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Structural Change of the Heme Pocket Due to Disulfide Bridge Formation Is Significantly Larger for Neuroglobin than for Cytoglobin

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Two new globins, neuroglobin (Ngb) and cytoglobin (Cygb), were recently added to the vertebrate globin family.[1] Cygb is expressed in all tissues studied thus far, whereas Ngb is predominantly expressed in the brain. The ferrous form of both proteins reversibly binds oxygen in competition with the internal E7 histidine ligand.[1,2] In the ferric form of the proteins, the heme iron is hexacoordinated with the distal HisE7 being the internal ligand.[2,3] Recently, mass spectrometry and kinetics studies revealed the formation of an intramolecular disulfide bond in human Ngb (hNgb) between the Cys at positions CD7 and D5.[4] The Ngb sequences available at present display conserved Cys at the positions CD7, D5, and G18/19 with two exceptions: the rodentia Ngb's, which are missing the CD7 Cys, and the zebrafish Ngb, which is missing the G18/19. The histidine binding affinity was found to decrease by a factor of 10 for the ferrous sulfur-bridged hNgb versus the reduced hNgb form, with a related increase in the O_2 affinity.[4] This finding suggests that the dioxygen release of hNgb is controlled by a reduction/oxidation mechanism of the disulfide bridge.[4] The Cys residues in Cygb occur at different positions than in Ngb (at B2 and E9), and they are less conserved within the cytoglobin family. Mass spectrometry and kinetics studies revealed the possibility of intramolecular disulfide bond formation between these two Cys with a similar, albeit smaller, effect on the histidine and O_2 affinity than observed for Ngb.[4] The change in histidine affinity of the ferrous forms of Ngb and Cygb upon disulfide bond formation suggests a significant change of the heme pocket structure. This deformation of the heme pocket should also be reflected in the ferric form of the proteins. Therefore, EPR studies of the ferric proteins were undertaken.

Our earlier studies revealed that the EPR spectra of ferric wild-type (wt) mouse neuroglobin (mNgb) are dominated by a rhombic spectrum ascribed to the low-spin (LS) HisF8−Fe^{3+}−HisE7 form (see Table 1, LS1).[3] Apart from the LS1 form, additional contributions to the EPR spectrum can be recognized (Figures 1 and 2): signals due to nonheme iron (marked with #), a minor impurity low-spin form (LS4: $g_3 = 2.62 \pm 0.02$, $g_2 = 2.36 \pm 0.02$, $g_1 = 1.92 \pm 0.05$) which was already observed by other researchers,[5] a HisF8−Fe^{2+}−NO contribution (marked with $) extensively discussed in our previous work,[3b] and a high-spin (HS) component (see below). Wt mNgb is missing the CD7 Cys and thus cannot form a disulfide bridge. As expected, mutation of the remaining Cys to Ser (mNgbΔcys) does not alter the dominant LS signal (Figures 1 and 2a,b, and Table 1).

The situation changes completely when ferric wt hNgb is investigated (Figure 2c). In this case, dominant contributions of two LS Fe(III) complexes (LS1, 62.5 ± 5%, and LS2, 37.5 ± 5%), with similar EPR parameters, can be observed (see splitting of g_3 signal in Figure 2c; Table 1). The EPR parameters are in agreement with two HisF8−Fe^{3+}−HisE7 forms with slightly altered structure as can be found from comparison with literature data on ferric heme proteins.[6] Upon mutation of the Cys to Ser (hNgbΔcys), the LS2 component disappears completely, suggesting that this can be ascribed to the protein conformation in which the disulfide bridge is present (Figure 2d). This is corroborated by the observation that upon addition of dithiothreitol (DTT) to wt hNgb the LS2 contribution disappears almost completely (Figure 2e). DTT will reduce the disulfide bridges,[4] but cannot reduce the Fe(III) to Fe(II). The remaining LS1 component is nearly identical to the one observed in mNgb, confirming that it is related to the reduced form of the protein (Figure 2a,b,d). Furthermore, it should be noted that the signal corresponding to an HS Fe(III) form of the protein (either the pentacoordinated HisF8−Fe^{3+} or the aquomet form of the protein[3]) is higher in hNgb than in the Ngb proteins that lack the ability to form a disulfide bridge (Figure 2). This suggests that the HS/LS ratio increases upon formation of the disulfide bridge or that, in other words, a larger fraction of the *ferric* protein has the E7His detached from the heme iron in the sulfur-bridged form. This is similar to the observation that *ferrous* sulfur-bridged hNgb has a lower affinity for binding of the E7His than its reduced form, suggesting a similar influence of the sulfur bridge on the ferric

Table 1. EPR Parameters of the Dominant LS Components in Ferric Ngb and Cygb Variants Obtained by Simulations from the CW EPR and Field-Sweep ESE (Electron Spin−Echo) Spectra (see also Supporting Information)

	g_1 (±0.08)	g_2 (±0.02)	g_3 (±0.02)	complex	reference
wt mNgb	1.29	2.15	3.12	LS1	3
mNgbΔcys	1.32	2.16	3.11	LS1	this work
wt hNgb	1.30	2.17	3.10	LS1	this work
	1.05	2.06	3.26	LS2	this work
hNgbΔcys	1.31	2.16	3.10	LS1	this work
wt hCygb	1.20	2.08	3.20	LS3	this work
hCygbΔcys	1.20	2.08	3.20	LS3	this work

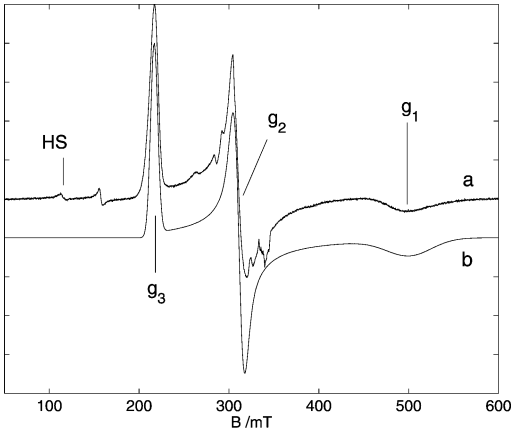

Figure 1. (a) CW EPR spectrum of ferric mNgbΔcys taken at 11 K. HS = high-spin form. (b) Simulation of the low-spin (LS1) form in (a), ascribed to the F8His−Fe(III)−E7His complex.

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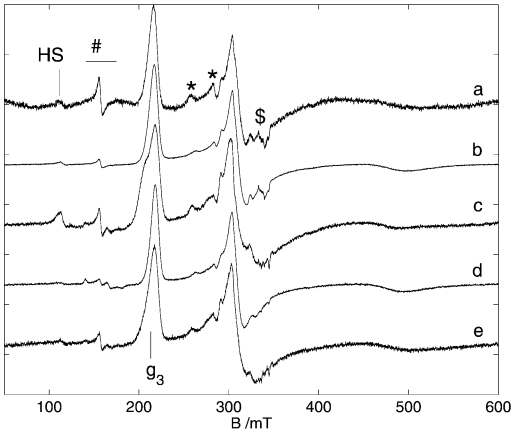

Figure 2. CW EPR spectra taken at 11 K of the ferric forms of (a) wt mNgb, (b) mNgbΔcys, (c) wt hNgb, (d) hNgbΔcys, and (e) hNgb + DTT. # indicates the signals from nonheme iron, * indicates the LS4 component, and $ marks the F8His−Fe^{2+}−NO form.

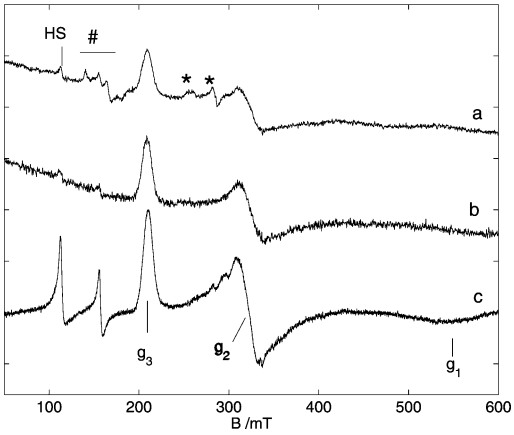

Figure 3. CW EPR spectra taken at 11 K of the ferric forms of (a) hCygbΔcys, (b) hCygbΔcys^{ref}, and (c) wt hCygb. # indicates the signals from nonheme iron, and * indicates the LS4 component.

and ferrous form of the proteins. Since the HS signal intensity is also influenced by the possible presence of free hemin in the sample and since at this stage we cannot oxidize the sulfur bridge in a controlled manner, this conclusion should be considered with care. Note also that the amount of HS is low in both cases (<6%).

Until now, no EPR studies on the ferric state of Cygb proteins have been performed. Native Cygb can be obtained in two ways: in a high yield by refolding the apoprotein isolated from inclusion bodies obtained from *Escherichia coli* expression followed by heme addition (indicated here as hCygb^{ref}) and in a low yield by directly providing *E. coli* with 2.5 mM δ-amino levulinic acid to properly fold and incorporate the heme group (indicated here as hCygb) (see Supporting Information for more details). Figure 3a,b shows the EPR spectra of hCygbΔcys (all Cys mutated to Ser) obtained using the two isolation pathways. Both spectra are dominated by the same LS contribution (LS3), typical of a HisF8−Fe^{3+}−HisE7 coordination (Table 1). A recent Raman and absorption spectroscopy study already predicted the hexacoordination of the heme iron.[2c] Note that the signal in the g_1 region is virtually unresolved due to a combination of a low protein concentration, high g strain, and baseline drift. To determine the g_1 value, field-sweep ESE spectra were recorded (see Supporting Information).

The second low-spin form LS4 observed in hCygbΔcys and also observed in the Ngb proteins disappears in hCygbΔcys^{ref} (Figures 2 and 3a, signals indicated with *). This corroborates the assignment to a minority impurity. In the EPR spectra of refolded mNgb, the LS4 form is also found to disappear (see Supporting Information).

Figure 3c shows the EPR spectrum of ferric wt hCygb. Comparison with the hCygbΔcys indicates that in both cases there is essentially only one dominant LS form present (LS3). This indicates that the change in the heme pocket structure upon disulfide bridge formation is minor compared to what is observed for the wt hNgb. This seems to agree with the fact that the observed change in histidine affinity is smaller for disulfide bridge formation in ferrous hCygb (reduction by factor <2) than in ferrous hNgb (reduction by factor 10).[4] Since the cysteines forming the disulfide bridge are found on different positions in the Ngb and Cygb sequences, a different effect of the disulfide bridge formation on the heme pocket structure is not unexpected. It should be noted that the HS Fe(III) component again decreases when the disulfide bridge is lost (Figure 3), possibly agreeing with the observed affinity changes for the ferrous form of the protein,[4] although other causes may lie at the heart of this signal decrease.

On the basis of the g values, the components LS1, LS2, and LS3 are expected to have a different heme pocket structure. Pulse EPR studies are in progress to unravel the structural differences.

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Supporting Information Available: All EPR spectra and simulations and experimental procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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