin, we generated recombinant mutants with cysteine 55 or 46 replaced by alanine (C55A and C46A), which slowed down the rate of nitrite reduction to similar rates observed with Ngb having fully reduced cysteines (Figure 2C). For a direct comparison we report the bimolecular reaction rates for WT and mutant Ngb proteins in Table 1 together with values for myoglobin and hemoglobin.

Physiological redox control of the C46-C55 disulfide bond regulates the rate of nitrite reduction to NO. We next determined if the formation of a disulfide bond between Cys 46 and 55 is redox-regulated within the physiological range of cellular redox state. We incubated wild type and C55A Ngb with increasing ratios of reduced/oxidized glutathione that established a gradient of ambient redox potentials (37). After 120 minutes incubation we removed glutathione anaerobically by passage through a G25 column and measured the rates of nitrite reduction (Figure 2D). We found that there was a sudden and substantial drop in the observed nitrite reductase rate constants ($k_{\text{obs}}$) with decreasing redox potential only for the wild type protein. Fitting the data to the Nernst equation provided a midpoint reduction potential of the C46/C55 thiol/disulfide redox couple of $-194 \pm 3 \text{ mV}$. This value is within the range of cellular redox potentials ($E. coli$ cytosol $E_0 = -280 \text{ mV}$ (37)).

To directly examine whether the cysteines redox state causes changes in heme pocket molecular and electronic structure we compared the NMR spectrum of wild type and C55A mutant met-Ngb (Figure 2E). Characteristic NMR signals for the heme methyls are visible in the spectral regions around 36 ppm, 23 ppm and 20 to 12 ppm and were assigned by comparison with the published spectra (38,39). The two spectra are largely similar but few marked differences in the positions of several heme methyl resonances (M8-B, M5-A, M1-A, M5-B) as well of several hyperfine shifted resonances between 18 and 12 ppm (region marked with an asterisk) are evident. Also some unassigned ring current shifted resonances around -2 ppm are different. We conclude that the thiol mutation C55A affects the geometry of the heme pocket environment.

Nicolis et al. reported that the oxidized disulfide-bridged (SS) met-Ngb exhibits a higher affinity for nitrite than the thiol reduced (SH) form (24) We then determined the nitrite affinity for the wild type SS- and SH-Ngb and for C55A mutant met-Ngb by difference spectra titration (Figure 2F). Due to the small absorbance changes, the calculated dissociation constants ($K_d$) reported in Table 2 show large standard deviations, nevertheless there is an apparent influence of the redox state of the cysteines on the distal histidine and nitrite affinity to the heme iron. During these
experiments we noticed that met-Ngb very slowly reacts with nitrite to produce nitrosyl-Ngb (bimolecular rate constants reported in Table 2). The slow rates of reaction produce a detectable spectroscopic effect only at high nitrite concentrations, approaching 0.1 M, and result in an artificial decrease of maximal absorbance difference that has previously been assigned to a second low-affinity binding constant (24). These experiments indicate that the redox state of cysteines C46 and C55 regulates both the five-to-six coordinate equilibrium and the rate of nitrite conversion to NO. Intriguingly, an analogous effect is observed with hemoglobin, in which oxidation of the cysteine 93 speeds up the rate of nitrite reduction to NO, and reduction slows the rate (40). This effect has been attributed to the effect of thiol oxidation on decreasing the heme redox potential.

The rate of nitrite reduction is maximal in the five-coordinate state of neuroglobin. To test the hypothesis that a change in the equilibrium between the five- and six-coordinate Ngb subpopulations mediates the control of the nitrite reduction rate, we generated recombinant Ngbss with His64 replaced by Leu or Gln. The absorbance spectra analysis of oxygenated and deoxygenated ferrous H64L and H64Q Ngb and the ferric species (Supplemental Figure 1) confirmed that both mutants are “locked” in the five coordinate conformation (41) and have very similar spectral characteristics to the classic five coordinate heme protein myoglobin (Supplemental Figure 1). We examined the reaction of nitrite with deoxygenated H64L Ngb in the presence of excess dithionite similarly to experiments with wild type Ngb but using 100 µM nitrite (Figure 3 A, B). To our surprise, the rate of deoxy-Ngb conversion to nitrosyl-Ngb was extremely fast, and the bimolecular rate constant was approximately 2000-fold higher than for the wild type Ngb. We then used fast mixing stopped-flow spectroscopy to determine the rates of the reaction in the range 10-1000 µM nitrite (Figure 3C). The observed rate constants increased linearly with increasing nitrite concentrations and the bimolecular rate constant derived from the linear least square fit was 259 ± 8 M⁻¹s⁻¹ at 25 °C, pH 7.4. Examination of the reaction at different pH values (Figure 3D) indicates that the reaction requires a proton similar to the reaction with wild type Ngb. Remarkably the rate increases above 2,500 M⁻¹s⁻¹ at pH 6.5 and 25 °C. The H64Q mutant showed a similar behavior, with a rate of 267 ± 16 M⁻¹s⁻¹ at 25 °C, pH 7.4 (Table 1, Supplemental Figure 3) and rates above 2,000 M⁻¹s⁻¹ at pH 6.5. These are the fastest reactions of nitrite with a heme-globin ever reported and confirm our hypothesis that the six-to-five coordinate heme pocket transition regulates the rate of nitrite reduction to NO.

We next determined the nitrite binding affinity for H64L and H64Q met-Ngb by fitting of the maximal changes in the Soret band as a function of nitrite concentration (Figure 3E, normalized difference spectra titration compared with wild type SS-Ngb). The calculated Kd values for these mutants are comparable to the wild type Ngb (Table 2) however the total maximal absorbance difference for H64L and H64Q mutants is more than 20-fold greater than wild type (Supplemental figure 4). These results suggest that in wild type Ngb His64 binding may outcompete nitrite binding even at high concentrations so only the ≈ 1% of five-coordinate molecules may bind nitrite, whereas for H64L and H64Q a fully bound situation is possible. The spectral changes for H64L and H64Q also indicate differences in the nitrite binding modes. For the H64L mutant there is a large increase in the Soret peak and little change in the 500-600 nm region. In the case of H64Q there is a decrease in the Soret peak accompanied by a shift to longer wavelengths and also changes in the 500-600 nm region that resemble the nitrite binding to Hb or Mb (Supplemental figure 4).

Finally we compared the reaction of 1 mM nitrite with our wild type Ngb (with oxidized and reduced cysteines), mutant H64L, H64Q and C55A Ngb (absorbance decrease traces of the Soret peak at 425 nm) in 0.1 M HEPES, pH 7.4. The relative percentage of the total absorbance change occurring in the first 60 minutes of the reaction is shown in Figure 3F (with H64L or H64Q Ngb normalized to 100%, wild type SS-Ngb is 38%, wild type SH-Ngb 20%, C55A Ngb 18% respectively). For both H64L and H64Q Ngb the reaction of the five-coordinate mutant proteins reached the end point in the first minute of the reaction and are expanded in the inset of Figure 3F.

Confirmation of reaction kinetics using electron paramagnetic resonance spectrometry. EPR spectrometry allows for direct measurement
of the paramagnetic NO-heme (iron-nitrosyl) species and provides confirmation of NO formation during the reaction of nitrite with Ngb. We evaluated the Fe(II)-NO build-up following reaction of 1 mM nitrite with wild type SS-Ngb, SH-Ngb and mutants H64L and H64Q Ngb (40 ± 5 µM) and compared it with the rate of iron-nitrosyl-myoglobin formation (Figure 4 A, B). EPR spectra analysis confirmed that the reduction of the cysteines (stabilizing the six-coordinate heme geometry) slowed the rate of iron-nitrosyl-Ngb formation, while replacement of the distal histidine with leucine (five-coordinate stabilization) dramatically increased the rate of NO formation. In particular, experiments using H64L and H64Q Ngb mutants and 1 mM nitrite were almost complete in one minute and to allow assessment of the reaction kinetics, lower concentrations of Ngb (10 µM) and nitrite (50 µM) were necessary (Figure 4 C, D). The calculated rates of nitrosyl-Ngb formation are similar to data obtained by absorbance spectrometry.

**Nitrite reduction by deoxyneuroglobin generates NO.** The reaction of nitrite with deoxy-Ngb generates NO and ferric Ngb. Although in our in vitro conditions deoxy-Ngb can recapture the NO, we next explored if free NO gas can escape at measurable rates. We mixed anaerobic Ngb (20 µM) and nitrite (1 mM) in a vessel purged with helium and carried in-line to a chemiluminescent NO analyzer. In these conditions the anaerobic mixture generated NO in gas phase (Figure 5A) and the rate of NO formation was again regulated by the cysteines 46-55 disulfide bond and by the heme pocket six-to-five coordination equilibrium. Figure 5B shows that the rate of NO detected was significantly decreased in reactions with six-coordinate C55A Ngb and increased in reactions with the five coordinate H64L Ngb, consistent with the hypothesis of six-to-five coordinate heme pocket control of nitrite reduction. Finally we incubated mutant H64L Ngb (30 µM) with increasing amounts of nitrite, starting with physiologically relevant concentrations (10 and 25 µM) up to 1 mM and we observed a rapid NO generation response roughly proportional to the nitrite concentration injected (Figure 5C).

**Comparison of mutant myoglobin and neuroglobin confirms unique fast reactivity of five-coordinate neuroglobin with nitrite.** In order to elucidate the factors that confer increased nitrite reductase activity to the Ngb H64L mutant we characterized the nitrite reductase activities of Mb mutants H64L and H64A and Ngb mutant H64Q (summarized in Table 1). Despite the structural similarities between the two globins, the difference in reactivity for comparable His64 mutations took opposite directions. Replacement of Mb His64 with Leu or Ala leads to decreased reactivity. This may be related to His64 stabilizing the heme ligands through hydrogen bonding as highlighted by previous reports (42,43). On the other hand Ngb H64Q shows similar rates to the H64L mutant. This indicates that the removal of the 6th ligand and formation of a stable 5-coordinate neuroglobin is the major determinant of the increased reactivity, and the residue polarity constitutes a lesser effect. Possible explanations for these effects are discussed below.

**Nitrite reduction by deoxyneuroglobin mediates NO signaling.** To test whether Ngb generated NO inhibits mitochondrial respiration during hypoxia, isolated rat liver mitochondria were placed in a sealed, stirred respirometer and substrates were added to stimulate respiration as previously described (19). Mitochondria were allowed to respire until the ambient oxygen tension dropped below detection level. At this point the respirometer is opened to air oxygen and cyanide is added to evaluate the time to complete inhibition of respiration, as determined by the increase in oxygen tensions measured with a Clark electrode (Figure 6A). The extent of mitochondrial inhibition for all experiments (without cyanide) is then compared to the effect of cyanide. We detected no significant inhibition of respiration when nitrite (20 µM) or purified wild-type Ngb (5 µM) alone were incubated with respiring mitochondria. However, when the same concentrations of nitrite and protein were allowed to react together we observed 78 ± 6 % inhibition of respiration. As expected the extent of inhibition was increased significantly by the H64L mutant Ngb (96 ± 2% inhibition) and decreased by the C55A mutant Ngb (62 ± 4 % inhibition) (Figure 6B). To evaluate this in cells, the neuronal cell line SHSY5Y were stably transfected using a lentivirus vector with GFP-tagged wild type and H64L mutant Ngb (Figure 6C) and were used to perform similar experiments. One million intact SHSY5Y cells were suspended in the respirometer and...
maximal respiration rate was stimulated by addition of the uncoupler FCCP. Then nitrite was added to cells transfected with GFP only (negative control) and cells expressing wild type Ngb or the H64L mutant Ngb. In Figure 6D we compare the extent of respiration inhibition to the cyanide effect (complete inhibition): cells with GFP only exhibited no significant inhibition but we observed about 15% and 40% inhibition respectively for wild type and H64L Ngb.

**DISCUSSION**

Altogether our experiments reveal that (1) neuroglobin can function as a nitrite reductase, producing NO from nitrite; (2) the redox state of the surface thiols of C46 and C55 modulates the heme coordination, nitrite affinity and NO generation and signaling; (3) The replacement of the His64 side chain locks the heme in a 5-coordinate geometry with increased reactivity towards nitrite; (4) under hypoxic conditions neuroglobin can inhibit mitochondrial respiration in the presence of nitrite.

We also show that the reaction of deoxy-neuroglobin with nitrite proceeds with stoichiometry formally similar to the reaction of myoglobin or hemoglobin (44). However, in neuroglobin the process is further regulated by the six-to-five coordinate equilibrium.

A mechanistic proposal is presented in scheme 1 where the first step of the reaction is the dissociation of His64 from the heme iron. The results obtained with Ngb H64L and H64Q mutants indicate that this process directly limits the nitrite reduction rate. The 5-coordinate heme can now bind to external ligands such as nitrite. As evidenced in recent reports (45,46) by experimental and density functional theoretical studies, nitrite may bind to the heme in either N-nitro or O-nitrito conformations (scheme 1, step 2). We cannot conclude from our results if either binding mode is preferred, and given the differences in the heme pocket of Ngb as compared to Mb (supplemental figure 5) the observations made in Mb may not extrapolate (47). Addition of a proton to the nitrite yields the nitrous acid bound species, those can be directly formed by nitrite at low pH. An alternative possibility is indeed the initial binding of HONO to five coordinate heme iron. In the N-bound route, loss of OH' and electron transfer yields the Fe(III)-NO species with subsequent NO dissociation. In the O-bound route, NO dissociates from the bound nitrous acid and Fe(III)-OH is formed. This met-hydroxide complex can be then protonated to the aquomet form. Independently of the route, the end products of the reaction are eventually the same: Fe(III) heme and NO. Interestingly, the dependence of this reaction on the concentration of proton is opposite to that of the reductive nitrosylation reaction, where the reaction rate increases with the concentration of OH' (48). These data show that the nitrite reductase reaction is the reverse of reductive nitrosylation. From the Scheme 1 the relationship between both reactions is apparent, although it must be noted that the N-bound and O-bound routes indicate two different reductive nitrosylation reactions, one involving Fe^{III}.NO + OH' and another with Fe^{III}.OH' + NO. It is conceivable that depending on the protein (and the concentrations of NO and OH'), one route may be more important than the other.

In myoglobin, the H64L mutation severely impairs ligand binding of cyanide and azide (42,49) as hydrogen bonding appears to be necessary to stabilize the ligand. The situation for neuroglobin appears to be very different and once the His64-heme interaction is disrupted the ligand affinity increases. Ngb H64L and H46Q show no significant differences in the nitrite reduction rates and thus nitrite reduction appears to be unrelated to hydrogen bonding. We speculate that the differences in the nitrite reactivity for Ngb and Mb/Hb (and the different requirements for ligand hydrogen binding) can be related to differences in the preferred nitrite-binding mode. This in turn may be related to the electronic properties of the heme. These concepts deserve further research; resolving the crystal structure of the nitrite bound ferric Ngb complex could provide definitive experimental insight into the nature of this rather complex nitrite-heme interaction.

**Modeling of nitrite reduction by five-coordinate neuroglobin.** Taking into account the proposed mechanism of reaction in scheme 1, we can expand and rewrite our reaction model as:

\[
\text{Fe}^{2+} (6c) \leftrightarrow \text{Fe}^{2+} (5c) \quad (\text{eq. 3})
\]

\[
\text{H}^+ + \text{NO}_2^- \leftrightarrow \text{HNO}_2 \quad (\text{eq. 4})
\]

\[
\text{Fe}^{2+} (5c) + \text{HNO}_2 \rightarrow \text{Fe}^{3+} + \text{NO}^+ + \text{OH}^- \quad (\text{eq. 5})
\]
Fe$^{+2}$ (5c) + NO$_2^-$ + H$^+$ → Fe$^{+3}$ + NO$^-$ + OH$^-$ (eq. 6)
NO$^-$ + Fe$^{+2}$ → Fe$^{+2}$ –NO (eq. 7)

Then the rate of reduction of nitrite by Ngb can be written as:

$$\frac{d[Ngb]}{dt} = \frac{k_0[H^+]/K_a + k'}{(1 + K_{His})} [Ngb][Nitrite]$$

(eq. 8)

where $K_{His}$ is the ratio of hexacoordinate to pentacoordinate Ngb (eq.3), $K_a$ is the equilibrium constant for nitrous acid and nitrite anion (about $10^{-3.15}$, eq. 4), $k_0$ is the rate of reaction of nitrous acid with Ngb (eq.5) and $k'$ is the rate that nitrite anion reacts with Ngb (eq. 6). This equation follows that used by Doyle for the reduction of nitrite by hemoglobin (50), combined with the idea that the heme must be at least transiently pentacoordinate for the reaction to occur. It describes the reaction to be bimolecular in nitrite and Ngb with the bimolecular rate constant $k$ being equal to the term multiplying the concentration of these reactants:

$$k = \frac{k_0[H^+]/K_a + k'}{(1 + K_{His})}$$

(eq. 9).

The rate of the reaction of Ngb with nitrite anion, like Hb, is small compared to that with nitrous acid except at very high pH. In principle, $k_0$ could be different for the WT and H64L or other Ngb mutants. However, if the only or dominating effect of the mutation is the formation of a permanently pentacoordinate heme ($K_{His} = \infty$), then one would expect $k_0$ to be the same for both species. On the other hand, if the mutation affects more than the heme coordination state (such as hydrophobicity in the heme pocket) then we would expect it to change $k_0$ as well.

In order to test the simple model and determine whether the H64L mutation does more than affect the coordination of the heme, we modeled our bimolecular rate constants as a function of pH. Figure 7A shows the data and fit for the H64L mutant where $K_{His}$ is taken as infinity. The least squares fit gave $k_0$ as $6.5 \times 10^6$ M$^{-1}$s$^{-1}$ and $k'$ as $49$ M$^{-1}$s$^{-1}$ confirming that $k_0$ dominates. Next we used these values to fit the WT data (Figure 7B), with the only free parameter now being $K_{His}$ which we found to be $2.4 \times 10^{-4}$ M$^{-1}$. The model fits the data very well and the value obtained for $K_{His}$ is very much in agreement with those determined previously (for example $3.3 \times 10^{-4}$ M$^{-1}$ (51)). Additional calculations with the H64Q mutant (supplemental figure 3) yielded values of $k_0$ as $4.6 \times 10^6$ M$^{-1}$s$^{-1}$ and $k'$ as $50$ M$^{-1}$s$^{-1}$, in remarkable agreement given the values determined for H64L. In conclusion, our results support the idea that the rate differences between WT and H64L/H64Q Ngb can be primarily determined by the heme coordination (i.e the six-coordinated to five-coordinated transition) and the nitrite reactivity of the WT enzyme could be vastly increased by changes in the $K_{His}$ parameter.

**Physiological relevance of the nitrite reductase activity of neuroglobin.** Neuroglobin has been shown to promote survival of neurons in hypoxic conditions, but its physiological function is still uncertain. Some of the proposed functions include oxygen supply, ROS/RNS detoxification, regulation of signaling pathways (10) and more recently inhibition of cytochrome c induced apoptosis (52). Ngb is expressed in metabolically active cells and organs (neurons, endocrine organs, retina, etc.) and has been hypothesized to interact with mitochondria and mediate cytoprotective responses to ischemic stress (53). NO binding to cytochrome c oxidase has been shown to reversibly inhibit electron transport at low oxygen tensions, in a process thought to contribute physiologically to hypoxic vasodilation and to the extension of oxygen diffusion gradients (54,55). There is also increasing evidence of neuroprotective effects of NO and nitrite/nitrate (56,57). We therefore hypothesized that the nitrite reductase activity of Ngb may regulate the hypoxic inhibition of cellular respiration by NO binding to cytochrome c oxidase. This pathway provides an alternative explanation of the protective action of neuroglobin.

NO can inhibit mitochondrial respiration and activate soluble guanylyl cyclase at picomolar concentrations. For this reason it is possible that changes in the disulfide bond may produce enough NO to sustain physiologically relevant NO synthesis rates (Figure 8).
Consistent with this thesis, our data (Figure 5) demonstrate an interaction between nitrite and deoxygenated neuroglobin that generates bioavailable NO. In Figure 6A using isolated mitochondria in the presence of nitrite we observe more effective inhibition of respiration with wild type Ngb, than the C55A mutant. On the other hand, the H64L and H64Q mutants indicate that fast rates of NO generation are within the reach of the enzyme. The extent of mitochondrial inhibition is dependent on the heme coordination structure of neuroglobin and intrinsic nitrite reductase activity. We speculate that the observed redox effects may be amplified by external effects such as changes in pH and protein-protein modifications, namely protein phosphorylation and/or protein-protein interactions, that further open up the enzyme under allosteric control.

In conclusion, the molecular examination of critical heme pocket and surface thiol amino acids, using site directed mutagenesis, provides a novel understanding of neuroglobin functionality as an enzyme with a redox regulated six-to-five coordinate iron heme transition that directs nitrite in the heme pocket for controlled electron and proton transfer reactions to form NO. The results presented in this study support the provocative hypothesis that the cellular six-coordinate heme globins, neuroglobin, cytoglobin, Drosophila melanogaster hemoglobin, and plant hemoglobins may subserve a function as primordial allosterically redox regulated NO signaling proteins. The identification of other allosteric regulators of the six-to-five coordination of the neuroglobin heme pocket may reveal new intracellular mechanisms for controlling NO signaling via nitrite reduction.
REFERENCES

1. Hankeln, T., Ebner, B., Fuchs, C., Gerlach, F., Haberkamp, M., Laufs, T. L., Roesner, A., Schmidt, M., Weich, B., Wystub, S., Saaler-Reinhardt, S., Reuss, S., Bolognesi, M., De Sanctis, D., Marden, M. C., Kiger, L., Moens, L., Dewilde, S., Nevo, E., Avivi, A., Weber, R. E., Fago, A., and Burmester, T. (2005) *J. Inorg. Biochem.* **99**, 110-119

2. Brunori, M., and Vallone, B. (2007) *Cell. Mol. Life Sci.* **64**, 1259-1268

3. Burmester, T., Haberkamp, M., Mitz, S., Roesner, A., Schmidt, M., Ebner, B., Gerlach, F., Fuchs, C., and Hankeln, T. (2004) *IUBMB Life* **56**, 703-707

4. Dewilde, S., Kiger, L., Burmester, T., Hankeln, T., Baudin-Creuza, V., Aerts, T., Marden, M. C., Caubergs, R., and Moens, L. (2001) *J. Biol. Chem.* **276**, 38949-38955

5. Uzan, J., Dewilde, S., Burmester, T., Hankeln, T., Moens, L., Hamdane, D., Marden, M. C., and Kiger, L. (2004) *Biophys. J.* **87**, 1196-1204

6. Capece, L., Marti, M. A., Bidon-Chanal, A., Nadra, A., Luque, F. J., and Estrin, D. A. (2008) *Proteins*

7. Krieg, J. M., Bhattacharyya, A. J., Nienhaus, K., Deng, P., Minkow, O., and Nienhaus, G. U. (2002) *Proc. Natl. Acad. Sci. U S A* **99**, 7992-7997

8. Kiger, L., Uzan, J., Dewilde, S., Burmester, T., Hankeln, T., Moens, L., Hamdane, D., Baudin-Creuza, V., and Marden, M. (2004) *IUBMB Life* **56**, 709-719

9. Giuffre, A., Moschetti, T., Vallone, B., and Brunori, M. (2008) *Biochem. Biophys. Res. Commun.* **367**, 893-898

10. Burmester, T., and Hankeln, T. (2009) *Journal of Experimental Biology* **212**, 1423-1428

11. Greenberg, D. A., Jin, K., and Khan, A. A. (2008) *Curr. Opin. Pharmacol.* **8**, 20-24

12. Khan, A. A., Wang, Y., Sun, Y., Mao, X. O., Xie, L., Miles, E., Graboski, J., Chen, S., Ellerby, L. M., Jin, K., and Greenberg, D. A. (2006) *Proc Natl Acad Sci U S A* **103**, 17944-17948

13. Krieg, J. M., Bhattacharyya, A. J., Nienhaus, K., Deng, P., Minkow, O., and Nienhaus, G. U. (2002) *Proc. Natl. Acad. Sci. U S A* **99**, 7992-7997

14. Kiger, L., Uzan, J., Dewilde, S., Burmester, T., Hankeln, T., Moens, L., Hamdane, D., Baudin-Creuza, V., and Marden, M. (2004) *IUBMB Life* **56**, 709-719

15. Giuffre, A., Moschetti, T., Vallone, B., and Brunori, M. (2008) *Biochem. Biophys. Res. Commun.* **367**, 893-898

16. Burmester, T., and Hankeln, T. (2009) *Journal of Experimental Biology* **212**, 1423-1428

17. Greenberg, D. A., Jin, K., and Khan, A. A. (2008) *Curr. Opin. Pharmacol.* **8**, 20-24

18. Khan, A. A., Wang, Y., Sun, Y., Mao, X. O., Xie, L., Miles, E., Graboski, J., Chen, S., Ellerby, L. M., Jin, K., and Greenberg, D. A. (2006) *Proc Natl Acad Sci U S A* **103**, 17944-17948

19. Wang, X., Liu, J., Zhu, H., Tejima, E., Tsuji, K., Murata, Y., Atochin, D. N., Huang, P. L., Zhang, C., and Lo, E. H. (2008) *Stroke* **39**, 1869-1874

20. Sun, Y., Jin, K., Mao, X. O., Zhu, Y., and Greenberg, D. A. (2001) *Proc. Natl. Acad. Sci. U S A* **98**, 15306-15311

21. Weiland, T. R., Kundu, S., Trent, J. T., 3rd, Hoy, J. A., and Hargrove, M. S. (2004) *J. Am. Chem. Soc.* **126**, 11930-11935

22. Nadra, A. D., Marti, M. A., Pesce, A., Bolognesi, M., and Estrin, D. A. (2008) *Proteins* **71**, 695-705

23. Garrocho-Villegas, V., Gopalasubramaniam, S. K., and Arredondo-Peter, R. (2007) *Gene* **398**, 78-85

24. Huang, Z., Shiva, S., Kim-Shapiro, D. B., Patel, R. P., Ringwood, L. A., Irby, C. E., Huang, K. T., Ho, C., Hogg, N., Schechter, A. N., and Gladwin, M. T. (2005) *J. Clin. Invest.* **115**, 2099-2107

25. Hogg, N., Schechter, A. N., and Gladwin, M. T. (2005) *J. Clin. Invest.* **115**, 2099-2107

26. Shiva, S., Huang, Z., Grubina, R., Sun, J., Ringwood, L. A., MacArthur, P. H., Xu, X., Murphy, E., Darley-Usmar, V. M., and Gladwin, M. T. (2007) *Circ. Res.* **100**, 654-661

27. Hendgen-Cotta, U. B., Merx, M. W., Shiva, S., Schmitz, J., Becher, S., Klare, J. P., Steinhoff, H. J., Goedecke, A., Schrader, J., Gladwin, M. T., Kelm, M., and Rassaf, T. (2008) *Proc. Natl. Acad. Sci. U S A* **105**, 10256-10261

28. Basu, S., Azarova, N. A., Font, M. D., King, S. B., Hogg, N., Gladwin, M. T., Shiva, S., and Kim-Shapiro, D. B. (2008) *J. Biol. Chem.* **283**, 32590-32597

29. Hamdane, D., Kiger, L., Dewilde, S., Green, B. N., Pesce, A., Uzan, J., Burmester, T., Hankeln, T., Bolognesi, M., Moens, L., and Marden, M. C. (2003) *J. Biol. Chem.* **278**, 51713-51721

30. Hamdane, D., Kiger, L., Dewilde, S., Green, B. N., Pesce, A., Uzan, J., Burmester, T., Hankeln, T., Bolognesi, M., Moens, L., and Marden, M. C. (2004) *Micron.* **35**, 59-62

31. Nicolis, S., Monzani, E., Ciaccio, C., Ascenzi, P., Moens, L., and Casella, L. (2007) *Biochem. J.* **407**, 89-99
25. Grubina, R., Huang, Z., Shiva, S., Joshi, M. S., Azarov, I., Basu, S., Ringwood, L. A., Jiang, A., Hogg, N., Kim-Shapiro, D. B., and Gladwin, M. T. (2007) *J. Biol. Chem.* **282**, 12916-12927
26. Burmester, T., Weich, B., Reinhardt, S., and Hankeln, T. (2000) *Nature* **407**, 520-523
27. Grassetti, D. R., and Murray, J. F., Jr. (1967) *Arch. Biochem. Biophys.* **119**, 41-49
28. Pesce, A., Dewilde, S., Nardini, M., Moens, L., Ascenzi, P., Hankeln, T., Burmester, T., and Bolognesi, M. (2003) *Structure* **11**, 1087-1095
29. Schwede, T., Kopp, J., Guex, N., and Peitsch, M. C. (2003) *Nucleic Acids Res.* **31**, 3381-3385
30. Yi, L., Jenkins, P. M., Leichert, L. I., Jakob, U., Martens, J. R., and Ragsdale, S. W. (2009) *J. Biol. Chem.* **284**, 20556-20561
31. Azarov, I., Huang, K. T., Basu, S., Gladwin, M. T., Hogg, N., and Kim-Shapiro, D. B. (2005) *J. Biol. Chem.* **280**, 39024-38032
32. Van Doorslaer, S., Dewilde, S., Kiger, L., Nistor, S. V., Goovaerts, E., Marden, M. C., and Moens, L. (2003) *J. Biol. Chem.* **278**, 4919-4925
33. Petersen, M. G., Dewilde, S., and Fago, A. (2008) *J. Inorg. Biochem.* **102**, 1777-1782
34. Grubina, R., Basu, S., Tiso, M., Kim-Shapiro, D. B., and Gladwin, M. T. (2007) *J. Biol. Chem.* **282**, 12916-12927
35. Salhany, J. M. (2008) *Biochemistry* **47**, 6059-6072
36. Wakasugi, K., Nakano, T., and Morishima, I. (2003) *J. Biol. Chem.* **278**, 36505-36512
37. Schafer, F. Q., and Buettner, G. R. (2001) *Free Radic. Biol. Med.* **30**, 1191-1212
38. Du, W., Syvitski, R., Dewilde, S., Moens, L., and La Mar, G. N. (2003) *J. Biol. Chem.* **278**, 36505-36512
39. Xu, J., Li, L., Yin, G., Li, H., and Du, W. (2009) *J. Inorg. Biochem.* **103**, 1693-1701
40. Crawford, J. H., Isbell, T. S., Huang, Z., Shiva, S., Shacko, B. K., Schechter, A. N., Darley-Usmar, V. M., Kerby, J. D., Lang, J. D., Jr., Kraus, D., Ho, C., Gladwin, M. T., and Patel, R. P. (2006) *Blood* **107**, 566-574
41. Nienhaus, K., Kriegl, J. M., and Nienhaus, G. U. (2004) *J. Biol. Chem.* **279**, 22944-22952
42. Ikeda-Saito, M., Hori, H., Andersson, L. A., Prince, R. C., Pickering, I. J., George, G. N., Sanders, C. R., 2nd, Lutz, R. S., McKelvey, E. J., and Mattera, R. (1992) *J. Biol. Chem.* **267**, 22843-22852
43. Biram, D., Garratt, C. J., and Hester, R. E. (1993) *Biochim. Biophys. Acta* **1163**, 67-74
44. Gladwin, M. T., Grubina, R., and Doyle, M. P. (2009) *Acc. Chem. Res.* **42**, 157-167
45. Yi, J., Safo, M. K., and Richter-Addo, G. B. (2008) *Biochemistry* **47**, 8247-8249
46. Basu, S., Grubina, R., Huang, J., Conradi, J., Huang, Z., Jeffers, A., Jiang, A., He, X., Azarov, I., Seibert, R., Mehta, A., Patel, R., King, S. B., Hogg, N., Ghosh, A., Gladwin, M. T., and Kim-Shapiro, D. B. (2007) *Nat. Chem. Biol.* **3**, 785-794
47. Copeland, D. M., Soares, A. S., West, A. H., and Richter-Addo, G. B. (2006) *J. Inorg. Biochem.* **100**, 1413-1425
48. Hoshino, M., Maeda, M., Konishi, R., Seki, H., and Ford, P. (1996) *J. Am. Chem. Soc* **118**, 5702-5707
49. Branccacio, A., Cutruzzola, F., Allocatelli, C. T., Brunori, M., Smerdon, S. J., Wilkinson, A. J., Dou, Y., Keenan, D., Ikeda-Saito, M., Brantley, R. E., Jr., and et al. (1994) *J. Biol. Chem.* **269**, 13843-13853
50. Doyle, M. P., Pickering, R. A., DeWeert, T. M., Hoekstra, J. W., and Pater, D. (1981) *J. Biol. Chem.* **256**, 12393-12398
51. Kiger, L., Uzan, J., Dewilde, S., Burmester, T., Hankeln, T., Moens, L., Hamdane, D., Baudin-Creuzoa, V., and Marden, M. C. (2004) *Iubmb Life* **56**, 709-719
52. Raychaudhuri, S., Skommer, J., Henty, K., Birch, N., and Brittain, T. (2010) *Apoptosis* **15**, 401-411
53. Liu, J., Yu, Z., Guo, S., Lee, S. R., Xing, C., Zhang, C., Gao, Y., Nicholls, D. G., Lo, E. H., and Wang, X. (2009) *J. Neurosci. Res.* **87**, 164-170
54. Brunori, M., Giuffre, A., Forte, E., Mastronicola, D., Barone, M. C., and Sarti, P. (2004) *Biochim. Biophys. Acta* **1655**, 365-371
55. Mason, M. G., Nicholls, P., Wilson, M. T., and Cooper, C. E. (2006) *Proc. Natl. Acad. Sci. U S A* **103**, 708-713

56. Presley, T. D., Morgan, A. R., Bechtold, E., Clodfelter, W., Dove, R. W., Jennings, J. M., Kraft, R. A., King, S. B., Laurienti, P. J., Rejeski, W. J., Burdette, J. H., Kim-Shapiro, D. B., and Miller, G. D. (2011) *Nitric Oxide* **24**, 34-42

57. Jung, K. H., Chu, K., Lee, S. T., Sunwoo, J. S., Park, D. K., Kim, J. H., Kim, S., Lee, S. K., Kim, M., and Roh, J. K. (2010) *Biochem. Biophys. Res. Commun.* **403**, 66-72

**ABBREVIATIONS**

Ngb, neuroglobin; Mb, myoglobin; Hb, hemoglobin; 4-PDS, 4,4’-dithiodipyridine; DTT, dithiothreitol; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; SHE, standard hydrogen electrode.

**ACKNOWLEDGEMENTS**

We thank Dr. Li Yi for helpful advice and discussions on disulfide couple redox potential determination and Dr. Yinna Wang for excellent technical assistance. Sperm whale myoglobins were a gift from John S. Olson (Rice University). This work was supported in part by NIH grants HL058091 to D.B.K.S. and GM084614 to C.H., and M.T.G. was supported by NIH grant HL098032, the Institute for Transfusion Medicine, and the Hemophilia Center of Western Pennsylvania (M.T.G and S.S.), and AHA 109SDG2150066 (S.S.).

**SUPPLEMENTARY MATERIAL**

The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1, Figures S1–S5 and the detailed derivation of the equation 8.
TABLE 1

Summary of bimolecular reaction rates for the reaction of heme containing deoxy-globins with nitrite (Hb = human hemoglobin, sw Mb = sperm whale myoglobin, Ngb = neuroglobin). All reactions were studied in sodium phosphate buffer 100 mM, pH 7.4. Hemoglobin values determined at 37 °C, Myoglobin and Neuroglobin values determined at 25 °C.

| Protein       | $k$ (M$^{-1}$s$^{-1}$)       |
|---------------|-----------------------------|
| Hb (T state)  | $\approx 0.12^a$            |
| Hb (R state)  | $\approx 6^a$               |
| Horse Mb      | $2.9 \pm 0.2$               |
| sw Mb WT      | $5.6 \pm 0.6$               |
| sw Mb H64A    | $1.8 \pm 0.3$               |
| sw Mb H64L    | very slow $^b$              |
| Ngb WT SS     | $0.12 \pm 0.02$             |
| Ngb WT SH     | $0.062 \pm 0.005$           |
| Ngb C55A      | $0.060 \pm 0.008$           |
| Ngb C46A      | $0.058 \pm 0.006$           |
| Ngb H64L      | $259 \pm 8$                 |
| Ngb H64Q      | $267 \pm 16$                |

$^a$ Values from reference (18)

$^b$ The reaction of Mb H64L is significantly (more than 10-fold) slower than wild-type or H64A sperm whale Mb and apparently independent of [NO$\cdot$] in the concentration range studied (1-5 mM)
TABLE 2

Nitrite dissociation constants ($K_d$) and bimolecular rate constants ($k$) for reactions of met-Ngb with nitrite determined at 25 °C in 200 mM phosphate buffer, pH 7.4.

| Protein | $K_d$ (NO$_2^-$) (mM) | rate of nitrite ferric heme reduction ($\text{M}^{-1}\text{s}^{-1}$) |
|---------|----------------------|-------------------------------------------------|
| NGB WT SS | 6.2 ± 2.1 | 0.0005 ± 0.0005 |
| NGB WT SH | 12.6 ± 3.3 | 0.0002 ± 0.0005 |
| NGB C55A | 30.1 ± 4.5 | 0.0002 ± 0.0004 |
| NGB H64L | 8.3 ± 1.8 | 0.032 ± 0.002 |
| NGB H64Q | 12.5 ± 1.8 | 0.016 ± 0.009 |
FIGURE LEGENDS

Figure 1. Anaerobic reaction of deoxyneuroglobin with nitrite in the absence and in the presence of dithionite. (A) Selected visible spectra of the reaction between 10 µM deoxyNgb and 10 mM nitrite at 1 min intervals. (B) Time-dependent changes of deoxyNgb (blue), iron-nitrosyl-Ngb (green) and total met-Ngb (red) concentration during the reaction. (C) (D) Respectively as in panels A and B for the reaction in the presence of 3 mM dithionite. (E) Plot of observed rate constants (k_{obs}) versus nitrite concentration, the second-order bimolecular rate constant obtained from the linear fit of the data is 0.12 ± 0.02 M^{-1}s^{-1} (F) Effect of pH on the nitrite reductase reaction rates. Inset: The bimolecular rate constant is linear with the proton concentration and it extends through the zero point (line shows linear regression analysis of the data). All measurements were made in 100 mM phosphate buffer and 25 °C as described in “Experimental procedures”.

Figure 2. Redox state of cysteines 46 and 55 modulates nitrite reductase reactivity. (A) Model of the wild-type human neuroglobin structure with indicated reduced cysteines C46, C55 and C120. (B) Determination of the number of reduced cysteines by the 4-PDS assay (see methods) (C) Comparison of the decrease of deoxy-Ngb and the formation of iron-nitrosyl Ngb over time for wild-type Ngb with oxidized (SS) and reduced (SH) thiol, C46A and C55A mutant Ngb (D) Observed nitrite reductase rate constants versus determined redox potentials. The midpoint redox potential of the thiol/disulfide couple in wild-type Ngb is −194 ± 3 mV. (E) Comparison of the NMR spectrum of wild type and C55A mutant met-Ngb. (F) Nitrite binding affinity constant determination by difference spectral titration for wild-type, DTT cysteines reduced and C55A mutant Ngb by differential spectra after indicated nitrite addition.

Figure 3. Kinetics of nitrite reaction with mutant H64L Ngb. (A) and (B) Spectrophotometric analysis of the anaerobic reaction of 10 µM H64L deoxy-Ngb with 100 µM nitrite at pH 7.4, 25°C and 3 mM dithionite. (C) Plot of k_{obs} versus nitrite concentration (10 µM - 1 mM) for H64L Ngb-mediated reduction of nitrite and formation of Ngb Fe(II)NO at pH 7.4 and 25 °C. The bimolecular rate constant derived from the linear fit of the data is 259 ± 8 M^{-1}s^{-1}. (D) Effect of different pH on the nitrite reductases rates. Inset: BRC is linear with the proton concentration (E) Nitrite binding affinity constant determination for H64L and H64Q (F) Comparison of representative traces of Ngb wildtype (with reduced and oxidized surface thiols) and mutants H64L and H64Q. The absorbance decreases of the Soret peak (425nm) are plotted as the percentage of the total absorbance change for human Ngb H64L measured at 25 °C, pH 7.4.

Figure 4. EPR spectroscopy. (A) and (C) EPR spectra showing Fe(II)-NO build-up following addition of amount of nitrite. (B) and (D) The rate of formation of iron-nitrosyl-heme (Fe^{2+}-NO) species measured by EPR. The concentrations were determined by performing the double integral calculation and comparing to standard samples.

Figure 5. Nitrite reduction by deoxyneuroglobin generates NO gas (A) Representative chemiluminescence traces of NO detection in gas phase released during the anaerobic reaction of nitrite with buffer only (blue) or 20 µM deoxyNgb wild type (black), H64L (red) or C55A (green). (B) Quantification of the rate of NO detected per min. (C) The nitric oxide signal measured during incubation of 30 µM H64L deoxyNgb and increasing concentrations of nitrite.

Figure 6. Deoxyneuroglobin nitrite reduction mediates intracellular NO signaling. (A) Traces of oxygen consumption by isolated mitochondria showing nitrite dependent inhibition of respiration; the early rise in oxygen tension indicates NO-dependent inhibition of cellular respiration which is maximal for cyanide (B) Comparison of percentage of extent of inhibition (cyanide defined as 100% inhibition) as measured in panel A for isolated mitochondria (C) Quantification of expression of GFP only, wild type Ngb and H64L mutant Ngb in lentivirus transfected and cloned SHSY5Y cells by Western blot of 4-15% SDS-polyacrylamide gradient gel (D) Mean extent of hypoxic inhibition of cellular respiration by
incubation of SHSY5Y cells expressing GFP, wild type Ngb or H64L Ngb with 20 µM nitrite (*P < 0.01, ** P < 0.001, compared with control).

**Figure 7. Fitting of bimolecular rate constants as a function of pH.** Equation 9 was used to fit the data with $K_a$ taken as $7 \times 10^{-4}$ M$^{-1}$. (A) The data for H64L were fit allowing $k_0$ and $k'$ to vary. (B) The data for WT were fit allowing only $K_{\text{His}}$ to vary using the values obtained from fitting of H64L data (Panel A) for the other parameters.

**Figure 8. Neuroglobin act as a nitrite reductase under oxidative stress conditions.** In normal conditions, cells keep a high concentration of reduced glutathione (GSH) and low oxidized glutathione (GSSG). In these circumstances the disulfide bond of Ngb is not formed and the protein has a low nitrite reductase activity (left). As oxidative stress conditions develop (right), reduced glutathione is consumed and the number of neuroglobin molecules with formed disulfide bonds increases. The production of NO from nitrite increases, causing the inhibition of respiratory enzymes and limiting oxygen consumption and ROS-producing reactions.

**Scheme 1. Mechanistic proposal for the reaction of neuroglobin with nitrite (N-bound vs O-bound routes).** The first step of the reaction is the dissociation of His64 from the heme iron (left). The 5-coordinate heme can now bind nitrite. Two binding modes for nitrite are possible, N-binding (top right section) or O-binding (bottom right section). Subsequent addition of a proton yields the nitrous acid bound species (those can be directly formed by nitrite at low pH). In the N-bound route, loss of $OH^-$ and electron transfer yields the FeIII-NO species with subsequent NO dissociation. In the O-bound route, NO dissociates from the bond nitrous acid and FeIII-OH is formed. This met-hydroxide complex can be then protonated to the aquomet form.
Figure 4

A

Intensity vs. Field (G)

B

Concentration (μM) vs. Time (s)

C

Intensity vs. Field (G)

D

Concentration (μM) vs. Time (s)

Legend:
- MnNO
- SS-NgbNO
- SH-NgbNO
- H64L-NgbNO
- H64Q-NgbNO

Details:
- 40μM Ngb wt, 1mM nitrite
- 40μM H64L Ngb, 50μM nitrite
Figure 5

A

B

C

[Graphs and data related to NO signal and production in different conditions shown in A, B, and C.]
Figure 8

Normal Cellular Function

GSH

GSSG

No disulfide bond
Low activity

Disulfide bond formed
High activity

Oxidative stress

Inhibition of mitochondrial respiration
oxygen consumption
and ROS production
Scheme 1
Human neuroglobin functions as a redox regulated nitrite reductase
Mauro Tiso, Jesus Tejero, Swati Basu, Ivan Azarov, Xunde Wang, Virgil Simplaceanu, Sheila Frizzell, Thottala Jayaraman, Lisa Geary, Calli Shapiro, Chien Ho, Sruti Shiva, Daniel B. Kim-Shapiro and Mark T. Gladwin

J. Biol. Chem. published online February 4, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M110.159541

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