Nitric Oxide Binding Properties of Neuroglobin

A CHARACTERIZATION BY EPR AND FLASH PHOTOLYSIS

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Neuroglobin is a recently discovered member of the globin superfamily. Combined electron paramagnetic resonance and optical measurements show that, in Escherichia coli cell cultures with low O₂ concentration overexpressing wild-type mouse recombinant neuroglobin, the heme protein is mainly in a hexacoordinated deoxy ferrous form (F8His-Fe²⁺-E7His), whereby for a small fraction of the protein the endogenous protein ligand is replaced by NO. Analogous studies for mutated neuroglobin (mutation of E7-His to Leu, Val, or Gln) reveal the predominant presence of the nitrosyl ferrous form. After sonication of the cells wild-type neuroglobin oxidizes rapidly to the hexacoordinated ferric form, whereas NO ligation initially protects the mutants from oxidation. Flash photolysis studies of wild-type neuroglobin and its E7 mutants show high recombination rates ($k_r$) and low dissociation rates ($k_{dis}$) for NO, indicating a high intrinsic affinity for this ligand similar to that of other hemoglobins. Since the rate-limiting step in ligand combination with the deoxy-hexacoordinated wild-type form involves the dissociation of the protein ligand, NO binding is slower than for the related mutants. Structural and kinetic characteristics of neuroglobin and its mutants are analyzed. NO production in rapidly growing E. coli cell cultures is discussed.

Two new globins, neuroglobin (Ngb)¹ and cytoglobin (Cygb), were recently added to the vertebrate globin family. Ngb is predominantly expressed in the brain and in other nerve tissues whereas Cygb is expressed in all tissues studied so far. Human Ngb and Cygb are composed of 151 and 190 amino acids, respectively, the Cygb being ~40 residues longer than standard globins because of the presence of amino- and carboxy-terminal extensions of ~20 residues each. Although both globins display the structural determinants of the globin fold (1), they share little sequence identity with vertebrate hemoglobin (Hb) and myoglobin (Mb) (2–7). In contrast with Mb, which is expressed in muscle tissue in the millimolar range, Ngb and Cygb are expressed at a much lower level (micromolar range) (2, 3).

Ngb and Cygb are hexacoordinated (hx), either in their ferrous or ferric forms, having the distal HisE7 as the internal ligand (3, 8, 9, 10). Flash photolysis studies of Ngb, at normal temperature, show high recombination ($k_r$) and low dissociation ($k_{dis}$) rates for O₂ and CO, suggesting a high intrinsic affinity for both ligands. However, since the rate-limiting step in ligand binding to the ferrous deoxy-hx form involves dissociation of the distal HisE7 residue, ligand binding in vivo is suggested to be low ($P_{50} = 1$ Torr) (6–9). The study of the ligand binding over a wide range of temperature reveals the presence of multiple, intrinsically heterogeneous distal heme pocket conformations in Ngb-CO (11). Distal heme pocket heterogeneity is also observed by Raman spectroscopy (8).

Cytosolic hxHbs are also observed in bacteria (12), unicellular eukaryotes (13), plants (14), and some invertebrates (15). It can be extrapolated that hxHbs are universally spread over the living world and thus may have essential function(s) in cell metabolism.

The physiological role of low expressed hxHb is not well understood. Several functions have been suggested. First, these might be proteins involved in O₂ scavenging under hypoxic conditions and supplying it for aerobic respiration (2, 6, 9). Second, they might function as terminal oxidases by reducing NADH under micro-aerobic conditions and enhancing such as the ATP production by glycolysis (16). Third, they could be O₂ sensor proteins activating other proteins with regulatory function (11, 12). Fourth, they may display as yet unknown enzymatic activities. Fifth, these might be involved in NO metabolism as shown for Mb (17–18), some flavo-Hbs (19) and truncated Hb (20), and the Ascarias (Nematoda) Hb (21).

Electron paramagnetic resonance (EPR) has been used for more than thirty years to analyze nitrosyl (NO) ligation to the heme group of different hemeproteins (22–31). The NO radical bound to the central Fe²⁺ ion in a hemeprotein forms a paramagnetic, low-spin ($S = ½$) complex, which can be detected by EPR. Furthermore, EPR techniques have been extensively used to study the oxidized ferric state of different heme proteins (32–35).

Here we present an extensive EPR analysis of recombinant wild-type (wt) and mutant mouse Ngb (mNgb) in its ferric and nitrosyl ferrous form in comparison with those of recombinant sperm whale (sw) wt and mutant Mbs. Furthermore, we give a
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EXPERIMENTAL PROCEDURES

Expression Cloning and Purification of Recombinant Wild Type and Mutant Mouse Ngb—Expression cloning and purification of wt and mutant mouse Ngb was performed as described previously (9). Briefly, the expression plasmids (cDNA of Ngb in pET3a) were transformed in Escherichia coli, strain BL21(DE3)pLysS, and grown at 25 °C in the presence of ampicillin, chloramphenicol, and δ-amino-levulinic acid. After induction, the cells were grown overnight. The cells were broken by freeze-thawing and sonication (9). The Ngb was purified from the clarified cell lysate by ammonium sulfate precipitation, DEAE-Sepharose ion exchange, and size exclusion chromatography. Substitution of the distal His (E7) by Leu, Val, or Gln, was performed on the recombinant Ngb using the QuickChange™ site-directed mutagenesis method (Strategene). The recombinant mutant Ngb was subsequently expressed and purified as wt Ngb.

Derivatization of Ngb—Purified ferric Ngb was converted to the ferrous form by addition of sodium dithionite. Excess reagent was eliminated by size exclusion chromatography (Amersham Biosciences PD10 column) under CO atmosphere (AtmosBag™, Aldrich) and using CO-saturated buffer (50 mM Tris-HCl, pH 7.5). Replacement of CO by NO was performed by incubating the ferrous Ngb-CO with 1 mM sperm oxygen meter (HANNA Instruments).

Mutant Mouse Ngb—mutant mouse Ngb was performed as described previously (9). Briefly, Ngb mutants were expressed and purified as described previously (37).

Analytical Techniques—Dissolved O2 was measured with a Hanna HI 9141 oxygen meter (HANNA Instruments). NO3− and NO2− were analyzed using a Dionex DX-120 ion chromatography equipped with an AS14 anion column (Dionex Benelux) (www.dionex.com). Graph equipped with an AS14 anion column (Dionex Benelux) was calibrated using a sample of DPPH (diphenylpicrylhydrazyl) and nitrite equilibrated under 1 atm of CO. The dithionite was added because nitric oxide formation. Immediately after mixing Ngb-CO with the NO

**Fig. 1.** X-band EPR spectra of samples of E. coli cell cultures overexpressing recombinant wild-type and mutant mouse neuroglobin taken before (A) and after (B) sonication. E. coli cell cultures overexpressing recombinant wt Ngb (a, e), recombinant E7-Val Ngb (b, f), recombinant E7-Gln (c, g), and recombinant E7-Leu (d, h). All spectra were measured at 13 K and with the same receiver gain. The spectra 1e–h are scaled in accordance with the amount of sample available for each measurement. The spin state of FeIII is indicated by: *, low spin; ***, high spin; **, non-heme.

Analytical Techniques—Dissolved O2 was measured with a Hanna HI 9141 oxygen meter (HANNA Instruments). NO3− and NO2− were analyzed using a Dionex DX-120 ion chromatography equipped with an AS14 anion column (Dionex Benelux) (www.dionex.com).

Optical Spectra—Spectral measurements were made with an SLM DW2000 spectrophotometer (Hewlett Packard). Under air, the samples (10 μM on a heme basis in 4 × 10−5 mm quartz cuvettes) oxidize within an hour, this form was taken to be the ferric state. 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.

Electron Paramagnetic Resonance—The EPR spectra were recorded on a Bruker ESP300E spectrometer (microwave frequency 9.43 GHz) equipped with a gas-flow cryogenic system, allowing operation from room temperature down to 2.5 K. All presented spectra were recorded with a microwave power of 10 milliwatts, a modulation frequency of 100 kHz, and a modulation amplitude of 0.5 milliTesla. The magnetic field was calibrated using a sample of DPPH (diphenylpicrylhydrazyl) and an NMR Gaussmeter (Bruker ER 035 M).

The EPR spectra were simulated using the EasySpin program (www.esr.ethz.ch/). The intensities of the spectra were determined by double-integration of the spectra after baseline correction. In relating the spectral intensities to relative spin concentrations the total protein concentration and volume were taken into account. Samples for the EPR measurements were taken at different stages in the isolation and purification process of the proteins.

Spectra and Ligand Binding Kinetics—For all the kinetic measurements the experimental conditions were 100 mM potassium phosphate, pH 7.0. The typical sample concentration was 10 μM on a heme basis, and the measurements were performed in a 4 × 10−5 mm quartz cuvette at 298 K.

**NO Binding Kinetics**—NO bihemoloc recombination rates (kobs) were measured after flash photolysis with 10-ns YAG laser pulses of 160 mJ at 532 nm (Quantel). The standard detection wavelength was 436 nm. Samples initially saturated with NO provide little bimolecular recombination; by contrast for CO the bihemoloc phase is generally the predominant pathway. We therefore first prepared a concentrated stock solution equilibrated under 0.01 atm of CO; the oxidized fraction was reduced by adding a slight excess of sodium dithionite (the final dithionite concentration was kept below 0.5 mM). Then the samples were diluted at least 50 times in the optical cuvette sealed with a rubber cap containing the phosphate buffer equilibrated under 0.009 atm of NO. The sample was introduced with a Hamilton syringe previously flushed with nitrogen. During all the experimental procedure great care was taken to work with NO without any oxygen contamination to avoid nitric oxide formation. Immediately after mixing Ngb-CO with the NO buffer, the sample was flashed by the laser pulse, and the NO rebinding was monitored. Indeed the NO bimolecular rate is faster than that of CO, allowing a measure of the NO association rate.

**NO Dissociation Kinetics**—Spectral measurements were made with a HP8453 diode array spectrophotometer. Optical changes were monitored in the Soret and visible regions.

During expression, the available O2 is completely consumed by the exponentially growing cells creating a micro-anaerobic environment (Table I). This explains the lack of FeII* globins in the EPR spectra. Nitrate consumption and nitrite production is obvious. Formation of volatile N-derivatives, including NO, is likely.
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The optical spectrum recorded directly in the living E. coli cell cultures overexpressing recombinant wt Ngb showed the typical features (α band: 560 nm, β band: 530 nm, Soret band: 426 nm) of a hz globin (His-Fe²⁺-Hiz) (9). When the cells are overexpressing the mutant Ngb proteins, the spectra become typical for hexacoordinated NO-heme proteins (α band: 571 nm, β band: 541 nm, Soret band: 417 nm) (38) (see Supplemental Data, Fig. S1). This assignment is corroborated by the comparison with the absorption spectra of purified ferrous Ngb in the hz deoxyform and bound to the external ligand NO (see Supplemental Data, Fig. S2). The combination of the optical and EPR data demonstrates that within the E. coli cell, recombinant wt Ngb occurs predominantly in its deoxy ferrous hexacoordinated form, whereby a small fraction of the hexacoordinated nitrosyl ferrous form is found. All recombinant mutant Ngbs under study are predominantly in the hz nitrosyl ferrous heme form.

Fig. 1B shows the EPR spectra of a batch of E. coli cells overexpressed with Ngb or its mutants after three freeze-thaw steps and sonication. Note that Fig. 1, A and B show different samples taken from a large batch of cells before and after sonication (see “Experimental Procedures”). The absolute spectral intensities of Fig. 1, A versus B should therefore not be compared; only the relative intensities of the spectra within one figure are relevant. In the case of the recombinant mutant Ngbs (Fig. 1B, f–h), the EPR spectra are virtually identical to those of the corresponding cell cultures (Fig. 1A, b–d). No significant contribution of ferric forms can be found. However, sonication of the cells overexpressing wt Ngb results in strong EPR contributions of Fe³⁺ complexes besides the spectrum of the NO-heme form (Fig. 1e). As shown earlier (9), one of the Fe³⁺ forms (indicated with an asterisk in Fig. 1e) can be assigned to the hexacoordinated ferric state of Ngb (His-His-Fe³⁺-Hiz). Again, the contribution of the hz His-Fe²⁺-NO form is found to be a factor 4–6 larger in the case of the mutants than of the wt. These results demonstrate that NO addition initially protects the Fe²⁺ center against oxidation. The hexacoordinated His-His-Fe²⁺-Hiz form is however less stable against oxidation.

In order to prove that the EPR spectra observed in Fig. 1A can indeed be ascribed to a nitroxide adduct of the Ngb proteins, the carboxyl ferrous forms of the proteins were reacted with the nitrosating reagent, spermine nonoate. The corresponding EPR spectra for wt and E7-Leu Ngb are depicted in Fig. 3A, a and b. As a comparison, the spectrum for the recombinant E7-Leu mutant of swMb, treated in the same way, is shown (Fig. 3A). Apart from EPR signals due to Fe³⁺ complexes, which were also present in the control measurements of the carboxyl ferrous proteins and which can be ascribed to an incomplete reduction of the Fe³⁺ proteins with sodium dithionite, clear signals similar to the ones observed in the E. coli cell cultures are observed. Both at pH 7.5 and 8.5, the Fe³⁺-NO concentration in E7-Leu Ngb was larger than the one in wt Ngb (the relative concentration was determined on the basis of the protein concentration, volume, and EPR signal intensity). The release of NO by spermine nonoate is controlled by the pH,
TABLE II

|                   | $g_1$ (±0.0002) | $g_2$ (±0.0009) | $g_3$ (±0.004) | $A_1$ (±3) | $A_2$ (±2) | $A_3$ (±5) | Ref. |
|-------------------|----------------|----------------|----------------|------------|------------|------------|------|
| Ngb               | 2.077          | 2.0037         | 1.970          | 32         | 65         | 40         |      |
| E7-Val Ngb        | 2.074          | 2.0035         | 1.973          | 36         | 66         | 40         |      |
| E7-Gln Ngb        | 2.070          | 2.0038         | 1.968          | 32         | 63         | 40         |      |
| E7-Leu Ngb        | 2.074          | 2.0030         | 1.968          | 36         | 66         | 40         |      |
| E7-Leu swMb       | 2.079          | 2.0038         | 1.968          | 38         | 63         | 40         |      |
| Mb                | 2.076          | 2.002          | 1.979          | 43.5       | 54         | 47.4       | 23   |
| aMb               | 2.07          | 2.004          | 1.97           | n.d.       | n.d.       | n.d.       | 28   |
| Cytochrome Oxidase| 2.091          | 2.006          | 1.980          | 30.6       | 59.9       | 30.6       | 24   |

whereby a higher release occurs at lower pH. The difference in the EPR intensities confirms the earlier observed differences (Fig. 1A) between the EPR spectra of E. coli cells overexpressing wt Ngb and its mutants.

Fig. 3B shows the NO-related signal in detail for E7-Val Ngb and E7-Leu Mb with the corresponding simulations. The simulations were done assuming an admixture of two species of rhombic (type I) and axial (type II) symmetry. The EPR parameters for type I are given in Table II, the axial $g$ tensor of type II was for all cases taken as $g_{z} = 2.035, g_{y} = 1.98$. The parameters are typical for a six-coordinated Fe(II) (heme)NO structure (22–31). The EPR spectra of the Ngb proteins could be simulated using a ratio of type I over type II ($n_{II}/n_{I}$) equal to 80/20%, whereby this ratio was 95/5% for E7-Leu Mb. Variations in the $n_{II}/n_{I}$ ratio have been reported earlier for different heme proteins (22, 26–30). Isolated NO-ligated alpha-beta-chains of Hb give $n_{II}/n_{I}$ ratios of 80/20 (10/90) at temperatures below 30 K (26, 28). For Mb at low temperature, $n_{II}/n_{I}$ ratios varying from 50/50% (28) to 70/30% (27) have been observed, depending on the type of Mb. For all hemoproteins, type II is found to dominate at high temperatures (>180 K). Furthermore, subtle variations of the $g$ tensors of type I were detected upon temperature increase (27, 30).

On the basis of extensive EPR and ENDOR studies, type I has been assigned to a hexacoordinated structure with the nitrosyl-histidine coordinated in a bent end-on orientation with the proximal histidine F8 as second axial ligand (23, 26, 28, 39). In this configuration the Fe-N(NO) bond does not coincide with the porphyrin plane normal (39). Density functional computations suggest that the NO is oriented toward a meso-C atom of the porphyrin ring (40). The nature of type II is still controversial, despite the large amount of spectral data available. Several authors have ascribed type II at low temperatures to a species where the Fe(NO) bond aligns with the normal of the porphyrin ligand (28, 26, 29, 39), although a topological isomer with the Fe atom displaced “below” the porphyrin ligand toward HisF8 has also been proposed (22). Recent density functional computations indicate that the observed axial $g$ tensor agrees either with a bent end-on orientation of NO where the NO ligand eclipses one of the equatorial Fe-Nporph bonds or with a partially dissociated hexacoordinated complex (distance (Fe-N(Im)) > 0.25 nm) with a freely rotating NO ligand (40). The latter may explain the changes in the $g$ tensor upon increase of the temperature, but at low temperatures (< 40 K), rotation of the NO ligand can be excluded. The computations excluded the “Fe-displacement” model.

**NO Affinity and Kinetics**—As for other heme proteins, NO binds with a high affinity to Ngb. Flash photolysis of the NO-bound form gave small signals on the μs timescale. We therefore injected Ngb-CO into an optical cuvette prepared under a partial NO atmosphere; these samples were immediately photodissociated to allow observation of the NO binding. As for other Hbs, the bimolecular association rate is high (Table III), approaching that for a diffusion-limited reaction.

The replacement of NO by CO allowed a measurement of the NO dissociation rate, which was quite low. Both the on- and off-rates of NO to pentacoordinated Ngb were similar for the series of mutants; thus the affinity for the pentacoordinated form $K_{penta}$ are similar. The main difference is therefore the competitive binding of the distal histidine for the wt Ngb. As for other external ligands, this will decrease the observed affinity by nearly a factor of 1000, as determined by the relation for competing ligands $K = K_{penta}/(1 + K_{His})$. This large reduction in affinity can also be explained as if there were two types of association by the external ligand: when the site is free (pentacoordinated form), there is a high rate of binding, whereas if the external ligand encounters the hexacoordinated form, it must wait for the histidine to dissociate, and the overall ligand replacement will occur on a much slower timescale.

**Analysis of the EPR Data of the Ferric Heme Proteins**—The ferric form of the wt Ngb has been characterized earlier by EPR (10). The study revealed the simultaneous presence, in a wide range of pH values, of two related structural forms. The dominant low-spin form (>90%) (LS1 (Table IV), indicated in Fig. 1e with an asterisk) could be attributed to a His-Fe$^{3+}$-His configuration. The high-spin form (HS1 (Table IV), indicated with a double asterisk in Fig. 1e) could be ascribed either to a hexacoordinated His-Fe$^{3+}$-H$_2$O form or to a pentacoordinated His-Fe$^{3+}$. The EPR spectra strongly suggested that an equilibrium exists between a low-spin species in which the E7 His residue is in a distal imide coordination at the iron atom, in a slightly tilted His-Fe$^{3+}$-His structure and a high-spin species, favored at low pH, in which the E7 His residue swings out from the heme pocket possibly allowing a water molecule to bind in the distal position. The equilibrium lies strongly on the side of the low-spin species. Ferrous Ngb was earlier found to remain hexacoordinated over a wide range of pH values (9). The fact that the distal histidine of Ngb can bind to the heme iron in both ferric and ferrous states is consistent with the distal histidine residing closer to the heme than seen in mammalian Hbs and Mbs, but similar to other non-mammalian Hbs and cytochromes. Indeed, comparison with the EPR spectrum of wt swMb (Fig. 2e) shows us that Mb is predominantly in the aquomet form (Hs5, Table IV) with a small contribution of a low-spin Fe$^{3+}$ species (LS4, Table IV), which can be attributed to a His-Fe$^{3+}$-OH$^{-}$ form that is only present at high pH (33) (see also Supplemental Data).

The signals marked by a triple asterisk in Fig. 1e, which can also be observed in the EPR spectra in Fig. 2A can be attributed to non-heme ferric residues (42) and will not be discussed further.
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The on- and off-rates were measured at 25°C. For Ngb the NO affinity depends on the competition between NO and His for the heme: \( K_{NO} = \frac{K_{NO}^{penta}}{1 + K_{His}} \) where \( K_{NO}^{penta} \) refers to the pentacoordinated form and \( K_{His} \) is determined in Ref. 9.

### TABLE III
Rates of NO binding to Ngb and sperm whale myoglobin

|          | \( k_{on} \) \( (s^{-1}) \times 10^2 \) | \( k_{off} \) \( s^{-1} \) | \( K_{NO}^{penta} \) \( = k_{off}/k_{on} \) | \( K_{His} \) | \( K_{NO} \) | Ref.   |
|----------|-------------------------------------|----------------|-------------------------------|------------|-----------|--------|
| Ngb      | 1.5                                 | \( 2 \times 10^{-4} \) | 750                           | \(~800\)   | \(~1\)    | this work |
| E7-Val Ngb | 2.7                                  | \( 2 \times 10^{-4} \) | 1000                          | 1000       | 1000      | this work |
| E7-Gln Ngb | 1.9                                  | \( 3 \times 10^{-4} \) | 900                           | 900        | 900       | this work |
| E7-Leu Ngb | 2.7                                  | \( 2 \times 10^{-4} \) | 1300                          | 1300       | 1300      | this work |
| Horse heart Mb | –                                    | \( 2.4 \times 10^{-4} \) | –                             | –          | –         | this work |
| SwMb     | 0.22                                | \( 0.98 \times 10^{-4} \) | 220                           | 220        | 37, 41   |        |
| E7-Val swMb | 2.7                                  | \( 11 \times 10^{-4} \) | 250                           | 250        | 37, 41   |        |
| E7-Gln swMb | 0.43                                | \( 1.1 \times 10^{-4} \) | 390                           | 390        | 37, 41   |        |
| E7-Leu swMb | 1.9                                  | \( 1.3 \times 10^{-4} \) | 1500                          | 1500       | 37, 41   |        |

### DISCUSSION

**Structural and Kinetic Analysis**—The EPR data and NO binding kinetics clearly show a different behavior of the wt Ngb versus its mutants E7-Leu, E7-Val, and E7-Gln. Fig. 4. shows schematically the reaction pathways under anaerobic conditions in the presence of NO (E. coli cell cultures) and under aerobic conditions in the absence of NO. As determined by EPR and optical spectroscopy, the wt Ngb is in the rapidly growing E. coli cells predominantly in the hexacoordinated His(F8)-Fe\(^{2+}\)-His(E7) configuration, whereas all mutants occur in the hx His(F8)-Fe\(^{2+}\)-NO form. This observation agrees with the fact that the NO affinity is about a factor 1000 lower for the wt Ngb than for the Ngb mutants (Table III). This results from the competition between NO and the distal histidine since the intrinsic NO affinities to the pentacoordinated form are comparable for the wt and mutant Ngb. However, the NO affinity is so high that the difference in values between wt Ngb and mutants (not versus at) probably does not matter. It is more likely that in hx wt Ngb the rate of NO binding is slower than in the mutants, and thus other NO consuming reactions can compete with wt Ngb for the ligand.

After sonication of the cells, the oxidation of the Ngb mutants is slow indicating that NO ligation initially protects the heme pocket against oxidation. The on-rate for NO binding to pentacoordinate E7-Leu Ngb is comparable with those for CO and O\(_2\) binding \((k_{on(CO)} = 7 \times 10^8 M^{-1} s^{-1}, k_{on(O_2)} = 2 \times 10^9 M^{-1} s^{-1})\), but the off-rate is considerably lower in the case of NO binding than for O\(_2\) binding \((k_{off(O_2)} = 200 s^{-1}, k_{off(NO)} \text{ not determined}) \) (9), which explains the observed protection against oxidation by NO. This protection is not given by the distal histidine ligation, as becomes obvious from the appearance of the EPR spectra of the His(F8)-Fe\(^{2+}\)-His(E7) form immediately after sonication of the cells (Fig. 1e). This agrees...
with the earlier determined high autodissociation rate of Ngb and with the fact that the NO dissociation rate constant is about a factor 6000 lower as the histidine dissociation rate constant \((k_{\text{off}} = 1.2 \text{ s}^{-1})\) (9). Our earlier EPR analysis of Ngb showed that ferrous Ngb is predominantly in the low-spin hexacoordinated form (Fig. 2a and Ref. 10). Besides the expected EPR signal of the high-spin pentacoordinated ferric heme complexes (HS2, HS3, HS4), unidentified signals of low-spin Fe\(^{3+}\) complexes could be observed in the EPR spectra of the purified Ngb mutants (Fig. 2A). Most of these signals were found to be buffer-dependent indicating that mutation of E7-His destabilizes the heme pocket.

It is interesting to note that all Ngb proteins under study show at 10–15 K the same ratio of type I versus type II ferrous nitrosyl-heme complexes (\(n_I/n_{II} = 80/20\%\)) as found for NO-ligated eHb. An extensive proton ENDOR analysis of the type I and type II forms in horse heart Mb at 10 K showed that for both isomers the E7-His and E11-Val residues are present in the heme pocket and stabilize the bound NO (29). Using electron spin echo envelope modulation (ESEEM) spectroscopy, an interaction with the \(N_2\) nitrogen of the distal E7-His could be found for NO-ligated heme proteins of type II, where this interaction was not observed for type I (28). This seems to correlate with our observation that at 13 K E7-Leu Mb is quasi in the pure type I form (95%), whereas this is only 50–70% for wt Mb (27, 28). The substitution of the distal histidine has a significant influence on the formation of the type II nitrosyl isomer in Mb. Furthermore, the stabilization of NO by a hydrogen-bonded \(N_2\) in the axial state II seems to agree with x-ray crystal structure (41). Although type I and type II species have been found in frozen solutions of NO-ligated tetraphenyl porphyrin imidazole (26), the ENDOR and ESEEM data on Hbs and Msbs seem to indicate that the variations of the NO binding geometry in heme proteins controlled by the heme’s protein surrounding. The ENDOR data on type I and type II suggest that the difference between the type I and type II form lies not only in the deviation of the Fe-N(NO) axis from the porphyrin normal, but also in a displacement of both distal and proximal histidine versus the heme plane (30, 39). Interestingly, mutation of the E7-His site in Ngb does not seem to influence the \(n_I/n_{II}\) ratio. From the observation of the hexacoordinated His-Fe-His configuration for Ngb we assume that the distal histidine resides closer to the heme in Ngb than in mammalian Hbs and Msbs. The current observations seem to indicate that in this geometry E7-His can influence less the equilibrium between the two NO binding modes than observed in Mb. This might also explain why the effect of the polarity of the E7 amino acid on the NO association rate constants is less pronounced for Ngb than for Mb (Table III).

**Biological Implications**—(i) NO production in \(E. coli\): during protein expression in \(E. coli\) under traditional laboratory conditions (250 ml of medium in a 1-liter Erlenmeyer; normal shaking), the metabolism of the cells gradually shifts from aerobic to anaerobic respiration because of the complete consumption of the initially available \(O_2\) by the increasing cell density. Under conditions of low \(O_2\), nitrate reductase is induced, and \(E. coli\) will reduce nitrate to nitrite and nitrite to NO using this enzyme. The concentrations of NO produced by \(E. coli\) in this way never exceed 300 \(\mu\text{M}\) despite large excesses of nitrite and formate and is self-limiting (45–48). The poisonous NO will be part of the mechanism for rapidly shutting down the TCA cycle and terminal oxidase in semi-anaerobic conditions (49). Excess NO will be detoxified by diverse systems including flavohemoglobin and flavinubredoxin (50, 51). During the expression of a hemoprotein at high concentration, NO will be scavenged by the heme-iron resulting in ferrous nitrosyl spectra. The degree of NO binding however will depend on the distal site conformation.

It is well documented that co-expression of \(Vitreoscilla\) Hb, in \(E. coli\), will increase bacterial protein expression (52–54). This effect is mainly attributed to an increased \(O_2\) supply to the bacteria by the presence of an oxygen carrier. However, in the light of our observations, the reduction of the nitrosative stress by the scavenging of NO by the heme iron of the \(Vitreoscilla\) Hb and thus a prolonged aerobic metabolism might be of more importance than the increased \(O_2\) supply (54, 55).

(ii) Ngb function: the involvement of Hb and Mb in the NO metabolism became clear only recently (17, 56). As a tissue hemoprotein, Mb functions as a scavenger of bioactive NO in cardio-myocytes by the reaction of MbO\(_2\) + NO to metMb + NO\(_2\), thereby effectively reducing the cytosolic NO concentration. Regeneration of metMb by metMb reductase to Mb and subsequent association with \(O_2\) leads to reformation of MbO\(_2\) available for another NO degradation cycle (17, 18). We therefore can ask the question whether Ngb is playing a similar role in neuronal tissue. Based on the presented EPR and kinetic data, this seems to be less probable. Indeed, the E7-His clearly hinders NO binding, in vitro, as compared with E7 mutants.

**Fig. 4. Reaction schemes for recombinant wild-type (A) and mutant (B) mouse neuroglobin inside and outside the \(E. coli\) cells.**
and protects as such the iron atom from oxidation. Therefore it seems unlikely that Ngb is a key molecule in NO metabolism. Other possible functions of Ngb must be explored further.

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