Biochemical Characterization and Ligand Binding Properties of Neuroglobin, a Novel Member of the Globin Family*

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Neuroglobin is a recently discovered member of the globin superfamily that is suggested to enhance the O₂ supply of the vertebrate brain. Spectral measurements with human and mouse recombinant neuroglobin provide evidence for a hexacoordinated deoxy ferrous (Fe²⁺) form, indicating a His-Fe²⁺-His binding scheme. O₂ or CO can displace the endogenous protein ligand, which is identified as the distal histidine by mutagenesis. The ferric (Fe³⁺) form of neuroglobin is also hexacoordinated with the protein ligand E7-His and does not exhibit pH dependence. Flash photolysis studies show a high recombination rate (kₘ) and a slow dissociation rate (kₐ) for both O₂ and CO, indicating a high intrinsic affinity for these ligands. However, because the rate-limiting step in ligand combination with the deoxy hexacoordinated form involves the dissociation of the protein ligand, O₂ and CO binding is suggested to be slow in vivo. Because of this competition, the observed O₂ affinity of recombinant human neuroglobin is average (1 torr at 37 °C). Neuroglobin has a high autoxidation rate, resulting in an oxidation at 37 °C by air within a few minutes. The oxidation/reduction potential of mouse neuroglobin (E₉₀ = −129 mV) lies within the physiological range. Under natural conditions, recombinant mouse neuroglobin occurs as a monomer with disulfide-dependent formation of dimers. The biochemical and kinetic characteristics are discussed in view of the possible functions of neuroglobin in the vertebrate brain.

In addition to the well known hemoglobins (Hbs)¹ and myoglobins (Mb), a third type of globin has recently been described in vertebrates that is predominantly expressed in the brain and other tissues (1). These neuroglobins (NGBs) consist of single chains with 151 amino acids (Mᵣ = 17,000) that share only little sequence similarity with the vertebrate globins (Mb < 21%; Hb < 25%). Nevertheless, all key determinants of genuine globins are conserved (2). Although NGB was initially discovered in mouse and man, recent data show its presence in many different mammalian species as well as in fish, suggesting the universal occurrence of NGB in vertebrate brains.²

Nerve-specific globins have been sporadically observed in mollusc, annelid, arthropod, and nemertean species (3–5). These invertebrate nerve globins reach high local concentrations up to the millimolar range, which may be sufficient to facilitate O₂ diffusion or store O₂ that supports cell function during temporary hypoxia (5). The latter assumption is supported by the observation that the nervous function in the mollusc Tellina alternata under anoxic conditions depends on the oxygenation of a nerve globin (6, 7). However, the estimated amount of NGB in the vertebrate brain under nonpathological conditions is only in the micromolar range and thus is much lower than that of a typical invertebrate nerve globin (1). The physiological role of such lowly expressed globins is not well understood. Wittenberg (8) proposed that cytoplasmic globins at low concentrations might support oxidative phosphorylation via O₂ delivery to an unknown mitochondrial terminus. Nevertheless, other globin functions are conceivable. For example, recent studies have demonstrated that some globins of bacteria, nematodes, and the human Mb may act as enzymes involved in the oxidation of nitric oxide (9–11).

The expression of an O₂-binding protein may have important implications for the function of the vertebrate brain (1, 12). The elucidation of the biochemical and kinetic properties of NGB is an essential prerequisite for the understanding of its role in the nervous system. Here we present a detailed biochemical and kinetic analysis of purified mouse and human recombinant neuroglobin.

EXPERIMENTAL PROCEDURES

Expression Cloning and Purification of Recombinant NGB—The expression plasmids (mouse and human NGB cDNA in pET3a; Ref. 1) were transformed into Escherichia coli strain BL21(DE3)pLysS. The cells were grown at 25 °C in TB medium (1.2% bacto-tryptone, 2.4% yeast extract, 0.4% glycerol, 72 mM potassium phosphate buffer, pH 7.5) containing 200 µg/ml ampicillin, 30 µg/ml chloromphenicol, and 1 mM β-ami-no-levulinic acid. The culture was induced at Aₖₒₒ = 0.8 by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.4 mM, and expression was continued overnight. The cells were...
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harvested and resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 mM dithiothreitol). The cells were then exposed to three freeze-thaw steps and were sonicated until completely lysed. The extract was clarified by low (10 min at 10,000 g) and high (60 min at 105,000 g) speed centrifugation and fractionated by ammonium sulfate precipitation. The 50% ammonium sulfate pellet containing the crude NGB was dissolved in 50 mM Tris-HCl, pH 8.5, dialyzed, and loaded onto a DEAE-Sepharose Fast Flow column equilibrated in the same buffer. After washing of the unbound material, the NGB was eluted with 200 mM NaCl. The NGB fractions were concentrated by Amicon filtration (PM10) and passed through a Sephacryl S 200 column. The fractions were pooled, concentrated, and stored at 4°C.

Mutagenesis of Recombinant NGB—A mutation was made on the recombinant mNGB resulting in the replacement of the distal, E7-His to Leu using the QuikChange™ site-directed mutagenesis method (Strategene). The recombinant mutant mNGB was subsequently expressed and purified as described above.

Spectra and Ligand Binding Kinetics—All ligand-binding experiments were performed on recombinant NGB in 100 mM potassium phosphate pH 7.0 at 25°C. The autoxidation kinetics and the O2 binding curve at equilibrium were monitored at 37°C.

Spectral measurements were made with an SLM DW2000 spectrophotometer. Under air, the samples (10 μM on a heme basis in 4 × 10−6 mM quartz cuvettes) oxidize within an hour; this form was taken to be the ferric state. The deoxy sample was obtained by equilibration under nitrogen and adding an excess of sodium dithionite. The oxidized species were reduced under air to record the oxy ferrous spectrum using the enzymatic system NADPH/ferrodoxin-NADP/ferrodoxin as described by Hayashi et al. (13). The spectra of the NGB directly within the E. coli cell culture were measured with the DW2a spectrophotometer (Amino) in the split beam mode, ranging from 350 to 650 nm.

The O2 equilibrium was determined by taking full spectra of samples in a tonometer equilibrated under a known O2 partial pressure. Each O2 level used a freshly reduced sample to avoid significant oxidation.

The spectrum of the deoxy ferrous hexacoordinated form was first recorded before addition of O2. After addition of O2 through a rubber septum cap vented by the reducing enzymatic system. After each experiment, CO was added to confirm that the sample was still in the reduced state, because the presence of an oxidized fraction will lead to an incorrect value of the O2 affinity.

O2 and CO bimolecular recombination rates (krec) were measured after photoysis with a 10-ns YAG laser pulse delivering 160 mJ at 532 nm (Quantel). The samples were equilibrated respectively under air or 1 atm of CO in 1- or 4-mm optical cuvettes with a detection wavelength at 436 nm. A typical kinetic curve is obtained from the preset molecular parameters (sedimentation coefficient, molar mass, 3-mm solution column), using the Yphantis method (16). After taking the equilibrium absorption profiles, the angular velocity ω was increased to high speed (45,000 rpm) for another 24 h so that all the protein material was sedimented. The absorption profiles were considered as the best estimate for the residual blank absorption and were subtracted from the sample absorption profiles to obtain the e values as a function of r. The standard equilibrium equation for a monomer of the holo- or apoprotein ligand was measured versus CO concentration. Indeed the first phase after CO photodissociation was independent of the CO concentration below 10% CO and was followed by a slow phase of protein ligand replacement by CO. This phase of replacement was calculated independently of the protein ligand dissociation. Assuming the absence of free binding sites and a negligible CO dissociation rate, the differential equations that give the rates of consumption/formation for protein ligand and CO form can be combined to obtain finally an irreversible first order reaction with an apparent rate constant (R) as follows.

$$R = \frac{k_{\text{HI}} \cdot k_{\text{CO}} [\text{CO}]}{k_{\text{HI}} + k_{\text{CO}} [\text{CO}]}$$  \hspace{1cm} (Eq. 1)

This reaction can also be followed by the stopped flow technique; for mixing with buffer equilibrated under 1 atm of CO, the rate of the replacement reaction approaches the dissociation rate of the protein ligand (k30). Computer simulations of the differential equations were carried out using a numerical integration procedure (MicroMath Scientific, Salt Lake City, UT).

O2 dissociation rates (kdiss) were measured by a replacement reaction technique. A sample with a slight excess of ligand was mixed with buffer containing a high concentration of a competing ligand. kdiss can be obtained from the observed replacement kinetics. Protein ligand and O2 dissociation was measured by the transition from the deoxy ferrous hexacoordinated form to the CO form as well as the transition from the ox to the CO form detected with a Biologic stopped flow apparatus. The samples were mixed with a buffered solution containing 20 mM sodium dithionite and equilibrated under 1 atm of CO gas. After mixing, the final CO concentration was around 0.7 mM. The kinetics at different wavelengths were followed from 2 to 10 s. CO dissociation from recombinant NGB was detected spectrally between 500 and 700 nm by measuring the kinetics of oxidation with 10 mM potassium ferricyanide using a diode array spectrophotometer, HP8453.

The autoxidation kinetics were followed in the visible spectral region (500–600 nm), because the spectra showed little change in the Soret region. The hexacoordinated ferric form was first enzymatically reduced, and the substrate was removed on a G25 column at 5°C. The kinetic of the kinetics was recorded using the diode array spectrophotometer. The aliquots were exposed to CO as a control of the fraction of ferrous heme, because CO binds only to the reduced form.

Potentialitometric Titration—Potentialitometric titration was done as reviewed by Wilson (14) and Dutton (15) in a DW-2a spectrophotometer (Amino) equipped with a magnetic stirrer accessory and a Phillips digital pH/mV meter PW 9408. Before and after each potentiometric titration, the electrode combination was calibrated by measuring the potential of a saturated solution of quinhydrone in 50 mM potassium hydrogen phthalate at 25°C (E° = +463 mV). Titration was carried out in the presence of 0.4 mM diaminodurene (E° = +275 mV), 0.1 mM trimethylhydroquinone (E° = +115 mV), 0.1 mM phenazine methosulfate (E° = +35 mV), 0.1 mM riboflavin-5′-monophosphate (E° = +465 mV), 0.4 mM 2-methyl-1,4-naphthoquinone (E° = −110 mV), 1.2 mM tetramethyl-p-benzozquinone (E° = +5 mV), 15 mM 2-hydroxy-1,4-naphthoquinone (E° = −145 mV), and 15 mM riboflavin-5′-monophosphate (E° = −219 mV).

O2 was excluded from the cuvette by flushing continuously with ultrapure argon (O2 < 0.1 ppm). Reductive titrations were performed by stepwise addition of anaerobic solutions of dithionite. In oxidative titrations the mNGB was reduced by the addition of NADH and dithionite before it was stepwise oxidized by the addition of an anaerobic solution of ferricyanide.

Concentrations of reduced mNGB were correlated to peak height of the a-band recorded in the dual beam mode. The absorbance at 540 nm was used as reference.

Equilibrium Ultracentrifugation and HPLC—The Beckman Optima XL-A analytical ultracentrifuge was used to perform sedimentation equilibrium experiments on recombinant mNGB samples in 150 mM Tris acetate pH 7.5 buffer with and without 20 mM dithiothreitol.

The run conditions (angular velocity ω and duration of run) were calculated from the preset molecular parameters (sedimentation coefficient, molar mass, 3-mm solution column), using the Yphantis method (16). After taking the equilibrium absorption profiles, the angular velocity ω was increased to high speed (45,000 rpm) for another 24 h so that all the protein material was sedimented. The absorption profiles were considered as the best estimate for the residual blank absorption and were subtracted from the sample absorption profiles to obtain the e values as a function of r. The standard equilibrium equation for a monomer of the holo- or apoprotein ligand was measured versus CO concentration. Indeed the first phase after CO photodissociation was independent of the CO concentration below 10% CO and was followed by a slow phase of protein ligand replacement by CO. This phase of replacement was calculated independently of the protein ligand dissociation. Assuming the absence of free binding sites and a negligible CO dissociation rate, the differential equations that give the rates of consumption/formation for protein ligand and CO form can be combined to obtain finally an irreversible first order reaction with an apparent rate constant (R) as follows.

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NGB near 423 nm is blue shifted relative to most pentacoordinated Hbs, which show peaks near 430 nm, and the shoulder in the Soret band suggests the presence of two forms.

Unlike most Mbs or Hbs, which display a transition from a water molecule to OH\(^{-}\) as sixth ligand at alkaline conditions, the spectrum of the ferric recombinant NGB is independent of pH. O\(_2\) and CO have a sufficiently high affinity to the Fe\(^{2+}\) to displace the protein ligand, but a pure oxy spectrum was difficult to obtain. The rapid oxidation rate (see below) interferes with oxygen binding measurements. The spectra of the hexacoordinated oxidized NGBs are similar to that of its ferrous oxy form (Fig. 1A). Because CO only binds to the ferrous form, the kinetics of oxidation were determined by the amplitude of the CO-binding signal. The NGBs showed a rapid autoxidation (Fig. 2) relative to Hbs and Mbs involved in O\(_2\) delivery. mNGB oxidized within a few minutes at 37 °C, about three times faster than the recombinant hNGB (Table I); at the moment no explanation is available for this difference.

Spectra were also recorded directly in living E. coli cell cultures. This clearly demonstrates that within the E. coli cell, recombinant mNGB occurs in its deoxy ferrous hexacoordinated form. However, it must be taken into account that the O\(_2\) concentration in rapidly growing E. coli cells is low, preventing oxidation of the iron atom.

**O\(_2\) and CO Affinity and Kinetics**—The O\(_2\) equilibrium curve was measured point by point, as described under “Experimental Procedures” (Fig. 3). A value of 1 torr at 37 °C for the recombinant hNGB was obtained compared with 2 torr at 25 °C previously reported for recombinant mNGB under conditions with some interference because of oxidation (1). The mNGB oxidized too rapidly to obtain reliable oxygen affinities. The binding rates provide an alternative method to estimate the
affinity; however, multiple protein conformations can complicate these calculations.

Ligand association rates were measured by flash photolysis and stopped flow mixing experiments. After photodissociation two kinetic phases were observed that are well separated in time. The rapid kinetics correspond to ligand recombination to the pentacoordinated form. The rebinding rates for O₂ were very high, approaching the diffusion limit for ligand binding. For samples equilibrated under air or 1 atm of CO, the ligand rebinding occurs on a microsecond time scale (Fig. 3).

In addition to the rapid ligand binding, a certain fraction of the photolyzed hemes will bind the internal protein ligand, in this case the distal E7-His; recovery to the preflash state may then take quite a long time for the ligand replacement reaction. By varying the CO concentration, the fraction of the two phases can be changed. At high CO levels, the CO on rate, with only a small fraction of histidine binding, was determined. As the CO level is decreased, the (concentration-independent) histidine on rate becomes competitive, and a larger fraction of the slow phase occurs. From the rate of this slow phase and direct measurements of the two on rates, the histidine off rate can be calculated (as described under “Experimental Procedures”).

The reaction scheme for the two phases can be described as follows.

\[
\text{REACTION 1}
\]

\[
\text{flash} \rightarrow \text{Fe-CO + His} \quad k_{\text{on}} \rightarrow \text{Fe + CO + His} \quad \text{His} \rightarrow \text{Fe-His + CO}
\]

If the “external” ligand CO does not recombine in the rapid (μs) phase, then the histidine will block the site for nearly 1 s. If an external ligand (for example oxygen or CO) is mixed by stopped flow with the deoxy ferrous hexacoordinated form, the observed ligand replacement requires about 1 s (Fig. 4). This corresponds to the slow replacement phase observed in the flash photolysis experiments. For mixing experiments with a high external ligand concentrations, the observed rate approaches that for the dissociation of the internal protein (histidine) ligand.

Ligand binding constants were extracted from these data by least square fits and are given in Table I. For each phase, it appears that two exponential terms are required for best simulations, especially for the ligand dissociation reactions. This may be due to the presence of two protein conformations (60–75% for the main component), but from the present data, one cannot conclude that there is a dynamic equilibrium between two states. The temperature or pH dependence of the kinetics could help resolve this question. The absence of cooperativity anti-cooperativity in the O₂ binding isotherm suggests a compensation between the hexacoordinated form and O₂ on and off rates in both conformations giving rise to similar O₂ binding affinities.

Mutation of the E7-His to E7-Leu increases the binding constants (Table I). Both O₂ and CO rebinding are very rapid, indicating little resistance by the protein for access to the ligand-binding site. This confirms again that the protein ligand is E7-His.

Reduction and Potentiometric Titration—Reduction of mNGB by ferredoxin was measured under anaerobic conditions, and the reduction velocity was slightly slower than that for horse heart Mb as a reference (Fig. 5A), which is in agreement with a slightly higher redox potential (Mb: \(E'_o = -291\) mV) under the conditions used.

The oxidation-reduction potential, \(E'_o\) of mNGB was determined by potentiometric titration. Fig. 5B presents the plot of the absorbance at 558 nm against the measured potential during the titration. An \(E'_o\) of \(-129\) mV (\(n = 3\)) for recombinant mNGB was calculated from the curve based on the Nernst equation fitted to the given data points (23).

Aggregation State of NGB—Both the ultracentrifugation experiments (Fig. 6) and the gel filtration (HPLC) studies (not shown) confirm that mNGB occurs mainly as monomers (\(M_r = 17,000\)), but aggregates are present as well. A significant dimer population (\(M_r = 34,000\)) is observed in addition to a small tetramer (\(M_r = 68,000\)) contribution. The addition of dithiothreitol to the sample abolished the presence of aggregates, suggesting that dimerization is based on disulfide bridging.

**DISCUSSION**

The spectral and ligand binding kinetics of ferrous NGB indicate a reversibly hexacoordinated Hb type with a His-Fe²⁺-

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**TABLE I**

Rates of ligand binding to NGB and other hexacoordinated hemoglobins

| CO     | KL    | \(k_{\text{on}}/h\) | \(P_{50}\) (torr) | Reference |
|--------|-------|----------------------|-------------------|-----------|
| Oxygen |       |                      |                   |           |
| hNGB   | 2000  | 4.5                  | 250 × 10⁶         | 0.8       | 3.2       | 65 × 10⁶ | 0.014 | 0.21 | 15 (5.7) | 4.2 | 19 | This study |
| mNGB   | 2000  | 1.2                  | 300 × 10⁶         | 0.4       | 1.3       | 72 × 10⁶ | 0.013 | 0.18 | 7.2 (40.5) | 29 | 19 | This study |
| mNGB E7-Leu | 700 × 10⁶ | 2000 × 10⁶ | 2800 | 2000 × 10⁶ | This study |
| Aphrodite aculeata Hb | 170 × 10⁶ | 300 × 10⁶ | 2118 | 21 × 10⁶ | 0.1 | 1.2 | 450 | 1.24 | 3 |
| Hordeum sp. Hb | 9.5 × 10⁶ | 0.0272 | 2.86 | 5.17 × 10⁶ | 0.0111 | 1.83 | 1.48 | 28 |
| Oryza sativa Hb1 | 68 × 10⁶ | 0.038 | 1.5 | 7.2 × 10⁶ | 0.001 | 0.14 | 4.0 | 34 |
| Arabidopsis Hb1 | 75 × 10⁶ | 0.12 | 1.6 | 0.51 × 10⁶ | 0.019 | 37 | 23 | 25 |
| Sperm whale Mb | 14 × 10⁶ | 12 | 857 | 0.51 × 10⁶ | 0.019 | 37 | 23 | 25 |
His binding scheme. In the absence of an external ligand, the protein will adopt this conformation. Upon the addition of either O2 or CO, there is a competition for binding to the iron atom between external ligands and the internal protein ligand. In terms of kinetics, two types of external ligand association must be considered, one to the pentacoordinated form and one to the hexacoordinated. In the latter case, the dissociation of the endogenous sixth ligand is the rate-limiting step. The two rates in the kinetic data suggest the existence of different conformations of the heme pocket for the pentacoordinated form. Based on the high association rates (k\text{on}) and low dissociation rates (k\text{off}), the intrinsic affinities for O2 and CO for the pentacoordinated form are quite high (Fig. 1 and Table I).

Several lines of experimental evidence indicate that the recombinant NGBs can be considered as representative for NGB in vivo. The spectrum of the deoxy ferrous recombinant mNGB (Fig. 1) is identical to the spectrum of NGB extracted from mouse brains (1), and globins with similar spectra are observed in invertebrate as well as in the nonsymbiotic plant Hbs (24, 25). Therefore, the hexacoordinated form of globins can be considered as a physiological one.

Our results are in full agreement with a recent characterization of the NGB by Raman spectroscopy (22). A heme pocket residue, most likely the distal His (E7), coordinates to the heme-Fe in the ferro and ferrous states. This residue is not protonated in the pH range 5–10. The endogenous ligand can be replaced by external ligands such as O2 and CO. The ferrous-CO complex suggests the presence of an open conformation, in which the bound CO is not interacting with a heme pocket residue, and a closed conformation, where a positively charged residue stabilizes the complex. An O2 off rate was predicted to be lower than that of Mb, whereas the O2 on rate will be limited by the displacement of the sixth ligand to the heme (22).

In contrast, our results are in disagreement with a recent study reporting on the ligand binding properties of hNGB (26). There is a major difference of a factor of 1000 in our values of the histidine dissociation rate, a difference that would propagate to the final estimated O2 affinity. Because the O2 and CO binding rates are similar, as well as the spectral form, it would appear that we are studying the same molecule, despite the independent preparations. The values of Trent et al. (26) suggest a K\text{H on} on the order of 1 and a very high O2 affinity, which is incompatible with the absorption spectrum showing predominantly the classical hexacoordinated form and with the observed oxygen affinity on the order of 1 torr (1). Our value of the histidine dissociation rate is 1000 times lower and indicates a high saturation level of the sixth ligand, compatible with the absorption spectrum, and an effective O2 affinity 1000 times lower, compatible with the observed affinity by equilibrium methods (Ref. 1 and Fig. 3).

We have measured more directly the replacement of the histidine by CO using the stopped flow technique. At high CO concentration the observed rate becomes limited by the histidine dissociation. We thus obtain a direct measurement that indicates that about 1 s is required, as opposed to the extraction of data on the microsecond time scale used by Trent et al. (26). Our flash photolysis data also show a very slow replacement reaction. For both methods, we observe a slow histidine dissociation compatible with the absorption spectrum, which shows a fully formed hexacoordinated shape (Fig. 4).

**Fig. 3.** Oxygen recombination kinetics after photolysis at pH 7 and 25 °C. The inset shows the Hill plot for oxygen equilibrium measured at 37 °C for the human recombinant neuroglobin.

**Fig. 4.** Transition from ferrous hexacoordinated to the CO form for human and mouse recombinant neuroglobin, measured by stopped flow.

**Fig. 5.** Determination of the oxidation/reduction potential of mouse recombinant neuroglobin. A, reduction of recombinant mouse neuroglobin by ferredoxin. B, redox titration plot of the absorption at 558 nm versus the potential measured during titration with sodium dithionite or FeCN.
residues of a fit to an isodesmic association of dimers. Considered as another method of fine tuning the O₂ affinity.

eral function. The competition between the external and inter-
general occurrence of hexacoordinated Hbs at low concentra-
the Protostomia/Deuterostomia split (1). The evolutionary origin of NGBs, having diverged from the other globins before the Protostomia/Deuterostomia split (1).

Despite the similarities between the nonsymbiotic plant Hbs and the NGBs, the observed oxygen affinity for the latter is much lower, because of compensation from the more tightly bound protein ligand (Table I).

With respect to the hexacoordinated conformation, the NGBs resemble the nonsymbiotic plant Hbs and the Hbs of the sea cucumber Caudina arenicola and the clam nerve-specific Hbs of Spisula and Tellina (7, 21, 27–29). A comparison with the hexacoordinated chloroplast Hb of Chlamydomonas eugametos is more difficult because this is a truncated Hb with an unusual helical structure (30–33). Among plants, the rice Hb1 has the typical His-Fe²⁺-His binding scheme, resulting in spectral characteristics strongly resembling those of cytochrome b₅ and the NGBs. The distal His (E7) is coordinated directly to the iron atom in both the ferrous and ferric forms, causing a bend of the E-helix (21). Dissociation of the distal His in favor of an external ligand occurs by an outward movement of the imidazole sidechain with concomitant upward and outward movement of the E-helix and a possible repacking of the CD corner and folding of the h-helix. This reorientation of the E-helix brings the distal residue in such a position that the formation of a strong hydrogen bridge with the bound O₂ becomes likely (21).

In contrast, formation of the hexacoordinated structure in C. arenicola Hb C is supposed to involve a movement of the heme group resulting in the dissociation of the E-E'-dimer interactions and a reorganization in the CD region (27).

The structural similarities between NGBs and nonsymbiotic Hbs, both displaying the same His-Fe²⁺-His coordination scheme, may extend to the functional properties of the molecules: (i) The residues important in the determination of the O₂ affinity (B10, E7, and E11) are identical in both groups, being Phe, His, and Val, respectively; (ii) NGBs and nonsymbiotic plant Hbs show a high k₅ₐ and a low kₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑἐ ᐅ

Currently, the biological function of the NGBs can only be hypothesized. As for the nonsymbiotic plant Hbs (21) and the high affinity nonvertebrate Hbs (25), several possibilities can be considered: (i) a function as an O₂ carrier and facilitating diffusion to the mitochondria within those cells that need an active aerobic metabolism; (ii) an enzymatic function comparable with, for example, NADH oxidases that facilitate the glycolytic generation of ATP; (iii) a function as an O₂ sensor participating in a signal transduction pathway that modulates the activities of regulatory proteins in response to changes in O₂ concentrations; and (iv) a function in the detoxification of NO.

A function as O₂ carrier might be supported by the low off rate of the NGBs as compared with Mb (Table I). This will prevent O₂ release except under conditions of high demand (hypoxia), whereas the hexacoordination will prevent rapid rebinding of the O₂, allowing it to be utilized by the mitochondria. hNGB is differentially expressed in brain, and its expression seems to be lowest in zones that are most sensitive to hypoxia (1, 12). Nonsymbiotic plant Hbs of class 1 are known to be induced by hypoxia and other stress factors (24, 35, 36), and it would not be surprising if this is also the case for NGBs. The observed O₂ affinity could be compatible with this role; the actual O₂ binding rates, coupled with competition from the histidine binding, would indicate a slower response time of the order of 1 s. As such, hexacoordination is indeed a way of fine tuning the O₂ affinity.

The low concentration of NGB in nonpathological brain tissue (in the micromolar range; Ref. 1) is hardly to be reconciled with an O₂ storage or carrier function. Indeed, the nerve Hbs of several invertebrates (3, 4, 6), which are definitively involved in O₂ transport, occur in much higher concentrations (millimolar concentrations) than the NGBs. Only in case of massive induction can it be envisaged that the concentration of NGB reaches levels that are sufficient to sustain an aerobic metabolism during temporary hypoxia.

Although the redox potential of mNGB (E'ₕ = −129 mV; Fig. 5) lies within the physiological range (NADH/NAD = −320 mV to ½ O₂/H₂O = +820 mV), the autoxidation of NGB is high (t₁/₂ = 11 min; Fig. 2) as compared with Hb and Mb. At first glance this also seems to be in conflict with a simple role as an oxygen reservoir, unless the oxidation rate of NGB under natural conditions is much lower, because of a potential efficient reducing system. Indeed a high autoxidation rate for NGB produces reactive oxygen species and requires a “NGB reductase” similar to the metMb reductase described in heart tissue (13). It cannot be excluded that in brain a comparable reducing enzyme is available as well. On the other hand, the NGB oxidation rate...
would be too slow for the high turnover required of an active redox protein such as the cytochrome P450.

O₂-consuming Hbs functioning as NO dioxygenases have been described for the flavohemoglobines (9) and the Ascaris perenteric fluid Hb (10). These Hbs typically have very high O₂ affinities because of a B10-Tyr and E7-Gln combination and a low autoxidation rate (Table I). NGB and nonsymbiotic plant Hbs, however, display moderate to high O₂ affinities and a low autoxidation rate (Table I). NGB and nonsymbiotic plant Hbs, however, display moderate to high O₂ affinities and a low autoxidation rate (Table I). NGB and nonsymbiotic plant Hbs, however, display moderate to high O₂ affinities and a low autoxidation rate (Table I). NGB and nonsymbiotic plant Hbs, however, display moderate to high O₂ affinities and a low autoxidation rate (Table I). NGB and nonsymbiotic plant Hbs, however, display moderate to high O₂ affinities and a low autoxidation rate (Table I). NGB and nonsymbiotic plant Hbs, however, display moderate to high O₂ affinities and a low autoxidation rate (Table I). NGB and nonsymbiotic plant Hbs, however, display moderate to high O₂ affinities and a low autoxidation rate (Table I). NGB and nonsymbiotic plant Hbs, however, display moderate to high O₂ affinities and a low autoxidation rate (Table I).

Finally, both NGB and nonsymbiotic plant Hbs are expressed at low levels, have a hexacoordinated His-Fe2⁺ binding scheme, and display the same B10-Phe and E7-His combination. Therefore, it seems difficult to attribute an enzymatic function in NO metabolism to the NGB. However, the detoxification of NO by Mb in skeletal and heart tissue, avoiding the inhibition of respiration, has recently been described (11).

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