Solution ^{1}H NMR Characterization of Equilibrium Heme Orientational Disorder with Functional Consequences in Mouse Neuroglobin

Weihong Du,[†] Ray Syvitski,[†] Sylvia Dewilde,[§] Luc Moens,[§] and Gerd N. La Mar*,[†]

Department of Chemistry, University of California, Davis 95616, and Department of Biomedical Sciences, University of Belgium, Antwerp Belgium

Received February 10, 2003; E-mail: lamar@indigo.ucdavis.edu

Neuroglobin (Ngb) is a recently discovered mammalian heme protein expressed predominantly in the brain.[1] It possesses all the determinants of a globin[2] (~17 kDa, His(F8), Phe(CD1), His(E7), etc.) but shares remarkably low sequence identity (21−25%)[1] with other globins. The highest sequence identity is found with the globin from the glial cells of *Aphrodite*.[3] Its spectral data indicate[4−7] a hexacoordinate heme in both reduced (Ngb) and oxidized (metNgb) forms. Although the role of Ngb in brain physiology is unknown at this time, its low expression levels[1] *in vivo* and its hexacoordination suggest possible roles other than O_2 transport/storage (i.e., O_2 sensor, NO detoxifier).[8] Sequence alignment and mutagenesis[4] point to the distal His(E7) as the sixth ligand. Endogenous ligation of the sixth position in vertebrate globins is unprecedented but has been recently established for nonsymbiotic plant hemoglobins (Hbs)[9,10] and some bacterial globins,[11,12] where the distal His(E7) provides the sixth ligand. It is now recognized that such endogenous ligation provides a new mechanism for modulating ligand affinity,[4,6,10] since the Fe−His(E7) bond must rupture to allow exogenous ligation (O_2, CO to Ngb, CN^- to metNgb). The intrinsic thermodynamic and dynamic stabilities of the Fe−His(E7) bond in these globins, and the factors that modulate them, are poorly understood at this time, but current structural studies are expected to shed light on the subject.[9,11] Functional studies have concluded that O_2 affinity of Ngb is similar to that of Mb, but two such studies differed by ~10^4 in the proposed intrinsic Fe−His(E7) bond rupture rate.[4,6] Moreover, both spectroscopic and functional data indicate an intrinsic heterogeneity.[4,5] We report here a ^{1}H NMR study of metNgb that (i) confirms His(E7) as the sixth ligand that makes an unstrained Fe−His(E7) bond, (ii) demonstrates that metNgb exhibits a ~2:1 equilibrium heme orientational disorder that equilibrates to ~1:1 upon ligation of cyanide, (iii) shows that the rate of CN^- ligation is extraordinarily slow and differs by a factor ~2 for the two heme orientations.

Resolved portions of the ^{1}H NMR spectra of metNgb[13] and ligated and equilibrated metNgbCN are illustrated in Figure 1,a and b, and Figure 2,a and d, respectively. Peaks are labeled M_i(m_i) for methyls and H_i(h_i) for single protons for metNgb (metNgbCN), where i is a heme position or residue number. The spectrum of metNgb resembles that of mammalian, heme orientationally disordered, bis-His ligated, ferricytochrome b_5,[14,15] both in the spectral dispersion and in number of resolved resonances. A heterogeneity in metNgb is evident in the presence of two sets of resonances, labeled superscript A or B; integration of H_i^B:H_i^A in Figure 2a yields a ~2:1 ratio of isomers that is invariant over 6 months. Similar integration of h^A:h^B peaks in equilibrated metNgbCN yield a ~1:1 ratio (Figure 2d).

Standard 2D NMR assignment protocols[15] (see Supporting Information for details) allow unambiguous assignment of the heme
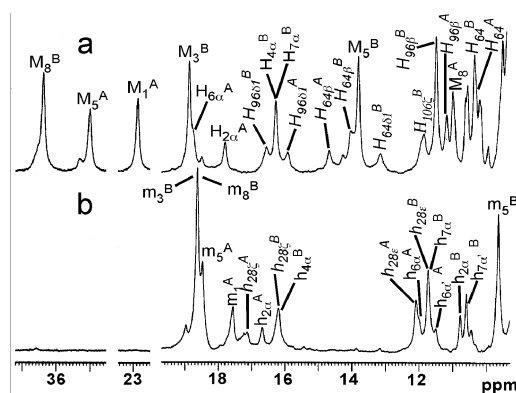
[†] University of California.
[§] University of Belgium.

Figure 1. Low-field portions of the 600 MHz ^{1}H NMR spectra in 50 mM phosphate, pH 7.5 at 30 °C of (a) metNgb in ^{1}H$_2$O and (b) metNgbCN in ^{2}H$_2$O. The heme and residue protons, i, are labeled $M_i^{A,B}$ ($m_i^{A,B}$), $H_i^{A,B}$ ($h_i^{A,B}$), for methyls (M) and single protons (H) for metNgb (M_i,H_i) and metNgbCN for heme orientations A and B in Figure 3.

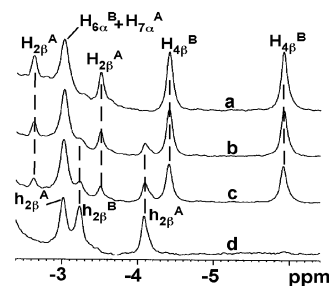**Figure 2.** Upfield portions of the ^{1}H NMR spectra in ^{2}H$_2$O, 50 mM phosphate, pH 7.5 at 30 °C of: (a) equilibrium metNgb, and (d) equilibrium metNgbCN. The initial time course of the ^{1}H NMR spectra after adding ~1:1 mol equiv KCN to metNgb is shown in (b) after 20 min and (c) after 2 h.

for each isomer, as labeled in Figures 1 and 2. Two hyperfine-shifted, partially resolved $NHCHCH_2$ spin systems for both isomers in metNgb, each exhibit dipolar contacts to a strongly relaxed (T_1 ~20 ms), hyperfine-shifted, labile proton. They are diagnostic for the $NHC_\alpha HC_\beta H_2$ and ring $N_\delta H$ of two ligated His,[15] as labeled in Figure 1a. One His is part (AMX_{i+3}) of a helical fragment Gly$_i$-X$_{i+1}$-X$_{i+2}$-AMX_{i+3} that identifies His96(F8). The other His participate in standard helical α_i-N_{i+3}, α_i-β_{i+3} contacts with the aliphatic AMX of an aromatic residue and is identified as His64(E7) that is in contact with the expected Phe61(E4). The hyperfine-shifted peaks for metNgb account for >95% of the protein (with negligible mono-His species[6,7]), exhibit essentially the same temperature dependence as observed for ferricytochrome b_5[14] and confirm a pure $S = ^1/_2$ ground state for metNgb. The comparable hyperfine shifts (mean $C_\beta H$ shifts of ~10 ppm (His96(F8)) and ~11 ppm (His64(E7)) for the two axial His in metNgb (see Supporting Information) argue against strain in the Fe−His(E7) bond.[15] Six moderately hyperfine-
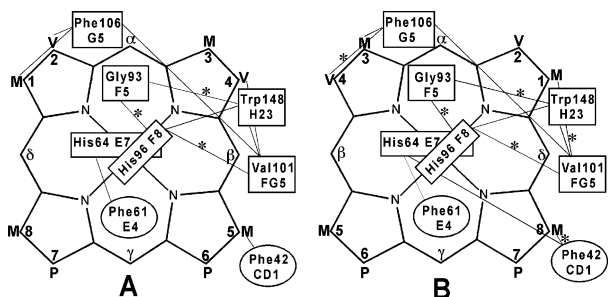
Figure 3. The heme orientations in Mb (A), and rotated 180° about the α–γ-meso axis (B). The heme-residues are shown by lines for metNgb; those detected for metNgbCN are shown by added asterisks.

shifted residues in metNgb (two in metNgbCN) were located (see Supporting Information) whose contacts to the heme, His96(F8) and His64(E7) uniquely identify them as Phe42(CD1), Phe61(E4), Val101(FG5), Phe106(G5), and Trp148(H23), with heme contacts as shown in Figure 3. The proximity of Val101(FG5) to 1-CH$_3$, 8-CH$_3$ in the major (B), and to 5-CH$_3$ and 4-vinyl in the minor isomer (A) of metNgb, comprise conclusive proof for heme disorder;[15] the A isomer has the orientation in Mb and Hb[2] (Figure 3A). Assignments in metNgbCN establish 1:1 ratio for the two heme isomers with orientations determined by heme contact with Phe42-(CD1) and Val101(FG5) as shown in Figure 3.

Addition of ∼15-fold excess CN$^-$ leads to ∼50% ligation within ∼ 10 min (not shown), but at different rates for the two isomers. As shown in Figure 2, a–c, addition of CN$^-$ to metNgb leads to loss of the metNgb peaks (Figure 2, a–c), but the intensity of H$_{2\beta}{}^A$ for metNgb is lost much faster than that of H$_{4\beta}{}^B$. Similarly, single proton peaks h$_{2\beta}{}^A$ and h$_{2\beta}{}^B$ for the two isomers of metNgbCN appear, but the intensity of h$_{2\beta}{}^A$ is initially ∼twice that of h$_{2\beta}{}^B$ (Figure 2, b,c). The latter observation dictates that the ligation rate of CN$^-$ is ∼twice as fast for metNgb with the A than with the B heme orientation. Even before CN$^-$ ligation is complete, the intensity of h$_{2\beta}{}^B$ relative to h$_{2\beta}{}^A$ increases (not shown) until equilibrium is reached where the ratio is 1:1 (Figure 2d). The data in Figure 2 allow three important conclusions: (1) the CN$^-$ ligation rate for metNgb is extraordinarily slow (∼10^{-2} s^{-1} with 15-fold excess CN$^-$) compared to other globins,[16] indicating that Fe–His-(E7) bond rupture in metNgb must be very slow; (2) the rates of CN$^-$ ligation and hence of Fe–His(E7) bond rupture depend on the heme orientation, with the rate twice as fast for the A as for the B heme orientation; and (3) the equilibrium constant for the two heme orientations is altered from ∼2:1 in metNgb to ∼1:1 upon displacing His(E7) by cyanide, with an equlibration half-life, similar to that of Mb.[17]

This is the first example of a mammalian globin that exhibits significant equilibrium heme orientational disorder, although several examples have been reported for globins from invertebrates and one fish.[15] Moreover, the orientational heterogeneity has functional consequences, with the ligation rate twice as fast for one than for the other isomer. It has been shown that the rate of heme binding by apoMb is independent of the Mb,[17,18] with the heme off-rate controlling Mb stability[18] and heme orientational preference.[17] Hence, the relative CN$^-$ binding rates parallel (but are much slower)

the expected relative heme off-rates for the two isomers, suggesting that similar structural factors control the heme affinity and HisE7 displacement rates. This microheterogeneity is a possible source of the reported heterogeneous ligand binding[4] and spectral hetero-geneity.[5] Last, while the exact reaction mechanism of CN$^-$ binding to metNgb requires additional studies,[16] the present results strongly suggest a very slow rupture of the Fe–His(E7) bond, which is more consistent with the reported ∼1 s^{-1} rate than the ∼10^4 s^{-1} rate for reduced Ngb.[4,6] More extensive NMR studies in progress are directed toward determining the nature of the differences in either molecular/electronic structure or in dynamic properties in the heme pocket that can account for the different reactivities for the alternate heme orientations. Solution ^{1}H NMR is both appropriate and highly unique in being able to address these questions. Preliminary results indicate that the heme orientation influences both the orientation of the His64(E7) and exchange rates of labile protons.

Supporting Information Available: Seven figures (1D, 2D NMR data for assignments in metNgb and metNgbCN) and two Tables (chemical shifts for metNgb and metNgbCN) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Burmester, T.; Weich, B.; Reinhardt, S.; Hankeln, T. *Nature* **2000**, *407*, 520–523.
(2) Dickerson, R. E.; Geis, I. *Hemoglobin: Structure, Function, Evolution and Pathology*; Benjamin-Cummings: Menlo Park, CA, 1983.
(3) Dewilde, S.; Blaxter, M.; Vanhauwaert, M. L.; Vanfleteren, J.; Esmans, E. L.; Marden, M. C.; Griffon, N.; Moens, L. *J. Biol. Chem.* **1996**, *271*, 19865–19870.
(4) Dewilde, S.; Kiger, L.; Burmester, T.; Hankeln, T.; Baudin-Creuza, V.; Aerts, T.; Marden, M. C.; Caubergs, R.; Moens, L. *J. Biol. Chem.* **2001**, *276*, 38949–38955.
(5) Couture, M.; Burmester, T.; Hankeln, T.; Rousseau, D. L. *J. Biol. Chem.* **2001**, *276*, 36377–36382.
(6) Trent, J. T. I.; Watts, R. A.; Hargrove, M. S. *J. Biol. Chem.* **2001**, *276*, 30106–30110.
(7) Nistor, S. V.; Goovaerts, E.; Van Doorslaer, S.; Dewilde, S.; Moens, L. *Chem. Phys. Lett.* **2002**, *361*, 355–361.
(8) Pesce, A.; Bolognesi, M.; Bocedi, A.; Dewilde, S.; Moens, L.; Hankeln, T.; Burmester, T. *EMBO Rep.* **2002**, *3*, 1146–1151.
(9) Hargrove, M. S.; Brucker, E. A.; Stec, B.; Sarath, G.; Arredondo-Peter, R.; Klucas, R. V.; Olson, J. S.; Phillips, G. N. J. *Structure* **2000**, *8*, 1005–1014.
(10) Trent, J. T. I.; Hvitved, A. N.; Hargrove, M. S. *Biochemistry* **2001**, *40*, 6155–6163.
(11) Lecomte, J. T. J.; Scott, N. L.; Vu, C.; Falzone, C. J. *Biochemistry* **2001**, *40*, 6541–6552.
(12) Wittenberg, J. B.; Bolognesi, M.; Wittenberg, B. A.; Guertin, M. *J. Biol. Chem.* **2002**, *277*, 871–874.
(13) Recombinant mouse neuroglobin was prepared and purified as described in detail previously.[4]
(14) Keller, R.; Groudinsky, O.; Wüthrich, K. *Biochim. Biophys. Acta* **1976**, *427*, 497–511.
(15) La Mar, G. N.; Satterlee, J. D.; de Ropp, J. S. In *The Porphyrins Handbook*; Kadish, K. M., Smith, K. M., Guilard, R., Eds.; Academic Press: San Diego, 1999; Vol. 5, pp 185–298.
(16) Dou, Y.; Olson, J. S.; Wilkinson, A. J.; Ikeda-Saito, M. *Biochemistry* **1996**, *35*, 7107–7113.
(17) Toi, H.; La Mar, G. N.; Margalit, R.; Che, C. M.; Gray, H. B. *J. Am. Chem. Soc.* **1984**, *106*, 6213–6217.
(18) Hargrove, M. S.; Barrick, D.; Olson, J. S. *Biochemistry* **1996**, *35*, 11293–11299; Hargrove, M. S.; Olson, J. S. *Biochemistry* **1996**, *35*, 11310–11318.

JA034584R