The Redox State of the Cell Regulates the Ligand Binding Affinity of Human Neuroglobin and Cytoglobin*

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

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

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

Ngb and Cygb are hexacoordinated (hx), either in their ferrous or in their ferric forms, having the distal HisE7 as the internal ligand. Flash photolysis studies of Ngb, at normal temperature, show high recombination (krec) and low dissociation (kdis) 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 (P50 = 1 torr) (6, 12), although a higher oxygen affinity (due to a lower affinity for the distal histidine) was reported (10–11).

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

The physiological role of hx-Hbs, mostly expressed at low level, is not well understood. Several roles have been suggested. First, these proteins may scavenge O2 under hypoxic conditions and supply it for aerobic respiration (6, 7, 17, 18). Second, they may function as terminal oxidases by oxidizing NADH under hypoxic conditions and hence enhance ATP production by glycolysis (19). Third, they could be O2 sensor proteins, activating other proteins with regulatory function (20, 21). Fourth, they may be involved in nitric oxide metabolism.

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

Initial ligand binding studies indicated a heterogeneity in the kinetics (12) or possible relaxation phenomena (10), and measurements over a wide range of temperatures suggested the presence of multiple conformations in Ngb-CO (21). Distal heme pocket heterogeneity in NGB was also observed by Raman spectroscopy and NMR (22–24). Unlike most other globins, NGB and CYGB contain 3 and 2 cysteine residues, re-
products were cloned in the pET3a vector using the NdeI and BamHI restriction sites. The clones were verified by sequencing before expression.

Analyses and Separation of Different Neuro- and Cytoglobin Subforms—Samples of NGB and CYGB were further purified with an Akta purifier system on a Hitrap DEAE-Sepharose column from Amersham Biosciences. This was done with or without the prior incubation in 1 mM dithiothreitol (DTT) for 1 h at 37 °C. Because of the low pl of the NGB and CYGB, the samples were loaded onto the column equilibrated with 20 mM phosphate buffer at pH 8.0. After a pH decrease to 6.5 (20 mM bis-tris-HCl), the protein elution was initiated at 100 mM NaCl from a selective salt gradient. Protein elution was monitored at 280 and 410 nm. The fractions of the protein were dialyzed against 100 mM phosphate buffer at pH 7, and its purity was assessed by spectroscopic analysis and by gel filtration on a Superose<sup>®</sup> 12 HR10/30.

Reaction and Binding Kinetics of Neuroglobin and Cytoglobin with Thiol Reagents—5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) was used to examine the reactivity of the sulfhydryl groups in the native NGB as described by Jocelyn (25). The reaction was performed at pH 7 in 100 mM potassium phosphate buffer and 100 μM DTNB with a protein concentration of 5–6 μM protein, determined from the Soret band for the carboxylated species using the extinction coefficient ε<sub>Soret</sub> = 190 mM<sup>-1</sup> cm<sup>-1</sup>. The absorption at 412 nm, corresponding to the TNB anion (ε<sub>412</sub> = 13.6 M<sup>-1</sup> cm<sup>-1</sup>), was continuously monitored over 4 h and corrected against a blank to which no protein was added. The reaction is TNBS<sub>SCys</sub> + SCys → TNBS<sub>Cys</sub> + S<sub>SCys</sub> where TNB is the species detected by visible spectroscopy.

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

Ligand Binding Kinetics—Spectral measurements were carried out with an HP8453 or Varian Cary 400 spectrophotometer. Laser flash photolysis and stopped-flow rapid mixing, the methods used to assess hexacoordination and bimolecular CO and O2 rate constants, have been photolysis and stopped-flow rapid mixing, the methods used to assess with an HP8453 or Varian Cary 400 spectrophotometer. Laser flash photolysis and stopped-flow rapid mixing, the methods used to assess hexacoordination and bimolecular CO and O2 rate constants, have been described previously (12). Photolysis was performed with 10-ns pulses at 532 nm. Detection of the sample absorption was in the Soret band, typically at 436 nm, using a 50-watt lamp and interference filters. Samples from 1 to 10 μM on a heme basis were in 4 × 10-mm quartz cuvettes.

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

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

Reduced samples were prepared by reacting the native samples (20 μM concentration) with 5 mM DTT in 10 mM ammonium bicarbonate (pH 8.0) for 10 min at 37 °C. The resulting solution was then diluted 5-fold in 1:1 acetonitrile:water containing 0.2% formic acid and infused directly into the mass spectrometer as described above.

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

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

RESULTS AND DISCUSSION

Occurrence of Cysteine Residues in Globins

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

The role of cysteine residues in globins is most likely the same as in proteins in general, namely, structural by the formation of intra- or intermolecular disulfide bonds or catalytic
by being part of an active site or a place of direct ligand binding. Intramolecular disulfide bonds are well documented in many proteins but so far have never been observed in vertebrate globins. Intermolecular disulfide bonds between globin chains are unknown in vertebrate Hbs and Mbs but are essential in some invertebrate extracellular Hbs to form giant aggregates (3.5 × 10^6 M_r; annelids, hexagonal bilayer Hbs), which are necessary to avoid elimination of the Hb molecule from the hemolymph by excretory processes (28, 29). The specific S-nitrosylation of vertebrate HbO_2 at Cys-93 of the chain is proposed to be part of a dynamic transport cycle of nitric oxide, leading to control of blood pressure and facilitating efficient delivery of oxygen to tissues (30).

The available Ngb sequences display conserved Cys at positions CD7, D5, and G18/19. There are two exceptions: the Rodentia Ngb are missing the CD7 Cys, and the zebrafish (Fig. 2) Ngb are missing the G18/19 Cys. Based on the crystal structure of the Ngb (31), sites CD7 and G19 host solvent-accessible residues, whereas the D5 residue is virtually solvent-inaccessible. The cysteine residues in CYGB occur at positions B2 and E9, different from those in Ngb, and are less conserved. Although the number of Ngb and Cygb sequences available is rather small, the relative conservation of the Cys residues may suggest a functional significance.

**Chromatographic Analysis of Human Neuro- and Cytoglobin**—Considering the potential effect of disulfide bonds in both molecules, the separation of "crude" Ngb and Cygb preparations into fractions by ion-exchange and gel filtration chromatography was performed. Crude recombinant Ngb was separated into three fractions, F1, F2, and F3, by DEAE ion exchange chromatography with gradient elution, with F1 being the major fraction. If the protein solution was first incubated in 1 mM DTT for 1 h at 37 °C, there was only one (monomeric) fraction, corresponding to F1, in the chromatography. This suggests the presence of a dimeric fraction in the crude extract, as confirmed by high performance liquid chromatography analysis.

The same preparation for Cygb, with or without DTT, gave mainly a band corresponding to a dimeric form. Dilution experiments did not show a transition to the monomeric form; these

![Fig. 4. Electrospray ionization mass spectra of Ngb.](image)

![Fig. 5. Amino acid sequence of Ngb and Cygb with indications of the tryptic fragments.](image)
results indicate a stable dimer not based on disulfide bonds. However, the mini Cygb, corresponding to the central globular fragment, was eluted as a monomer.

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

The NGB mutant Cys(G19) → Ser with only two cysteines (CD7 and D5) showed essentially no reaction with DTNB (Fig. 3, Table I), under normal or denaturing conditions, indicating that cysteines CD7 and D5 were involved in a disulfide bond. The presence of a CD7/D5 disulfide bond was also recently suggested (32). Overall, these binding studies indicated the presence of disulfide bonds, but more information was necessary to fully determine all the possible bonding patterns, including dimers via intermolecular bonds.

WT mouse Ngb, or mutants with either the D5 or the G19 cysteine changed to serine, show nearly 100% reactivity with DTNB. Note that cysteine D5 reacted more slowly than that at position G19 (Fig. 3), consistent with results from the x-ray structure, indicating that the D5 site is not directly solvent-accessible (31). Iodoacetamide accessibility for the cysteines in NGB and CYGB was studied using ESI-MS and will be discussed in the next section.

**Mass Spectrometry Assessment of Disulfide Bonds and Accessibility of Cysteines to Iodoacetamide**

**Human Neuroglobin**—To rigorously confirm the presence of disulfide bonds, the fractions obtained by DEAE-Sepharose chromatography were analyzed by ESI-MS with the following results. Fraction F1 (Fig. 4a) gave the mass of the dominant component as 16930.9 Da, agreeing, within experimental error,

with the sequence mass of NGB with one disulfide bond (16931.4 Da). Also present was a minor component (~10%; 33891.4 Da) that was 30.3 Da higher in mass than expected for a disulfide-linked dimer of NGB (33860.9 Da).

Upon reduction with DTT, the mass of the monomer in-

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**Fig. 6.** Partial electrospray ionization mass spectra from an 80-min tryptic digest of NGB before (a) and after (b) reduction with DTT.

**Fig. 7.** Electrospray ionization mass spectra of CYGB before (a) and after (b) reduction with DTT and after oxidation of the reduced sample (c). Myoglobin was introduced with the samples to internally calibrate the mass scale. MCE, mercaptoethanol.
creased to 16933.2 Da (Fig. 4b), agreeing, within experimental error, with the sequence mass of NGB with all Cys free (16933.5 Da). Concomitantly, the minor dimer fraction disappeared. Fractions F2 and F3 comprised 50% of NGB (16931.3 and 16931.2 Da, respectively) and ~50% of a component of 33862.8 and 33861.9 Da, respectively, in agreement with the sequence mass of a disulfide linked dimer of NGB (data not shown).

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

The disulfide bond between CD7-Cys and D5-Cys was verified by reducing an aliquot of the digest solution with DTT when the (T5 + T6 – 2H) peptide disappeared with the concomitant appearance of the T5 and T6 peptides (Fig. 6b). Furthermore, the T13 dimer disappeared concomitantly with the appearance of T13 monomer.

The intramolecular disulfide bond apparently perturbs the three-dimensional structure of the CD-D region in the crystallized protein. The WT NGB was difficult to crystallize; the initial structure was obtained for the mutant without cysteines (CCC→GSS) bearing the Cys-46→Gly, Cys-55→Ser, Cys-120→Ser mutations (31). Analysis of the mutated protein structure (i.e. in the absence of any intramolecular disulfide bonds) shows that two of the four crystallographically independent NGB molecules have disordered CD-D regions (residues 44–54), whereas the remaining two display a rather extended conformation for the CD-D region (31), deviating remarkably from that of homologous heme proteins. Although the crystal structure observed for the latter two mutated NGB molecules does not suggest the formation of a CD7-D5 intramolecular disulfide, based on simple geometrical considerations, it should be considered that the local structure of the protein may have been altered by the introduction of a Gly residue at site CD7. Under this hypothesis, it appears structurally plausible that local conformational rearrangements may lead the CD7 and D5 residues to a closer mutual location, allowing formation of the disulfide bond.

If a conformational transition in NGB between an oxidized (i.e. hosting an intramolecular disulfide bond) form and a reduced (i.e. disulfide free) NGB form can be envisaged, such a transition could affect the location of the neighboring E-helix, thus allowing fine modulation of the availability of the endogenous HisE7 ligand for heme hexacoordination. It can thus be proposed that the redox state of the cell acts on the rupture/formation of the intramolecular CD7-D5 disulfide bond, thus initiating a conformation change that affects the overall oxygen affinity of NGB and/or other functional properties as well.

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

Which cysteine residues are involved in the formation of...
disulfide bonds can also be inferred from their accessibility and potential for derivatization by iodoacetamide. Derivatization of NGB with iodoacetamide to S-carboxyamidomethylate the free Cys, even in the presence of 8 M urea, was unsuccessful. Derivatization of DTT-reduced NGB was partly successful without denaturation, showing mainly NGB + Cam and NGB + 2Cam (~50% as intense as NGB + Cam) but no NGB + 3Cam. When the procedure was undertaken in 8 M urea, derivatization was more successful, but all three cysteines were never completely derivatized. Fig. 4e shows ESI-MS spectra from a second preparation of NGB after reduction and S-carboxyamidomethylation in 8 M urea. Four species are present at masses 16931.4, 16990.4, 17047.5, and 17104.4 Da, corresponding to oxidized NGB (4%) and NGB with 1Cam (45%), 2Cam (39%), and 3Cam (12%), respectively. A tryptic digest of the same NGB solution showed that T5, T6, and T13 were ~94, 54, and 38% S-carboxyamidomethylated, respectively.

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

**Human Cytoglobin**—Analysis of native reconstructed CYGB by ESI-MS demonstrated that both Cys residues were disulfide-linked to 2-mercaptoethanol (derivatized CYGB (21556.9 Da) = underivatized CYGB (21404.7 Da) + 2 × 76.1 Da) (Fig. 7). This derivatization with 2-mercaptoethanol results from the reconstruction procedure used. Upon reduction with DTT, the mass decreased to 21404.7 Da, in agreement with the sequence having 2 Cys free (Fig. 7). These observations also underscore the importance of using mass spectrometry to check the authenticity of recombinant protein samples and stress the disadvantage of using 2-mercaptoethanol as a reducing agent. After removal of the DTT, air oxidation of the reduced CYGB (pH 8, 37 °C) resulted in a progressive decrease of the mass over 7 h, from 21404.7 to 21402.6 Da, implying the formation of a disulfide bond. No evidence was found for CYGB dimers that would indicate the formation of interchain disulfide bonds. The existence of an intrachain disulfide bond was substantiated by the analysis of a tryptic digest of oxidized CYGB (Fig. 8). In the spectrum, ions were present corresponding to the peptides expected from the sequence with free Cys except T14, which contains E9-Cys (Fig. 5). Peptide T8, containing B2-Cys, was present, but at a low level. In addition, there were triply charged ions at m/z 706.72 and 749.41, corresponding to (T8 + T14 – 2H) and (T8 + T (13–14) – 2H) of calculated m/z 706.69 and 749.39, respectively (Fig. 8a).

Upon reduction of the digest with DTT, these triply charged ions disappeared concomitantly with a 10-fold increase in the intensity of doubly charged ions, corresponding to the T8 peptide (Fig. 8b), together with the appearance of singly charged ions, corresponding to the T14 peptide (Fig. 8b, inset) and T (13–14) peptide (not shown). In contrast to NGB, both Cys in reduced CYGB reacted readily with iodoacetamide to form S-carboxyamidomethylated CYGB without denaturing in 8 M urea.

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

**Ligand Binding of Human Neuro- and Cytoglobin**—The kinetics of rebinding of NGB and CYGB to CO shows the form expected for hexacoordinated globins. The rapid bimolecular phase corresponds to a competitive binding of CO and the distal (E7) histidine; the slow phase corresponds to the replacement reaction of the distal histidine by CO. Reduction of the disulfide bonds with DTT leads to a change in the kinetics; there is less slow phase, and the replacement reaction is much slower. The solid lines are simulations using the model for competitive ligand binding. B, kinetics of the slow phase (involving histidine dissociation) showing the large decrease in rate upon addition of DTT; data, presented on a log-linear scale, were normalized to the amplitude for the slow phase.

**Fig. 9. Photodissociation kinetics.** A, the kinetics of ligand rebinding to NGB for samples equilibrated under 0.1 atm CO. The detection wavelength was 436 nm. The biphasic shape is characteristic of the hX globins; data are presented on a log-log scale with time in s. The rapid phase reflects competitive binding of CO and the distal (E7) histidine; the slow phase corresponds to the replacement reaction of the distal histidine by CO. Reduction of the disulfide bonds with DTT leads to a change in the kinetics; there is less slow phase, and the replacement reaction is much slower. The solid lines are simulations using the model for competitive ligand binding. B, kinetics of the slow phase (involving histidine dissociation) showing the large decrease in rate upon addition of DTT; data, presented on a log-linear scale, were normalized to the amplitude for the slow phase.
break the S–S bonds (Fig. 9). The dominant change is in the histidine dissociation rate, which decreases by nearly an order of magnitude (Table II). Since the overall $O_2$ affinity depends on the histidine binding, this leads to an effective decrease in $O_2$ affinity. A similar shift was observed for NGB with mutated cysteines. Thus breaking the S–S bond by DTT or elimination of the bond by genetic engineering results in a low $O_2$ affinity conformation of NGB.

For WT NGB, this transition is reversible; however, we have never obtained 100% of the kinetics corresponding to the S–S state NGB. Attempts to purify or oxidize the protein resulted in samples that show at most 90% of the high $O_2$ affinity form; perhaps even with the disulfide bond, the protein is not completely in a single conformation. This means that the NGB studied so far was always a variable mixture of several forms: with and without the disulfide bond. This would explain the heterogeneity reported in our earlier study (12) and would correspond to some of the multiple conformations reported by Kriegel et al. (21).

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

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

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

**A Hypothetical Model for the Heme-Cysteine Coupling in Neuroglobin**

Structural analysis by mass spectrometry clearly shows the presence in NGB of a disulfide bond between CD7-Cys and D5-Cys. Substitution of CD7-Cys and D5-Cys by Ser and eliminating as such the disulfide bond, or its reduction by DTT, lowers the distal histidine dissociation rate by a factor of 10 with a consequent lowering of the $O_2$ affinity by the same factor. This suggests the existence of two major conformations for the protein: one with the disulfide bond present, resulting in a slightly stressed protein, and one without the disulfide bond. Breaking the disulfide bond allows a shift in the orientation of the E-helix, resulting in a lowered affinity and a release of $O_2$.

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

The scheme for this hypothetical model for NGB function is presented in Fig. 10. In this model, formation and cleavage of a disulfide bond influences the functional characteristics of the protein. The same may be true for CYGB, although much less is currently known about the physiological role of this globin. This mechanism would explain how different globins could coexist; specific partner molecules would provoke release of the oxygen, as opposed to a simple Hb-O\(_2\) equilibrium with the local environment.

Until recently, the disulfide bonds present in mature proteins were thought to be inert and remain unchanged during the life of the protein. However, it now appears that this is not necessarily the case: disulfide bonds can be cleaved and re-formed, and this event may have significant consequences for protein function (34). Examples of disulfide bond cleavage/formation that have an effect on the protein function, and the factors facilitating it, are: thrombospondin-1-protein disulfide isomerase (35), plasmin/annexin II (36), and CD4/thioredoxin (37). Our model for the regulation of the oxygen binding affinity of NGB and the redox state of the cell via disulfide bond formation that have an effect on the protein function, and the protein function (34). Examples of disulfide bond cleavage/formation that have an effect on the protein function, and the factors facilitating it, are: thrombospondin-1-protein disulfide isomerase (35), plasmin/annexin II (36), and CD4/thioredoxin (37). Our model for the regulation of the oxygen binding affinity of NGB and the redox state of the cell via disulfide bond formation, in agreement with a role of NGB in cellular oxygen homeostasis.

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