A distal ligand mutes the interaction of hydrogen sulfide with human neuroglobin

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Hydrogen sulfide is a critical signaling molecule, but high concentrations cause cellular toxicity. A four-enzyme pathway in the mitochondrion detoxifies H₂S by converting it to thiosulfate and sulfate. Recent studies have shown that globins like hemoglobin and myoglobin can also oxidize H₂S to thiosulfate and hydropolysulfides. Neuroglobin, a globin enriched in the brain, is reported to bind H₂S tightly and is postulated to play a role in modulating neuronal sensitivity to H₂S in conditions such as stroke. However, the H₂S reactivity of the coordinately saturated heme in neuroglobin is expected a priori to be substantially lower than that of the 5-coordinate hemes present in myoglobin and hemoglobin. To resolve this discrepancy, we explored the role of the distal histidine residue in muting the reactivity of human neuroglobin toward H₂S. Ferric neuroglobin is slowly reduced by H₂S and catalyzes its inefficient oxidative conversion to thiosulfate. Mutation of the distal His⁶⁴ residue to alanine promotes rapid binding of H₂S and its efficient conversion to oxidized products. X-ray absorption, EPR, and resonance Raman spectroscopy highlight the chemically different reaction options influenced by the distal histidine ligand. This study provides mechanistic insights into how the distal heme ligand in neuroglobin caps its reactivity toward H₂S and identifies by cryo-mass spectrometry a range of sulfide oxidation products with 2–6 catenated sulfur atoms with or without oxygen insertion, which accumulate in the absence of the His⁶⁴ ligand.

Neuroglobin is a member of the globin family (1) that is expressed primarily in neuronal tissues (2) but also in other metabolically highly active endocrine tissues, such as the adrenal gland and the pancreatic islets of Langerhans (3, 4). Although the physiological role of neuroglobin is still elusive, early studies suggested that it might be involved in oxygen supply (1, 5). However, neuroglobin is generally expressed at low levels and exhibits a high autoxidation rate, producing reactive oxygen species, which makes its role as an O₂ carrier unlikely (6). Neuroglobin can also scavenge reactive oxygen species and reduce nitrite to NO under hypoxic conditions (7–9).

In contrast to hemoglobin and myoglobin, two histidine residues coordinate the heme cofactor in neuroglobin in the ferrous and ferric states (Fig. 1A). The crystal structure of neuroglobin reveals a high structural similarity to myoglobin, despite <25% amino acid sequence similarity between the proteins. The structure reveals a large hydrophobic cavity, which connects the proximal and distal sides of the heme (7). His⁶⁴ and His⁹⁶ coordinate the heme on the distal and proximal sides, respectively (10). Exogenous ligands like O₂, CO, and NO bind to ferrous neuroglobin (Fe²⁺-Ngb)² on the distal side, and binding is limited by dissociation of His⁶⁴, which is slow (6, 11–14). Limited characterization of the binding of sulfide to ferric neuroglobin (Fe³⁺-Ngb) has been reported; the bimolecular rate constant for H₂S binding to neuroglobin was 35- and ~1000-fold slower than for myoglobin and hemoglobin, respectively, at pH 5.5, where the comparisons were made (15). The ability of exogenous ligands to bind to the 6-coordinate heme in neuroglobin has been attributed to the electronic properties of the heme ligand environment and the flexibility of the helix bearing the distal heme ligand (16).

H₂S is a signaling molecule (17, 18), which is synthesized endogenously (19–22) but is toxic at high concentrations by virtue of its inhibition of cytochrome c oxidase in the electron transfer chain (23). H₂S is detoxified via a mitochondrial sulfide oxidation pathway comprising four enzymes that convert it to thiosulfate and sulfate. The electrons released by sulfide quinone oxidoreductase, which catalyzes the first step in the mitochondrial sulfide oxidation pathway (24, 25), are transferred to complex III, thus coupling H₂S oxidation to the mitochondrial electron transfer pathway (26). Recently, we have described a hemeprotein-dependent alternative to the mitochondrial pathway for catalytic H₂S oxidation. Both ferric myoglobin and ferric hemoglobin oxidize sulfide to thiosulfate and to hydropolysulfides (27, 28).

The abbreviations used are: Fe²⁺-Ngb, ferrous neuroglobin; Fe³⁺-Ngb, ferric neuroglobin; Cys-SSH, cysteine persulfide; ESI, electrospray ionization; MWCO, molecular weight cut-off; XAS, X-ray absorption spectroscopy; EXAFS, extended X-ray absorption fine structure; XANES, X-ray absorption near edge structure; CSE, cystathionine γ-lyase.
**Reaction of $H_2S$ with neuroglobin**

$H_2S$ functions as a neuromodulator (29) and also protects neurons from oxidative stress (30). Several differences appear to exist in the enzymes involved in $H_2S$ homeostasis in brain versus liver, where $H_2S$ metabolism has been studied in greater detail. Thus, whereas γ-cystathionase is the major contributor to $H_2S$ generation via the transsulfuration pathway in liver (31), cystathionase β-synthase appears to assume this role in brain (32). The rate of $H_2S$ consumption in brain is ~6-fold slower than in liver and is insensitive to stigmatellin, an inhibitor of complex III (33). Similarly, when $[^{35}S]H_2S$ was used to track sulfide oxidation in brain homogenate, an insensitivity of sulfate and thiosulfate formation to stigmatellin was observed (33).

Very low levels of mRNA (and protein) encoding sulfide quinone oxidoreductase are found in brain (33). These observations raise questions about the mechanism by which $H_2S$ is oxidized in the brain and the possible involvement of hemoproteins such as neuroglobin in catalyzing $H_2S$ oxidation via a non-canonical pathway.

In this study, we have examined the interaction of human neuroglobin with $H_2S$ and find that it can oxidize $H_2S$ to thiosulfate, albeit inefficiently. The reaction of $H_2S$ with Fe$^{II}$-Ngb results in slow reduction of the heme iron to the ferrous state. Replacement of the distal histidine ligand by alanine greatly accelerates sulfide oxidation by neuroglobin and supports its oxidation to thiosulfate and to a wide range of cationated sulfur products.

**Results**

**Purification and reaction of neuroglobin with sulfide**

Wild-type and H64A human neuroglobin were expressed as fusion proteins with an N-terminal glutathione S-transferase (GST) tag that was cleaved by thrombin as described under “Experimental procedures.” The proteins were judged to be >95% pure by SDS-PAGE analysis (Fig. 1B).

Human Fe$^{II}$-Ngb exhibits a UV-visible spectrum typical of a bis-histidine coordinated hemeprotein with a Soret peak at 412 nm and a broad α/β band with a peak at 532 and a shoulder at 560 nm (Fig. 2A). Upon the addition of Na$_2$S, the Soret peak shifts to 415 nm with a concomitant decrease in intensity, whereas the α/β bands remain broad with a peak at 540 nm and a shoulder at 575 nm (Fig. 2A). The spectrum of sulfide-treated neuroglobin under aerobic conditions appears to be a mixture of species and resembles that of O$_2$-bound ferrous neuroglobin, which displays absorbance maxima at 413, 543, and 571 nm (34, 35). However, accumulation of O$_2$-Fe$^{II}$-Ngb is excluded because similar spectral changes were induced by sulfide under anaerobic conditions with the exception that a slight increase in the intensity at 558 nm was observed, indicating the presence of a small amount of Fe$^{II}$-Ngb (not shown).

The H64A mutation resulted in a shift in the Soret peak from 412 to 406 nm (at pH 7.4) compared with wild-type neuroglobin, and the α/β bands were at 565 and 531 nm (Fig. 2B). The addition of sulfide shifted the Soret band to 422 nm and the α/β bands to 570 and 543 nm (red spectrum). Similar absorbance changes were observed under anaerobic conditions (not shown). The maximum at 422 nm slowly decreased in intensity and slightly blue-shifted to 419 nm over the next 30 min (blue spectrum). A slight increase in intensity was observed at ~620 nm, suggesting that a small amount of sulfonuroglobin had formed from the addition of sulfur to the porphyrin ring (Fig. 2B, inset).

A pH dependence for binding of exogenous ligands to neuroglobin has been reported previously and ascribed to protonation of the distal histidine, which would facilitate its dissociation (15, 36). We therefore examined the pH dependence of sulfide binding to wild-type and H64A neuroglobin (Fig. 3). The interaction of Fe$^{II}$-Ngb with sulfide exhibited biphasic kinetics at all pH values and was monitored at 412 or 406 nm for wild-type and H64A neuroglobin, respectively. From the pH dependence of the initial velocity for sulfide binding, $K_{p}$ values of 7.19 ± 0.04 and 6.85 ± 0.07 for wild-type and H64A neuroglobin, respectively, were obtained (Fig. 3, A and B).

Because these values are essentially the same within experimental error, it rules out the contribution of the distal His$^{64}$ ligand to the observed $pK_a$.

Replots of the $k_{obs}$ (for the fast phase) on sulfide concentration yielded the following parameters for wild-type neuroglobin: $k_{on} = 13.8 ± 0.7 \, M^{-1} \, s^{-1}$ and $k_{off} = 5.1 ± 0.3 \times 10^{-3} \, s^{-1}$ at pH 7.4 and 25 °C, yielding a $K_B = 370 \, \mu M$ (Fig. 3C). The $k_{obs}$ for the slow phase ($5.60 \times 10^{-4} \, s^{-1}$) was independent of pH and sulfide concentration. The kinetics of sulfide binding to H64A Fe$^{II}$-Ngb were considerably faster than for the wild-type protein and had to be monitored by stopped-flow spectroscopy. Analysis of the data for H64A neuroglobin yielded the following parameters: $k_{on} = 58.8 ± 3.8 \times 10^{3} \, M^{-1} \, s^{-1}$, $k_{off} = 3.7 ± 0.4 \, s^{-1}$, and $K_B = 63 \, \mu M$ at pH 7.4 and 25 °C (Fig. 3D).

**Sulfide oxidation products of neuroglobin**

$H_2S$ disappeared slowly when mixed with 50 μM Fe$^{II}$-Ngb under aerobic conditions. After 1 h, 328 ± 6 μM sulfide and 73 ± 4 μM O$_2$ were consumed, whereas 18 ± 6 μM thiosulfate (corresponding to 36 μM sulfur) and 72 ± 13 μM hydro polysulfides were formed, accounting for ~33% of the total sulfur (Fig. 4A). The H64A mutant was more active in oxidizing sulfide. After 1 h, 859 ± 28 μM sulfide and 227 ± 4 μM O$_2$ were consumed, whereas 194 ± 8 μM thiosulfate and 152 ± 6 μM protein-bound polysulfides were formed, accounting for ~63% of...
the total sulfur (Fig. 4B). Under anaerobic conditions, the concentration of H₂S lost was equal to the concentration of heme with both wild type and the H64A neuroglobin (Fig. 4, C and D).

**Cryo-mass spectrometric characterization of sulfide oxidation intermediates**

The products of the reaction between neuroglobin and H₂S were detected by low-temperature ESI-TOF-MS, which preserves weak coordination species. Significant differences were observed between the mass spectra of wild-type and H64A neuroglobin following H₂S treatment (Fig. 5, A and B). Analysis of the Δ(m/z) between the control and H₂S-treated samples revealed the actual speciation of the reaction products (Table 1). Whereas a limited number of sulfide oxidation products were observed with wild-type neuroglobin (SO²⁻, SO₄²⁻, and S₂O₅²⁻), a complex array of products was seen with the H64A mutant with 2–6 sulfur atoms (HS⁻, HS₂⁻, HS₃⁻, HS₄⁻, HS₅⁻, HS₆⁻) and variously oxygenated derivatives (Table 1).
To investigate whether the observed species are covalently attached either to the heme or to the protein, samples were mixed with acetonitrile (with 0.5% formic acid) to denature the protein and sprayed into the mass spectrometer. No differences in the heme or protein mass spectra were observed (not shown), demonstrating that the sulfide oxidation products were not covalently attached to the heme or to the protein.

**EPR spectroscopy**

The EPR spectrum of wild-type FeIII-Ngb at 10 K is complex (Fig. 6) and displays a low-spin ferric signal with $g = 3.09, 2.17$, and 1.48, which is in good agreement with published data (37). The presence of a high-spin signal ($g = 6.0$ and 1.99) could be due to the presence of a small population of FeIII-Ngb with a disulfide bridge between Cys$^{46}$ and Cys$^{55}$, which promotes His$^{64}$ dissociation from the iron (38). In the presence of sulfide, a rhombic EPR signal was seen within 5 min with $g = 2.48, 2.21$, and 1.85 (Fig. 6) representing $\sim 28\%$ of the initial spin concentration. The broad positive feature at $g \approx 1.35$ might represent an integer spin (open shell $S = 0$) species or a ferric heme with very small $d_{xy}$ $d_{yz}$ splitting, as seen with the cyanide adduct of myoglobin (39). However, its origin is presently unknown. With longer incubation times, the EPR signal decayed further (supplemental Fig. S1).

The EPR spectrum of the H64A mutant showed a $g = 5.87$ axial high-spin signal (Fig. 7). Treatment of H64A neuroglobin with sulfide for 5 min led to the quantitative conversion of the high-spin to a low-spin species, comprising at least two overlapping EPR signals. Simulations were used to deconvolute the spectra to a major signal ($\sim 80\%$) with $g = 2.54, 2.24$, and 1.87 and a minor signal ($\sim 20\%$) with $g = 2.58, 2.21$, and 1.83 (Fig. 7 and supplemental Figs. S2 and S3). The EPR signal decreased in...
intensity with time, and at 60 min, ~50% of the original spin concentration was observed (supplemental Fig. S1). The third EPR signal with g values of 2.48, 2.29, 1.85 were dominant at this time point.

Resonance Raman analysis of sulfide oxidation by Fe\textsuperscript{III}-Ngb

The high-frequency region of the resonance Raman spectrum comprises in-plane porphyrin vibrational modes that hold information about the oxidation, coordination, and spin states of the heme iron. The resonance Raman spectrum of Fe\textsuperscript{III}-Ngb (0 min) shows bands at 1375 cm\textsuperscript{-1} (ν\textsubscript{1}), characteristic of Fe\textsuperscript{III}, and 1507 cm\textsuperscript{-1} (ν\textsubscript{3}), characteristic of low-spin Fe\textsuperscript{III} (Fig. 8), which are similar to previously reported spectra of human (40) and murine (41) neuroglobin. Following the addition of sulfide, a gradual shift of ν\textsubscript{1} to 1362 cm\textsuperscript{-1} along with the appearance of ν\textsubscript{3} at 1482 cm\textsuperscript{-1} was observed, indicating the presence of ferrous heme, probably in the low-spin state (42). Increased fluorescence associated with the samples as the reaction progressed (especially between 30–60 min) resulted in the loss of spectral quality. The spectra obtained under aerobic and anaerobic conditions were identical (not shown), ruling out the formation of O\textsubscript{2}-Fe\textsuperscript{II}-Ngb, which is also not expected to accumulate due to its susceptibility to autoxidation (6).

The H64A mutant shows the oxidation state marker at 1375 cm\textsuperscript{-1} (ν\textsubscript{1}) and the spin state marker at 1475 cm\textsuperscript{-1} (ν\textsubscript{3}), indicating the presence of high-spin Fe\textsuperscript{III} heme. This is similar to the resonance Raman spectrum of the H64V mutant reported previously at pH 6.4 with bands at 1374 cm\textsuperscript{-1} and 1484 cm\textsuperscript{-1} (40). In contrast to the wild-type protein, sulfide-treated H64A neuroglobin showed persistence of the oxidation state marker at 1375 cm\textsuperscript{-1} over 60 min (Fig. 9). However, the ν\textsubscript{3} marker at 1475 cm\textsuperscript{-1} decreased in intensity, whereas a band at 1498 cm\textsuperscript{-1}...
Reaction of $H_2S$ with neuroglobin

XAS characterization of neuroglobin treated with sulfide

Extended X-ray Absorption Fine Structure (EXAFS) analysis of wild-type Fe$^{III}$-Ngb in the presence and absence of sulfide reveals the presence of 6-coordinate iron (Table 2). Fe$^{III}$-Ngb shows a first inflection point edge energy at 7125.5 ($-H_2S$) and 7124.4 eV ($+H_2S$). In comparison with the edge energies of the standards, ferrous sulfate (7122.9 eV) and ferric sulfate (7126.3 eV), the neuroglobin samples appear to consist of a mixture of ferrous and ferric iron (Fig. 12A). The pre-edge peak area of 9.16 $10^{-2}$ eV (neuroglobin) and 8.16 $10^{-2}$ eV (neuroglobin + sulfide) is consistent with a pseudosymmetric 6-coordinate iron center. The EXAFS data for both samples are best fit with six nitrogen ligands with Fe–N bond lengths of 1.99 Å (Fig. 13A). In addition, there are three Fe-C vectors that are due to single and multiple scattering signals of the porphyrin ring (Table 2). Sulfur coordination to the heme iron was not detected with wild-type neuroglobin.

The XAS spectra of H64A Fe$^{III}$-Ngb with and without sulfide show first inflection point energies at 7124.9 eV ($-H_2S$) and 7124.0 eV ($+H_2S$) (Fig. 12B). The EXAFS spectrum of H64A neuroglobin is best fit with five nitrogen ligands at a distance of 1.98 Å and three Fe-C vectors with values similar to those seen with wild-type neuroglobin (Fig. 13B). In the $H_2S$-treated sample, in addition to Fe–N bonds with a 1.99-Å length, a sulfur ligand at an Fe–S distance of 2.23 Å was detected (Table 2), which is considerably longer than observed in $H_2S$-treated myoglobin (2.16 Å) (28) but similar to the 2.25-Å bond length predicted for an FeN$_5$S$_1$ species from the Cambridge structural database. Only two Fe-C vectors at bond distances of 3.02 and 4.08 Å were observed in H64A neuroglobin treated with sulfide.

Reaction of Fe$^{III}$-Ngb with cysteine persulfide

The transsulfuration pathway enzymes generate cysteine persulfide (Cys-SSH) from cysteine (46), which is more reactive than $H_2S$ and might play a role in sulfide signaling. To test its reactivity toward neuroglobin, Cys-SSH was generated in situ by reaction of γ-cystathionase with cystine (46). Under aerobic conditions, a small decrease in the 412 nm Soret absorption was seen, which was accompanied by small increases at 538 and 575 nm (Fig. 14A). In contrast, marked changes were observed in the spectrum of sulfide treated H64A neuroglobin (Fig. 14B). The Soret peak shifted from 406 to 422 nm with a simultaneous decrease in intensity, and the α/β bands increased in intensity at 543 and 570 nm. Under anaerobic conditions, the Soret peak of the wild-type protein shifted to 424 nm, and the β/α bands sharpened at 530 and 558 nm appear, indicating conversion to Fe$^{II}$-Ngb (Fig. 14C). The spectrum of H64A neuroglobin was similar under aerobic and anaerobic conditions (Fig. 14, B and D).

Discussion

Neuroglobin is a divergent member of the globin superfamily whose cellular role continues to be a matter of debate (47). It is up-regulated in response to hypoxia and plays a role in protect-
ing neurons against hypoxic damage (48). Neuroglobin is unusual in several respects, including the presence of a commodious cavity around its 6-coordinate heme (10) that is not present in hemoglobin or myoglobin (Fig. 1A). Spectroscopic studies have revealed the presence of heme orientational isomers and complex kinetics for binding exogenous ligands, which is slowed by displacement of the endogenous distal heme ligand, His64 (6, 12, 40, 41, 49). The oxidation state of a pair of cysteine residues (Cys46 and Cys55 in the human sequence), modulates the O2 affinity of neuroglobin, decreasing it 10-fold when the cysteines are reduced (38).

The interaction between neuroglobin and CO, reactive oxygen and reactive nitrogen species that accumulate during ischemia reperfusion has been studied (6, 41, 50). In contrast, the interaction between neuroglobin and H2S, which also accumulates during hypoxia (51) due to inhibition of the O2-dependent route for its disposal, has been minimally investigated (15). Treatment of FeIII-Ngb with NaHS under anaerobic conditions reportedly resulted in a shift in the Soret maximum from 412 to 426 nm (changes in the visible region of the spectrum were not reported) (15). This spectral change was unlike what we observed under similar conditions (Fig. 2) but like the changes that we saw when anaerobic FeIII-Ngb was mixed with Cys-SSH (Fig. 1C). The spectrum of FeIII-Ngb exposed to Cys-SSH under anaerobic conditions showed absorption maxima at 424, 530, and 558 nm, consistent with reduction to FeII-Ngb. Hydrodisulfides are better reducing agents than the corresponding thiols and can reduce heme iron (52). Hence, it is likely that Cys-SSH reduced FeIII-Ngb via an outer-sphere process under these conditions. These results lead us to conclude that the interaction of FeIII-Ngb with spurious sulfide oxidation products was characterized previously (15). In contrast to Na2S used

Figure 7. EPR spectroscopic characterization of H64A FeIII-Ngb following the addition of Na2S. Shown is the spectrum of H64A FeIII-Ngb (500 μM) in aerobic 100 mM HEPES, pH 7.4, and 20% glycerol (v/v) after the addition of Na2S (10 mM) at 25 °C at the indicated times. The EPR settings are described under “Experimental procedures.” The signal marked with an asterisk represents adventitious iron. R.U., relative units.
as a sulfide source in our study, NaHS, used in the previous study, is known to be highly contaminated with oxidized sulfide products (53).

When Fe\textsuperscript{III}-Ngb was treated with H\textsubscript{2}S, we observed small changes in the Soret peak shifted from 412 to 415 nm (Fig. 2A). The resonance Raman spectra showed a slow conversion from the Fe\textsuperscript{III} to Fe\textsuperscript{II} species, and a mixture of oxidation states was present at 30 min (Fig. 8). In contrast, the EPR spectrum showed a rapid loss of paramagnetic signal at 5 min, and 

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\frac{1}{10} \text{of the original spin concentration remained at 30 min. Collectively, these spectroscopic data suggest the presence of a mixture of (a) diamagnetic and paramagnetic states and (b) Fe\textsuperscript{III} and Fe\textsuperscript{II} species, albeit their relative proportions as reported by EPR and resonance Raman spectroscopy appear to be different. The X-ray absorption near edge structure (XANES) data are consistent with the presence of a mixture of Fe\textsuperscript{III} and Fe\textsuperscript{II} oxidation states both in the presence and absence of sulfide, which indicates the occurrence of photoreduction (Table 2). The EXAFS data, however, do not support direct sulfur coordination to the iron in wild-type neuroglobin (Fig. 13A). It is important to note that the samples for XAS data collection were prepared under very different conditions in which the concentration of protein was high relative to O\textsubscript{2}, as described under “Experimental procedures.” The low-energy Raman data show little change upon exposure of wild-type neuroglobin to sulfide, providing further evidence that sulfide is not directly coordinated to the heme iron.

The chemistry of heme-dependent sulfide oxidation to a mixture of products is complex and poorly understood. To explain the spectroscopic data, we postulate that sulfide binding to the heme pocket in neuroglobin induces O\textsubscript{2}-dependent formation of reactive sulfur/oxygen radical species that is spin-coupled to Fe\textsuperscript{III}-Ngb, accounting for the initial loss of the EPR signal but retention of the Fe\textsuperscript{III} oxidation state marker as the
major species at 5 and 15 min (Fig. 8). Detection of reactive sulfur/oxygen species by cryo-MS (Table 1) and limited O₂ consumption during the reaction (Fig. 4A) are consistent with this model. Spectroscopic studies provide evidence for the electrostatic stabilization of CO bound to neuroglobin as an Fe=C=O–X⁻ species in which X⁻ could be His⁶⁴ and/or Lys⁶⁷ (41). These residues could potentially also stabilize sulfide and reactive sulfur species intermediates that are formed in the heme pocket. An alternative explanation to account for the apparent discrepancy at the early time points between the EPR (showing predominance of diamagnetic heme) and resonance Raman (showing predominance of ferric heme) data is formation of FeII-O₂-Ngb. In analogy to FeII-O₂-myoglobin (54), FeII-O₂-Ngb should exhibit a ferric ν₂ signature while being EPR-silent. However, a significant accumulation of an FeII-O₂ species seems unlikely because similar absorbance changes were seen under aerobic and anaerobic (not shown) conditions and because FeII-O₂-Ngb undergoes fairly rapid autoxidation (6).

Prolonged incubation (>60 min) of FeIII-Ngb with sulfide resulted in the full conversion of ferric to ferrous heme, as indicated by the 1362 cm⁻¹ oxidation state marker in the resonance Raman spectrum (Fig. 8). However, the UV-visible spectra at 30 and 60 min (not shown) were the same and do not correspond to the spectrum described for deoxy-ferrous neuroglobin (i.e. FeII-Ngb), which exhibits a Soret peak at 426 nm and sharp 6 and 5 bands at 528 and 558 nm (6). These results indicate that the 415 nm spectrum obtained in the presence of sulfide (Fig. 2A) represents a mixture of species, which cannot be assigned at present. We can, however, rule out the presence of a dominant iron-coordinated sulfur species, which is associated with a significant red shift in the Soret peak. Such peak shifts were seen with myoglobin (409 nm) and hemoglobin (405 nm) together with sharpening of the 6/6 bands (27, 28). As described below, spectral changes similar to those of myoglobin and hemoglobin are observed with the H64A mutant of neuroglobin.

The bimolecular rate constant for the interaction of sulfide with FeIII-Ngb (13.8 M⁻¹ s⁻¹ at pH 7.4 and 25 °C) is considerably smaller than for sulfide binding to hemoglobin (3.2 × 10³ M⁻¹ s⁻¹) or myoglobin (1.6 × 10⁶ M⁻¹ s⁻¹). The difference in the k₉₀ values for sulfide binding to the three globins is probably...
due to the difference in their heme iron coordination states. We note that reduction of the Cys46–Cys55 disulfide bond in neuroglobin stabilizes the 6-coordinate state of the heme iron and decreases its affinity for exogenous ligands (38). Under the conditions of our experiment, where the sulfide concentration was high, the Cys46–Cys55 pair should have been in the reduced dithiol state.

Sulfide oxidation products (i.e. thiosulfate and polysulfides) were detected, albeit in lower amounts with neuroglobin (Fig. 4A) than seen with myoglobin and hemoglobin (27, 28). These results suggest that sulfide oxidation and oxygenation products can be formed even in the absence of direct coordination of the sulfur to the heme. However, we cannot rule out that a small proportion of FeIII-Ngb exists with direct sulfide coordination and is responsible for the observed oxidation products, which do not accumulate above background levels in the absence of O2 (Fig. 4C).

Overall, the reaction and reactivity of sulfide with neuroglobin are substantially different from those of hemoglobin and myoglobin (27, 28), which we attribute to the bis-histidine coordination of the heme iron in neuroglobin. To investigate the influence of the distal histidine on sulfide interaction with neuroglobin, His64 was replaced by alanine. The latter substitution resulted in a Soret peak shift from 412 nm in wild-type FeIII-Ngb to 406 nm. In the presence of sulfide, the Soret peak of the mutant shifted from 406 to 422 nm, and sharpening of the bands at 541 and 572 nm was seen (Fig. 2B).

Coordination of thiols to ferric hemes generally lowers the thiol pKa by 4 units (55), predicting a pKa of ~3 for sulfide bound to heme. Hence, HS~–FeIII-H64A Ngb is expected to be the initial complex that is formed with a Soret peak at 422 nm.

The EXAFS data provide evidence for sulfur coordination to iron with an Fe–S bond distance of 2.23 Å (Table 2). As with the wild-type protein, a mixture of iron oxidation states was observed by XANES spectroscopy for the mutant with and without H2S treatment (Fig. 12B), indicating photoreduction. The H64A mutant showed evidence for sulfur coordination to ferric iron in the presence of Cys-SSH (Fig. 14, B and D). The Soret peak shifted from 406 to 422 nm, and the a/β bands were observed at 570 and 543 nm. It is unclear whether Cys-SSH was directly coordinated to

| Sample                | Nearest-neighbor ligand environment | Long-range ligand environment |
|-----------------------|-------------------------------------|------------------------------|
|                       | Atom a  | R c | C.N. d  | σ² e | F f  |
| WT Ngb                | N 1.99 | 6   | 2.73    |      |      |
| Wt Ngb + H₂S         | N 1.99 | 6   | 3.61    |      |      |
| H64A Ngb             | N 1.98 | 5   | 2.95    |      |      |
| H64A Ngb + H₂S       | N 1.99 | 4   | 2.58    |      |      |

| Sample                | Atom a  | R c | C.N. d  | σ² e | F f  |
|-----------------------|---------|-----|---------|------|------|
| WT Ngb                | C 3.03  | 6   | 0.93    | 0.27 |
| Wt Ngb + H₂S         | C 3.41  | 3   | 2.23    |      |      |
| H64A Ngb             | C 4.05  | 6   | 4.05    |      |      |
| H64A Ngb + H₂S       | C 3.04  | 6   | 2.18    | 0.36 |

Table 2
Summary of the iron EXAFS simulations for human neuroglobin samples

| Atom b  | R c | C.N. d  | σ² e | F f  |
|---------|-----|---------|------|------|
| N       | 2.23 | 1       | 3.96 |
| S       | 2.23 | 3.96    |      |      |

| Atom b  | R c | C.N. d  | σ² e | F f  |
|---------|-----|---------|------|------|
| N       | 3.04 | 6       | 1.42 | 0.47 |
| C       | 4.08 | 6       | 0.42 |

Figure 12. Normalized iron XANES spectra of FeIII-Ngb. A, normalized iron XANES of wild-type neuroglobin with (blue) and without (red) sulfide treatment. B, normalized iron XANES of H64A neuroglobin with (blue) and without (red) sulfide treatment. In both panels, the XANES spectra of neuroglobin are compared with the FeSO4 (green) and FeSO4 (black) standards.
the iron in this sample or whether \( \text{H}_2\text{S} \) produced by spontaneous decomposition of Cys-SSH (Reaction 1) as reported previously (46), was coordinated to the ferric iron.

Interestingly, the spectrum of H64A Fe\(^{III}\)-Ngb in the presence of cysteine was similar to that observed in the presence of Cys-SSH (not shown), indicating that the distal heme pocket of neuroglobin can accommodate cysteine and, presumably, Cys-SSH, supporting direct coordination of the persulfide to the ferric iron.

In contrast to myoglobin and hemoglobin, the 422 nm Soret band observed with H64A neuroglobin was not stable and, after \( \sim 5 \) min, started to shift to 419 nm with a slight increase in intensity (Fig. 2B). It is unclear what the 419 nm band represents, and it is likely to be a mixture of species. We speculate that the accumulation of catenated sulfur oxidation products coordinated to the heme iron might induce the slight blue shift. The H64A mutant exhibited a 4000-fold greater reactivity toward \( \text{H}_2\text{S} \) (\( k_{\text{on}} = 58.8 \pm 3.8 \times 10^3 \text{M}^{-1} \text{s}^{-1} \)) than wild-type neuroglobin. A similar enhancement of nitrite reduction was reported by mutation of the distal histidine residue to leucine or glutamine (7). The 700-fold higher \( k_{\text{off}} \) for sulfide for H64A versus wild-type neuroglobin is consistent with the role of the distal histidine residue in stabilizing sulfide, as was previously noted with CO in the closed conformation of neuroglobin (41).

The EPR spectrum of the H64A mutant shows high-spin iron, consistent with the presence of 5-coordinate ferric heme (Fig. 7). The addition of sulfide resulted in the appearance of a complex mixture of low-spin ferric species within 5 min and a progressive loss of spin intensity, indicating conversion to an EPR-silent species. Multiple species are present in the EPR spectrum of sulfide-treated H64A neuroglobin (also seen in the 30- and 60-min spectra of the wild-type protein (Fig. 6)). This heterogeneity probably reflects distinct stable conformational substates of the distal pocket that have been spectroscopically characterized previously (12). Additionally, with the H64A mutant, the heterogeneity could reflect the presence of a mixture of oxidized sulfur species coordinated to the ferric iron. Similar EPR spectra have been reported for the multiheme protein SoxXA involved in bacterial sulfur oxidation (55). The complexity in SoxXA spectrum arises from the simultaneous presence of three hemes that are coordinated by histidine on one side and to a thiol, persulfide, or methionine on the other (56).

Interestingly, the resonance Raman spectra of the H64A mutant indicates that the iron remains in the ferric state upon sulfide addition for the duration of the experiment (Fig. 9), whereas the EPR spectrum shows a loss of \( \sim 50\% \) of the spin concentration at 60 min (Fig. 7). However, the low quality of the resonance Raman spectra due to the high background fluorescence does not allow us to unequivocally exclude the presence of a small amount of ferrous heme between 15 and 60 min. In the low-energy region of the resonance Raman spectrum, a prominent 495 cm\(^{-1}\) band was observed upon the addition of sulfide (Fig. 11). We assign this band to the S–S vibration of an iron-bound hydropolysulfide product. This assignment is based on its similarity to the 498 cm\(^{-1}\) band in a Cys-SS-ligated [2Fe-2S]\(^{2+}\) cluster in the FNR transcriptional factor, which was assigned using \(^{34}\)S isotope labeling to the S-S stretching mode of the persulfide (57). In the resonance Raman spectrum of Na\(_2\)S\(_3\), a 482 cm\(^{-1}\) band was assigned to the S-S vibration (58).

The H64A mutant catalyzed production of higher concentrations of sulfide oxidation products paralleled by higher \( \text{O}_2 \) consumption compared with wild-type neuroglobin (Fig. 4B).

2Cys-S-SH→Cys-S-S-S-Cys+H\(_2\)S

\[ \text{REACTION 1} \]

Figure 13. Comparison of iron EXAFS and Fourier transforms of the EXAFS data for wild-type and H64A neuroglobin. A, raw \( k^3 \)-weighted EXAFS data (a) and phase-shifted Fourier transforms of the EXAFS data (b) for wild-type Fe\(^{III}\)-Ngb are shown. EXAFS and Fourier transforms for Fe\(^{III}\)-Ngb + sulfide are shown in c and d, respectively. B, comparison of iron EXAFS and Fourier transforms of the EXAFS data for H64A neuroglobin. Raw \( k^3 \)-weighted EXAFS data (a) and phase-shifted Fourier transforms of the EXAFS data (b) for H64A Fe\(^{III}\)-Ngb are shown. EXAFS and Fourier transforms for H64A Fe\(^{III}\)-Ngb + H\(_2\)S are shown in c and d, respectively.
On the other hand, only stoichiometric loss of sulfide was observed under anaerobic conditions and corresponded to the heme iron concentration (Fig. 4D). MS analysis revealed a rich mixture of sulfide oxidation chemotypes with 1–4 oxygens and hydropolysulfides with 2–6 sulfur atoms (Table 1). Whereas the reaction of wild-type neuroglobin was associated with oxygenation at a single sulfur (HSO\(^{-}\), HSO\(_4^{-}\), H\(_2\)S\(_2\)O\(_3\)\(^{-}\)), the H64A mutant was associated with hydropolysulfides (H\(_2\)S, H\(_2\)S\(_2\), H\(_2\)S\(_3\), H\(_2\)S\(_4\), H\(_2\)S\(_5\), and H\(_2\)S\(_6\)) with a subset containing oxygenated sulfur (HSO\(_3\), H\(_2\)S\(_2\)O\(_3\), and H\(_2\)S\(_4\)O\(_3\)). In fact, the only product observed in common between wild-type and H64A neuroglobin was thiosulfate (HS\(_2\)O\(_3\)).

The combination of spectroscopic approaches used in this study allows us to conclude that the reaction of Fe\(^{III}\)-Ngb with sulfide is very different from that of the ferric forms of myoglobin, hemoglobin, and H64A neuroglobin and also different from that previously reported for neuroglobin (15). We speculate that with wild-type neuroglobin, outer-sphere electron transfer reactions involving sulfide, oxygen, and ferric iron lead to the limited formation of sulfide oxidation products. The redox potentials for the S\(^{-}\)/H\(^{+}\)/HS\(^{-}\) couple and the \(\frac{1}{2}\)O\(_2\)/2H\(^{+}\)/H\(_2\)O couple are +920 mV (59) and +815 mV versus the standard hydrogen electrode at pH 7. The redox potential for the Fe\(^{3+}\)/Fe\(^{2+}\) couple in neuroglobin is −129 mV (6). The large excess of sulfide over Fe\(^{III}\)-Ngb probably allows the mismatch in the redox potentials to be overcome in the in vitro experiments but suggests that this reaction is unlikely to occur in vivo.

In contrast, persulfides are more reactive toward ferric neuroglobin, and under anaerobic conditions, accumulation of Fe\(^{II}\)-Ngb is observed (Fig. 14).

In summary, our results indicate that Fe\(^{III}\)-Ngb is relatively inert toward oxidizing sulfide, limiting the potential biological relevance of this interaction, especially when the cysteines are in the reduced dithiol state. The reactivity of wild-type neuroglobin toward H\(_2\)S could, however, increase under cellular redox conditions that result in oxidation of both the cysteine pair and the heme iron. In the dithiol state, the O\(_2\) affinity of human neuroglobin is reduced 10-fold primarily due to a decrease in the dissociation rate of the distal histidine ligand (38). By analogy, the presence of the disulfide bond should increase the affinity of Fe\(^{III}\)-Ngb for H\(_2\)S. Preservation of the disulfide bond in the presence of H\(_2\)S is experimentally difficult to achieve in vitro, although it might be possible in vivo. Under these conditions, the interaction of H\(_2\)S with neuroglobin is expected to be enhanced and could lead to sulfide oxidation. Unlike the globins with 5-coordinate hemes, including the H64A mutant of neuroglobin, accumulation of sulfide-coordinated heme is not observed with wild-type neuroglobin. The array of catenated sulfide oxidation products generated that were trapped by cryo-MS provides evidence for the rich
Reaction of H₂S with neuroglobin

ferric iron-dependent sulfide oxidation chemistry that can be catalyzed by iron-containing proteins. In contrast to H₂S, Cys-SSH results in reduction Fe⁺⁻⁻Ngb and accumulation of the bis-His coordinated ferrous species under anaerobic conditions.

Experimental procedures

Materials

Na₂S nonahydrate, l-cystine, glutathione, H₂S solution (0.8 m) in tetrahydrofuran, meta-phosphoric acid, and potassium ferricyanide were from Sigma. Monobromobimane was from Molecular Probes (Grand Island, NY). Dithiothreitol, isopropyl β-d-thiogalactopyranoside was from Gold Biotechnology (St. Louis, MO), and δ-aminolevulinic acid was from Frontier Scientific (Logan, UT). Glutathione-agarose was from Thermo Scientific (Waltham, MA). All other chemicals were reagent grade and were purchased from Fisher. Recombinant human cystathionine γ-lyase (CSE) was prepared as described previously (60). Protein molecular weight standards were from Bio-Rad.

Purification of human neuroglobin

A thrombin-cleavable GST tag was introduced at the N terminus of neuroglobin. For this, the neuroglobin cDNA was amplified by PCR using a pET28a plasmid containing the human neuroglobin cDNA as template (generously provided by Dr. Gladwin, University of Pittsburgh), using the following primers: 5′-TTATAAGGATCCCAGGAAGGTTTGGGGG-3′ containing the BamHI restriction site and 5′-TTATCTCTGAGTTACTGCGC-3′ with the XhoI restriction site. The PCR product was cut with BamHI and XhoI and ligated into the pEGX19 plasmid. The H64A mutation was introduced by site directed mutagenesis using the QuikChange kit with the following primers: 5′-TCGCTGATGTTTCTGGACGCAATCAGGAAGGTTGATGCT-3′ and 5′-AGCATC-ACCTCTCTGATGTTTGGGGTGTTACTGCGC-3′. The presence of the mutation was confirmed by nucleotide sequencing (University of Michigan DNA Sequencing Core).

GST-tagged neuroglobin was expressed in Escherichia coli BL21(DE3) as described previously with some modifications (8). Briefly, starting cultures were grown overnight at 37 °C in Luria-Bertani medium containing 100 μg/ml ampicillin. The initial culture (1 ml) was transferred to 1 liter of Terrific Broth medium (with 100 μg/ml ampicillin) and grown at 37 °C until the A₆₀₀ reached 0.6 – 0.8. Then 0.4 mM δ-aminolevulinic acid was added, protein expression was induced by Dr. Gladwin, University of Pittsburgh), using the following conditions.

Dependence of sulfide-binding kinetics on pH

The interaction between wild-type neuroglobin (5 μM) in 100 mM HEPES, pH 7.4, and Na₂S (0.05–1.5 mM) was monitored at 25 °C under aerobic conditions. Binding of sulfide to H64A neuroglobin was carried out under anaerobic conditions using an Applied Photophysics stopped-flow spectrophotometer (SX.MV18) placed inside a glove box (Vacuum Atmospheres Co., Hawthorne, CA) with an O₂ concentration below 0.7 ppm. A solution of H64A neuroglobin (5 μM) in 100 mM HEPES, pH 7.4, was mixed with Na₂S solution (0.025–0.5 mM) to final concentrations ranging from 25 to 500 μM at 25 °C. Biphasic time-dependent changes in absorbance at 412 nm (wild-type neuroglobin) and 406 nm (H64A neuroglobin) were observed and fitted using Equation 1 to obtain the rate constants associated with each reaction phase. In Equation 1, A₁ and A₂ represent the amplitude changes associated with the two phases, k₁ and k₂ represent the corresponding rate constants, and y₀ is the final absorbance.

$$y = y_0 + A_1 e^{-k_1 x} + A_2 e^{-k_2 x} \quad (Eq. 1)$$

The values for k₁, k₂, and Kᵣ were obtained by replotting the dependence of k₉ on the concentration of sulfide.

Dependence of sulfide-binding kinetics on pH

Neuroglobin (5 μM) in 100 mM aerobic buffer, pH ranging from 5.5 to 9.0 was mixed with 1 mM Na₂S solution in the same buffer. Tris was used for pH 9.0, 8.5, 8.0, and 7.8; HEPES for pH 7.75, 7.5, and 7.0; and MES for 6.5, 6.0, and 5.5. The change in absorbance was monitored at 412 nm following the addition of sulfide, and the kinetic data were fit to Equation 1. Due to the faster kinetics of sulfide binding at lower pH, the experiments between pH 5.5 and 6.5 were performed on a stopped-flow spectrophotometer. The pH dependence of sulfide binding to H64A neuroglobin was monitored in a stopped-flow spectrophotometer at 406 nm. The data were fitted to Equation 1 to obtain an estimate of kᵣ, the rate constant for the fast phase. The
Reaction of neuroglobin with cysteine persulfide

Neuroglobin (10 μM wild type or H64A mutant) was mixed with CSE (2 μM) in aerobic 100 mM HEPES buffer, pH 7.4, at 37 °C. Persulfide formation was initiated by 1 mM cysteine, and the absorbance was monitored from 300 to 750 nm. For monitoring the reaction under anaerobic conditions, it was set up similarly but in an anaerobic chamber, and anaerobic reagents were used.

Analysis of neuroglobin-catalyzed sulfide oxidation

Oxygen consumption was monitored using a Clark O2 electrode. For this, neuroglobin (25 μM) in 100 mM HEPES buffer, pH 7.4, was placed in a 1.5-ml Gilson type chamber, and Na2S (1 mM in the same buffer) was injected into the chamber. O2 consumption was recorded using a chart recorder.

The sulfide oxidation products, sulfite and thiosulfate, were detected using an HPLC method, and polysulfides were measured using cold cyanalysis as described previously (27). For this, neuroglobin (50 μM, 1 ml) was incubated with or without Na2S (1 mM) in aerobic 100 mM HEPES, pH 7.4, at 25 °C in a closed 1.5-ml sample tube. Aliquots were removed at the desired time points. For HPLC analysis, aliquots (45 μl) were mixed with 2.5 μl of 1 M Tris, pH 9.0, and 2.5 μl of 60 mM monobromobimane in DMSO in black 0.5-ml sample tubes and incubated at room temperature for 10 min. Then 100 μl of meta-phosphoric acid (16.8 mg/ml in H2O) was added, the mixture was centrifuged for 3 min at 15,900 × g, and the supernatants were stored at −80 °C until analysis.

HPLC analysis was performed using a Zorbax Eclipse XDB C-18 column (4.6 × 150 mm) using the following solvent system: Buffer A, 100 mM ammonium acetate, pH 4.75, 10% MeOH (v/v); Buffer B, MeOH + 10% 100 mM ammonium acetate, pH 4.75 (v/v). The following gradient was used for sample elution: 0–10 min, linear gradient of 0–20% B; 10–15 min, linear gradient of 20–50% B; 15–20 min, isocratic 50% B; 20–22 min, 50–100% B; 22–27 min, isocratic 100%; 27–29 min, linear 100 to 0% B; 29–35 min, isocratic 100% A. The bimane adducts of sulfite and thiosulfate were detected by fluorescence emission at 490 nm following excitation at 390 nm. The products were quantified using calibration curves generated with the respective standards.

For determining polysulfide concentrations, 100-μl aliquots were incubated with 50 μl of 62.5 mM potassium cyanide in 125 mM ammonium hydroxide for 45 min at 25 °C. Then 150 μl of Goldstein solution (1.25 g of Fe(NO3)3·9H2O in 50 ml of 17% HNO3) was added, mixed, and centrifuged for 5 min at 15,900 × g. The absorbance at 460 nm was recorded, and the concentration of polysulfides was determined using a calibration curve generated with potassium thioctonate.

EPR spectroscopy

EPR spectra were recorded on a Bruker EMX 300 equipped with a Bruker 4102-ST cavity and an Oxford liquid helium cryostat. Spectra were measured at 10 K with the following parameters: 9.34-GHz microwave frequency; power, 10 milliwatts; modulation amplitude, 7.5 G; modulation frequency, 100 kHz; 5000-G sweep width centered at 3000 G; conversion time, 164 ms; time constant, 82 ms. FeII-Ngb (400 μM) and H64A FeII-Ngb (500 μM) samples were prepared aerobically in 100 mM HEPES, pH 7.4, containing 20% glycerol. Na2S prepared in the same buffer was added to a final concentration of 10 mM. At the desired time points, 300 μl of the solution was transferred to an EPR tube, and the tube was sealed with a silicon septum and frozen in liquid nitrogen. Low spin signals were quantified using a 1 mM copper EDTA solution. Low spin (S = ½) signals were fitted with the Easyspin toolbox (61) using the parameters described in supplemental Tables S1 and S2.

Resonance Raman spectroscopy

The resonance Raman measurements were performed using the 413.13 nm excitation line from a Kr+ gas ion laser (Spectra Physics Beam Lok 2060-RS). The frozen samples were prepared in EPR tubes, which were placed in a liquid N2-filled Dewar for all measurements or stored in a Dewar filled with liquid N2 in the dark until further use. Raman spectra were recorded at 77 K using an Acton two-stage TriVista 555 monochromator connected to a liquid nitrogen-cooled CCD camera (Princeton instruments Spec-10:400B/LN). The experiments were performed at different exposure times and accumulations due to the background fluorescence associated with neuroglobin, which increased with the reaction time. Typical laser powers were in the 10–30-milliwatt range. Relative wavenumbers (Raman shift) were calibrated using sodium sulfate (Na2SO4).

Mass spectrometry

MS measurements were performed on a UHR-TOF Bruker Daltonik maXis 4G (Bremen, Germany), coupled to a Bruker cryospray unit, an ESI-TOF-MS with a resolution of at least 40,000 full width at half-maximum. Detection was in the positive-ion mode. The flow rates were 300 μl/h. The drying gas (N2) was held at 10 °C, and the spray gas was held at 10 °C. The machine was calibrated before every experiment by direct infusion of the Agilent ESI-TOF low concentration tuning mixture, which provided an m/z range of singly charged peaks up to 2,700 Da in both ion modes. Wild-type or H64A neuroglobin (50 μl of 400 μM) in 200 mM ammonium carbonate buffer, pH 7.4, was mixed with 4 mM H2S (from 0.8 M H2S in tetrahydrofuran), diluted 10-fold with nano-pure water, and sprayed continuously into the MS. The spectra were recorded over 45 min. As a control, covalent modification of the protein or the heme cofactor was investigated by mixing 50 μM neuroglobin in 20 mM ammonium carbonate buffer, pH 7.4, with 500 μM H2S for 45 min. Samples were then mixed with acetonitrile (50/50, v/v) and formic acid (0.5%) and sprayed at 200 °C with a flow rate of 180 μl/h.
**Reaction of H$_2$S with neuroglobin**

**XAS analysis**

Samples were prepared by mixing wild-type or H64A neuroglobin (1 mM in iron concentration) in 100 mM HEPES, pH 7.4, with H$_2$S (20 mM final concentration) in tetrahydrofuran and incubated for 30 min at 25 °C. An equivalent volume of tetrahydrofuran only was added to the control sample. Glycerol (30% (v/v) final concentration) was added to the samples, which were then loaded into Kapton-wrapped lucite XAS sample cells and frozen quickly in liquid N$_2$ until data collection at the beamline.

XAS data were collected at the Stanford Synchrotron Radiation Laboratory on beamlines 7-3 and 9-3. Beamline 7-3 is equipped with a single rhodium-coated silicon mirror and an Si[220] double crystal monochromator, and harmonic rejection was achieved by detuning the monochromator 50%. The 9-3 beamline is equipped with an Si[220] double crystal monochromator and a harmonic rejection mirror, so spectra were collected under fully tuned conditions. Samples were maintained at 10 K using Oxford Instrument continuous-flow liquid helium cryostats at both beamline locations. Protein fluorescence excitation spectra were collected using a 30-element germanium solid-state array detector at 7-3 and a 100-element germanium solid-state detector at 9-3. XAS spectra were measured using 5-eV steps in the pre-edge regions (6900–7094 eV), 0.25 eV steps in the edge regions (7095–7135 eV), and 0.05 Å$^{-1}$ increments in the EXAFS region (to $k = 13.5$ Å$^{-1}$), integrating from 1 to 20 s in a $k^2$-weighted manner for a total scan length of ~40 min. X-ray energies were calibrated by collecting an iron foil absorption spectrum simultaneously with collection of protein data. The first inflection point for the iron foil edge was assigned at 7111.3 eV. Each fluorescence channel of each scan was examined for spectral anomalies before averaging, and spectra were closely monitored for photoreduction. Spectra collected at 7-3 represent the average of 7–8 scans, whereas spectra collected at 9-3 represent the average of 4–5 scans.

XAS data processing and analysis were performed following protocols outlined previously (62). EXAFS spectra were simulated using both filtered and unfiltered data; however, simulation results are presented only for fits to raw (unfiltered) data. Simulation protocols and criteria for determining the best fit have been described previously (63).

**Author contributions**—M. R., J. K., B. E. L., and M. R. F. performed the experiments. N. L. helped analyze the resonance Raman data and co-wrote the manuscript. T. S. helped analyze the XAS data and co-wrote the manuscript. All authors analyzed the experiments performed in their laboratories. M. R. and R. B. were primarily responsible for writing the manuscript. All authors edited and approved the final version of the manuscript.

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**References**

1. Burmester, T., Weich, B., Reinhardt, S., and Hankeln, T. (2000) A vertebrate globin expressed in the brain. *Nature* **407**, 520–523
2. Fabrizius, A., Andre, D., Laufs, T., Bicker, A., Reuss, S., Porto, E., Burmester, T., and Hankeln, T. (2016) Critical re-evaluation of neuroglobin expression reveals conserved patterns among mammals. *Neuroscience* **337**, 339–354
3. Reuss, S., Saaler-Reinhardt, S., Weich, B., Wystub, S., Reuss, M. H., Burmester, T., and Hankeln, T. (2002) Expression analysis of neuroglobin mRNA in rodent tissues. *Neuroscience* **115**, 645–656
4. Geuens, E., Brouns, L., Flamez, D., Dewilde, S., Timmermans, J. P., and Moens, L. (2003) A globin in the nucleus! *J. Biol. Chem.* **278**, 30417–30420
5. Burmester, T., and Hankeln, T. (2009) What is the function of neuroglobin? *J. Exp. Biol.* **212**, 1423–1428
6. Dewilde, S., Kiger, L., Burmester, T., Hankeln, T., Baudin-Creuzva, V., Aerts, T., Marden, M. C., Caubergs, R., and Moens, L. (2001) Biochemical characterization and ligand binding properties of neuroglobin, a novel member of the globin family. *J. Biol. Chem.* **276**, 38949–38955
7. Tiso, M., Tejeiro, J., Basu, S., Azarlov, I., Wang, X., Simpleanu, V., Frizzell, S., Jayaraman, T., Geary, L., Shapiro, C., Co, H., Shiva, S., Kim-Shapiro, D. B., and Gladwin, M. T. (2011) Human neuroglobin functions as a redox-regulated nitric oxide reductase. *J. Biol. Chem.* **286**, 18277–18289
8. Tejeiro, J., Sparacino-Watkins, C. E., Ragireddy, V., Frizzell, S., and Gladwin, M. T. (2015) Exploring the mechanisms of the reductase activity of neuroglobin by site-directed mutagenesis of the heme distal pocket. *Biochemistry* **54**, 723–733
9. Fordel, E., Thijs, L., Martinet, W., Lenjou, M., Laufs, T., Van Bockstaele, D., Moens, L., and Dewilde, S. (2006) Neuroglobin and cytochrome overexpression protects human SH-SY5Y neuroblastoma cells against oxidative stress-induced cell death. *Neurosci. Lett.* **410**, 146–151
10. Pesce, A., Dewilde, S., Nardini, M., Moens, L., Ascenzi, P., Hankeln, T., Burmester, T., and Bolognesi, M. (2003) Human brain neuroglobin structure reveals a distinct mode of controlling oxygen affinity. *Structure* **11**, 1087–1095
11. Trent, J. T., 3rd, Watts, R. A., and Hargrove, M. S. (2001) Human neuroglobin, a hexacoordinate hemoglobin that reversibly binds oxygen. *J. Biol. Chem.* **276**, 30106–30110
12. Kriegel, J. M., Bhattacharyya, A. J., Nienhaus, K., Deng, P., Minkow, O., and Nienhaus, G. U. (2002) Ligand binding and protein dynamics in neuroglobin. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 7992–7997
13. Van Doorslaer, S., Dewilde, S., Kiger, L., Nistor, S. V., Goovaerts, E., Marden, M. C., and Moens, L. (2003) Nitric oxide binding properties of neuroglobin. A characterization by EPR and flash photolysis. *J. Biol. Chem.* **278**, 4919–4925
14. Nienhaus, K., Kriegel, J. M., and Nienhaus, G. U. (2004) Structural dynamics in the active site of murine neuroglobin and its effects on ligand binding. *J. Biol. Chem.* **279**, 22944–22952
15. Brittain, T., Yosaatmadja, Y., and Henty, K. (2008) The interaction of human neuroglobin with hydrogen sulfide. *IUBMB Life* **60**, 135–138
16. Pesce, A., De Sanctis, D., Nardini, M., Dewilde, S., Moens, L., and Hankeln, T., Burmester, T., Ascenzi, P., and Bolognesi, M. (2004) Reversible hexa- to penta-coordination of the heme Fe atom modulates ligand binding properties of neuroglobin and cytochrome. A characterization by EPR and flash photolysis. *J. Biol. Chem.* **279**, 4919–4925
17. Kimura, H. (2002) Hydrogen sulfide as a neuromodulator. *Mol. Neurobiol.* **27**, 13–19
18. Kabil, O., and Banerjee, R. (2010) The redox biochemistry of hydrogen sulfide. *J. Biol. Chem.* **285**, 21903–21907
19. Chiku, T., Padovani, D., Zhu, W., Singh, S., Vitvitsky, V., and Banerjee, R. (2009) H$_2$S biogenesis by cystathionine-γ-lyase leads to the novel sulfur metabolites, lanthionine and homolanthionine, and is responsive to the grade of hyperhomocysteinemia. *J. Biol. Chem.* **284**, 11601–11612
20. Singh, S., Padovani, D., Leslie, R. A., Chiku, T., and Banerjee, R. (2009) Relative contributions of cystathionine-β-synthase and γ-cystathionase to H$_2$S biogenesis via alternative trans-sulfuration reactions. *J. Biol. Chem.* **284**, 22457–22466
21. Shibuya, N., Tanaka, M., Yoshida, M., Ogasawara, Y., Togawa, T., Ishii, K., and Kimura, H. (2009) 3-Mercaptopruvate sulfurtransferase produces...
hydrogen sulfide and bound sulfane sulfur in the brain. Antioxid. Redox Signal. **11**, 703–714

22. Yadav, P. K., Yamada, K., Chiku, T., Koutmos, M., and Banerjee, R. (2013) Structure and kinetic analysis of H2S production by human mercaptopyruvate sulfurtransf erase. J. Biol. Chem. **288**, 20002–20013

23. Petersen, L. C. (1977) The effect of inhibitors on the oxygen kinetics of cytochrome c oxidase. Biochim. Biophys. Acta **460**, 299–307

24. Hildebrandt, T. M., and Grieshaber, M. K. (2008) Three enzymatic activities catalyze the oxidation of sulfide to thiosulfate in mammalian and invertebrate mitochondria. FEBS J. **275**, 3352–3361

25. Libiad, M., Yadav, P. K., Vitvitsky, V., Martinov, M., and Banerjee, R. (2014) Organization of the human mitochondrial H2S oxidation pathway. J. Biol. Chem. **289**, 30901–30910

26. Goubern, M., Andriamihaja, M., Nübel, T., Blachier, F., and Bouillaud, F. (2007) Sulfide, the first inorganic substrate for human cells. FASEB J. **21**, 1699–1706

27. Vitvitsky, V., Yadav, P. K., Kurthen, A., and Banerjee, R. (2015) Sulfide oxidation by a noncanonical pathway in red blood cells generates thiosulfate and polysulfides. J. Biol. Chem. **290**, 8310–8320

28. Bostelaar, T., Vitvitsky, V., Kumutima, J., Lewis, B. E., Yadav, P. K., Brunold, T. C., Filippovic, M., Lehnhert, N., Stemmler, T. L., and Banerjee, R. (2016) Hydrogen sulfide oxidation by myoglobin. J. Am. Chem. Soc. **138**, 8476–8488

29. Abe, K., and Kimura, H. (1996) The possible role of hydrogen sulfide as an endogenous neuromodulator. J. Neurosci. **16**, 1066–1071

30. Kimura, Y., Dargusch, R., Schubert, D., and Kimura, H. (2006) Hydrogen sulfide protects HT22 neuronal cells from oxidative stress. Antioxid. Redox Signal. **8**, 661–670

31. Kabil, O., Vitvitsky, V., Xie, P., and Banerjee, R. (2011) The quantitative significance of the transsulfurization enzymes for H2S production in murine tissues. Antioxid. Redox Signal. **15**, 363–372

32. Enokido, Y., Suzuki, E., Iwasawa, K., Namekata, K., Okazawa, H., and Fago, A., Hundahl, C., Dewilde, S., Gilany, K., Moens, L., and Weber, R. E. (2004) Allosteric regulation and temperature dependence of oxygen binding to human neuroglobin: implications for the multiple ligand binding steps. J. Biol. Chem. **279**, 5803–5811

33. Sun, Y., Jin, K., Mao, X. O., Zhu, Y., and Greenberg, D. A. (2001) Neuroglobin monoxide-sensitive hydrogen sulfide pathway. FEBS Lett. **488**, 48–54

34. Jeyarajah, S., Proniewicz, L. M., Bronder, H., and Kincaid, J. R. (1994) Low frequency vibrational modes of oxygenated myoglobin, hemoglobin, and modifiedderivatives. J. Biol. Chem. **269**, 31047–31050

35. Smith, A. T., Pazicni, S., Marvin, K. A., Stevens, D. J., Paulsen, K. M., and Burstyn, J. N. (2015) Functional divergence of heme-thiolate proteins: a classification based on spectroscopic attributes. Chem. Rev. **115**, 2532–2558

36. Jeyarajah, S., Proniewicz, L. M., Barner, H., and Kincaid, J. R. (1994) Low frequency vibrational modes of oxygenated myoglobin, hemoglobin, and modified derivatives. J. Biol. Chem. **269**, 31047–31050

37. Smith, A. T., Pazicni, S., Marvin, K. A., Stevens, D. J., Paulsen, K. M., and Burstyn, J. N. (2015) Functional divergence of heme-thiolate proteins: a classification based on spectroscopic attributes. Chem. Rev. **115**, 2532–2558

38. Reijerse, E. J., Sommerhalter, M., Hellwig, P., Quentmeier, A., Rother, D., Laurich, C., Bothe, E., Lubitz, W., and Friedrich, C. G. (2007) The unusual redox centers of SoxXA, a novel c-type heme-enzyme essential for chemotrophic sulfur-oxidation of Paracoccus pantotrophus. Biochemistry **46**, 7804–7810

39. Zhang, B., Crank, J. C., Subramanian, S., Green, J., Thomson, A. J., Le Brun, N. E., and Johnson, M. K. (2012) Reversible cycling between cysteine persulfide-ligated [2Fe-2S] and cysteine-ligated [4Fe-4S] clusters in the FNR regulatory protein. Proc. Natl. Acad. Sci. U.S.A. **109**, 15734–15739

40. Janc, G. J., Downey, J. R., Roduner, E., Wasilczuk, G. J., Coutts, J. W., and Eluard, A. (1976) Raman studies of sulfur-containing anions in inorganic polysulfides: sodium polysulfides. J. Inorg. Chem. **15**, 1759–1763

41. Das, T. N., Huie, R. E., Neta, P., and Padmaja, S. (1999) Reduction potential of the sulphydryl radical: pulse radiolysis and laser flash photolysis studies of...
Reaction of \( \text{H}_2\text{S} \) with neuroglobin

of the formation and reactions of \( \text{`SH} \) and \( \text{HSSH} \) in aqueous solutions. *J. Phys. Chem. A* **103**, 5221–5226

60. Zhu, W., Lin, A., and Banerjee, R. (2008) Kinetic properties of polymorphic variants and pathogenic mutants in human cystathionine \( \gamma \)-lyase. *Biochemistry* **47**, 6226–6232

61. Stoll, S., and Schweiger, A. (2006) EasySpin, a comprehensive software package for spectral simulation and analysis in EPR. *J. Magn. Reson.* **178**, 42–55

62. Cook, J. D., Bencze, K. Z., Jankovic, A. D., Crater, A. K., Busch, C. N., Bradley, P. B., Stemmler, A. J., Spaller, M. R., and Stemmler, T. L. (2006) Monomeric yeast frataxin is an iron-binding protein. *Biochemistry* **45**, 7767–7777

63. Cotelesage, J. J., Pushie, M. J., Grochulski, P., Pickering, I. J., and George, G. N. (2012) Metalloprotein active site structure determination: synergy between X-ray absorption spectroscopy and X-ray crystallography. *J. Inorg. Biochem.* **115**, 127–137