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Short communication

Proton-linked subunit heterogeneity in ferrous nitrosylated human adult hemoglobin: an EPR study

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Abstract

The effect of pH on the X-band electron paramagnetic resonance (EPR) spectrum of ferrous nitrosylated human adult tetrameric hemoglobin (HbNO) as well as of ferrous nitrosylated monomeric α- and β-chains has been investigated, at −163 °C. At pH 7.3, the X-band EPR spectrum of tetrameric HbNO and ferrous nitrosylated monomeric α- and β-chains displays a rhombic shape. Lowering the pH from 7.3 to 3.0, tetrameric HbNO and ferrous nitrosylated monomeric α- and β-chains undergo a transition towards a species characterized by a X-band EPR spectrum with a three-line splitting centered at 334 mT. These pH-dependent spectroscopic changes may be taken as indicative of the cleavage, or the severe weakening, of the proximal HisF8-Fe bond. In tetrameric HbNO, the pH-dependent spectroscopic changes depend on the acid–base equilibrium of two apparent ionizing groups with pK_a values of 5.8 and 3.8. By contrast, the pH-dependent spectroscopic changes occurring in ferrous nitrosylated monomeric α- and β-chains depend on the acid–base equilibrium of one apparent ionizing group with pK_a values of 4.8 and 4.7, respectively. The different pK_a values for the proton-linked spectroscopic transition(s) of tetrameric HbNO and ferrous nitrosylated monomeric α- and β-chains suggest that the quaternary assembly drastically affects the strength of the proximal HisF8-Fe bond in both subunits. This probably reflects a 'quaternary effect', i.e., structural changes in both subunits upon tetrameric assembly, which is associated to a relevant variation of functional properties (i.e., proton affinity).

Keywords: Ferrous nitrosylated tetrameric human adult hemoglobin; Ferrous nitrosylated monomeric α- and β-chains; Proton-linked subunit heterogeneity in ferrous nitrosylated human adult tetrameric hemoglobin; EPR spectroscopy

The heme-ligand binding and spectroscopic properties of human adult tetrameric hemoglobin (Hb) are modulated physiologically by heterotropic effectors, such as 2,3-D-glycerate bisphosphate, inorganic phosphate, chloride, carbon dioxide, and protons. A similar effect has been reported for organic phosphates (e.g., inositol hexakisphosphate), dodecylsulfate, and synthetic compounds displaying pharmaceutical activity (e.g., bezafibrate, clofibrate, chlorpromazine, proflavine, salicylate, and trifluoperazine). Heterotropic effectors bind to Hb not only to the so-called '2,3-D-glycerate bisphosphate' pocket (i.e., at the dyad axis, in between the

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β-chains), but also to different multiple functionally-linked interaction sites (see Refs. [1–23]).

In the present study, a detailed investigation on the effect of pH (between 7.3 and 3.0) on the X-band electron paramagnetic resonance (EPR) spectroscopic properties of ferrous nitrosylated human adult tetrameric Hb (HbNO) as well as of ferrous nitrosylated monomeric α- and β-chains, at −163 °C, is reported. NO binding to Hb is of physiological relevance. In fact, ferrous oxygenated Hb facilitates NO scavenging, yielding ferric (or met) Hb and nitrate; ferric Hb is reduced back to the ferrous derivative by met-Hb-reductase (see Ref. [24]). Moreover, NO facilitates O_2 uptake stabilizing the high affinity form (i.e., the R-state) of ferrous Hb by nitrosylation of the Cysβ 93 residues (one for each β-chain). The denitrosylation of the Cysβ 93 residues shifts the Hb tetramer towards the low affinity form (i.e., the T-state) facilitating the O_2 release (see Ref. [25]). The present results indicate that the quaternary assembly in HbNO drastically affects the pK_a values for the protonation of the HisF8 Nε atom and the cleavage, or the severe weakening, of the proximal HisF8-Fe bond with respect to the isolated nitrosylated α- and β-chains.[1]

Ferrous oxygenated tetrameric Hb was prepared and stripped of any cation and anion (e.g., allosteric effectors) bound to the protein as previously reported [2,6,8]. The tetrameric Hb concentration was determined on the basis of $\varepsilon = 1.33 \times 10^5\ M^{-1}\ cm^{-1}$ (at 430 nm); the extinction coefficient ε is expressed on the molar heme basis and refers to ferrous deoxygenated tetrameric Hb [2,6,8].

Ferrous oxygenated *p*-chloromercuribenzoate-reacted monomeric α- and β-chains were prepared and stripped of any cation and anion (e.g., allosteric effectors) bound to the protein as previously reported [2,6,8]. The concentration of monomeric α- and β-chains was determined on the basis of $\varepsilon = 1.11 \times 10^5\ M^{-1}\ cm^{-1}$ (at 431 nm); the extinction coefficient ε is expressed on the molar heme basis and refers to ferrous deoxygenated α- and β-chains [2,6,8].

Tetrameric HbNO as well as ferrous nitrosylated *p*-chloromercuribenzoate-reacted monomeric α- and β-chains have been obtained, under anaerobic conditions, by sequential addition of sodium dithionite (final concentration 15 mg/ml) and potassium nitrite (final concentration 5 mg/ml) to ferrous oxygenated tetrameric Hb and to monomeric α- and β-chain solutions [11,15].

All chemicals (from Merck AG, Darmstadt, Germany, or Sigma–Aldrich, St. Louis, MO, USA) were of analytical grade and were used without further purification.

X-band EPR spectra of tetrameric HbNO and of ferrous nitrosylated *p*-chloromercuribenzoate-reacted monomeric α- and β-chains were collected between pH 3.0 and 7.3 (5.0×10^{-2} M phosphate or acetate buffer), at −163.0 °C. Setting conditions were: 9.42 GHz microwave frequency, 20 mW microwave power, and 0.10 mT modulation amplitude.

The protein samples for X-band EPR experiments were prepared by rapid mixing and rapid freezing 0.30 ml of the tetrameric HbNO or of the ferrous nitrosylated monomeric α- and β-chain solutions with 0.30 ml of the buffer solution (1.0×10^{-1} M) at the appropriate pH or 0.03–0.3 ml of the NaOH solution (5.0 M) (see Refs. [26,27]). Rapid freezing of protein solutions, inside the EPR tube, was carried out by employing cold isopentane (see Refs. [26,27]). The X-band EPR spectra (obtained between pH 3.0 and 7.3) were fully developed within the dead time of the rapid mixing and rapid freezing apparatus (~0.05 s). The dead time of this apparatus was determined by extrapolation at time zero of the dependence of the X-band EPR signal for the reduction of Fe^{3+} to Fe^{2+} ions by quinol on the mixing and freezing time (see Refs. [26,27]).

For the neutral-to-acid pH titration, the tetrameric HbNO or the ferrous nitrosylated monomeric α- and β-chain solution (0.30 ml) at low ionic strength (3.0×10^{-3} M phosphate buffer, pH 7.0) was mixed with the high ionic strength buffer solution (0.30 ml of 1.0×10^{-1} M phosphate buffer between pH 3.0 and 4.0 and between pH 5.5 and 7.5, or 0.30 ml of 1.0×10^{-1} M acetate buffer between pH 4.0 and 6.0). Therefore, X-band EPR spectra have been obtained at the same ionic strength. The pH value of the final ferrous nitrosylated heme-protein solutions, obtained by mixing the high ionic strength buffer solution (1.0×10^{-1} M phosphate or acetate buffer, pH range 3.0–7.5) with the ferrous nitrosylated heme-protein solution (3.0×10^{-3} M phosphate buffer, pH 7.0), was determined at 20.0 °C, after recording the X-band EPR spectrum at −163.0 °C. Only if the pH value of the final ferrous nitrosylated heme-protein solution at 20.0 °C corresponded to that of the initial heme-protein-free high ionic strength buffer solution at 20.0 °C (± 0.1 pH unit), the X-band EPR spectrum was analyzed (see Refs. [28,29]).

For the acid-to-neutral pH back titration, aliquots (0.03–0.3 ml) of a 5.0 M degassed NaOH solution were injected into the ferrous nitrosylated heme-protein sample (0.6 ml), which had previously been lowered to pH 3.0 and characterized by X-band EPR spectroscopy. Then, the X-band EPR spectrum of the alkalinized ferrous nitrosylated heme-protein solution was recorded at −163.0 °C. Furthermore, the pH value of the alkalinized ferrous nitrosylated heme-protein solution was determined at 20.0 °C (see Refs. [28,29]).

Under all the experimental conditions, no significant ion effects were observed for buffers overlapping in pH values.

[1] Amino acid residues have been identified by their three-letter code and their topological position within the classical globin fold [5].

The molar fraction of the five-coordinate form of tetrameric HbNO and of ferrous nitrosylated monomeric α- and β-chains (i.e., Y) was determined by subtracting from the experimentally observed X-band EPR spectrum obtained between pH 3.0 and 7.3 a given percentage of the experimentally observed X-band EPR spectrum of the six-coordinated species obtained at pH 7.0 or 7.3. X-band EPR spectra deconvolution has been performed by using the MatLab (MathWorks Inc., Natick, MA, USA) software.

Around neutrality, the X-band EPR spectrum of tetrameric HbNO and of ferrous nitrosylated monomeric α- and β-chains displays a rhombic shape (Fig. 1 and Table 1). The X-band EPR spectrum of HbNO has been associated to the high affinity six-coordinate form (i.e., the R-state) of the ligated tetramer (see Refs. [1,3–6,8–17,30]).

Lowering the pH from 7.3 to 3.0, tetrameric HbNO and ferrous nitrosylated monomeric α- and β-chains undergo a transition towards a species characterized by a X-band EPR spectrum with a three-line splitting ($A_3 = 1.65$ mT) centered at 334 mT (Fig. 1 and Table 1). Such a behavior is reminiscent to that of HbNO in the presence of allosteric effectors and drugs, around neutrality, which has been attributed to the shift of the conformational equilibrium toward the low affinity form (i.e., the T-state) of the ligated tetramer (see Refs. [1,3–6,8–17]). The pH- and allosteric effector-dependent spectroscopic changes occurring in tetrameric HbNO and in ferrous nitrosylated monomeric α- and β-chains (Fig. 1 and Table 1) are similar to those reported for monomeric, dimeric and tetrameric ferrous nitrosylated heme-proteins (see Refs. [12,28,29,31–40]) and may reflect the cleavage, or the severe weakening, of the proximal HisF8-Fe bond (see Refs. [1,3–6,8–17,41,42]).

Fig. 2 shows the pH dependence of the molar fraction of the low affinity five-coordinate form of tetrameric HbNO (i.e., the T-state) and of ferrous nitrosylated monomeric α- and β-chains (i.e., *Y*). Data reported in Fig. 2 (panel a) have been analyzed according to Eq. (1) [43,44]:

$$Y = \{[H^+]/K_{a1} + 2 \times [H^+]^2/(K_{a1} \times K_{a2})\}/2 \times \{1 + [H^+]/K_{a1} + [H^+]^2/(K_{a1} \times K_{a2})\}, \quad (1)$$

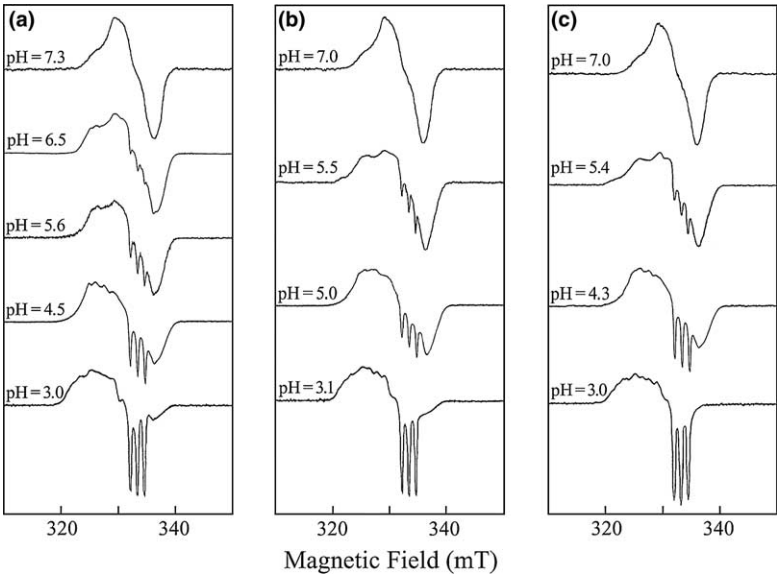

Fig. 1. Effect of pH on the X-band EPR spectra of tetrameric HbNO (panel a) and of ferrous nitrosylated monomeric α- and β-chains (panel b and c, respectively). The concentration of tetrameric HbNO and of ferrous nitrosylated monomeric α- and β-chains was 1.0×10^{-3} M. For details, see text.

Table 1
Effect of pH on the X-band EPR parameters of tetrameric HbNO and ferrous nitrosylated monomeric α- and β-chains

Heme-protein	pH	A_3 (mT)	g_1	g_2	g_3
Tetrameric HbNO	7.3 (R-state)	u.s.	2.060	1.986	2.006
	3.0 (T-state)	1.640	2.095	2.055	2.009
Ferrous nitrosylated monomeric α-chains	7.0	u.s.	2.063	1.983	2.007
	3.1	1.660	2.100	2.060	2.010
Ferrous nitrosylated monomeric β-chains	7.0	u.s.	2.058	1.985	2.006
	3.0	1.650	2.099	2.058	2.010

u.s., unresolved signal.

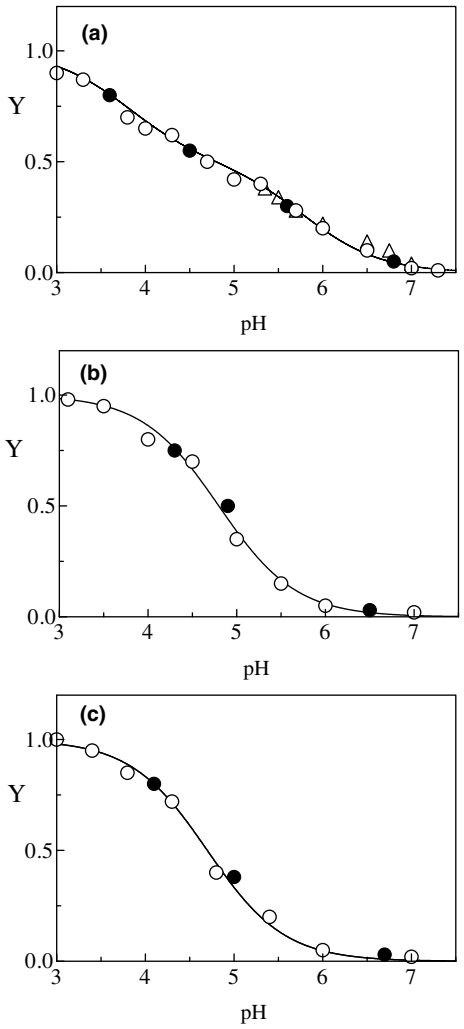

Fig. 2. Effect of pH on the X-band EPR spectra of tetrameric HbNO (panel a) and of ferrous nitrosylated monomeric α- and β-chains (panel b and c, respectively). Open and filled symbols indicate neutral-to-acid and acid-to-neutral pH-jump experiments, respectively. Triangles indicate data obtained from [12]. The continuous line in panel A was calculated according to Eq. (1) with $pK_{a1} = 5.8$ and $pK_{a2} = 3.8$ (Table 2). The continuous lines in panels b and c were calculated according to Eq. (2) with $pK_a = 4.8$ and 4.7, respectively (Table 2). For details, see text.

where K_{a1} and K_{a2} are the equilibrium dissociation constants for the reversible H^+ binding to the two classes of binding sites present in tetrameric HbNO (i.e., ferrous nitrosylated monomeric α- and β-chains in the nitrosylated tetramer). According to Eq. (1), the amplitude of the pH-dependent spectroscopic changes of the two classes of H^+ binding sites present in tetrameric HbNO is identical within the experimental error over the whole magnetic field range explored. Data reported in Fig. 2 (panels b and c) have been analyzed according to Eq. (2) [43,44]:

$$Y = 1/\{1 + K_a/[H^+]\}, \quad (2)$$

where K_a is the equilibrium dissociation constant for the reversible H^+ binding site to the a single class of binding sites per monomer (i.e., ferrous nitrosylated monomeric α- or β-chain). Eqs. (1) and (2) have been used to generate the continuous lines shown in Fig. 2; the agreement with the experimental data is fully satisfactory (Fig. 2), giving us confidence that the correct assumptions were made underlying Eqs. (1) and (2).

In ferrous nitrosylated monomeric α- and β-chains, the spectroscopic transition reflecting the cleavage, or the severe weakening, of the proximal HisF8-Fe bond (Figs. 1 and 2) can be described by a single protonating group, characterized by a pK_a value of 4.7 and 4.8, respectively (Table 2). By contrast, in tetrameric HbNO the pH-dependent spectroscopic transition shown in Figs. 1 and 2 requires two protonating groups, with $pK_{a1} = 5.8$ and $pK_{a2} = 3.8$ (Table 2). Therefore, tetrameric HbNO displays first a spectroscopic transition with $pK_{a1} = 5.8$, already reported previously (see Ref. [12]), which is referable to conformational changes involving among others the cleavage, or the severe weakening, of the proximal HisF8-Fe bond of α-chains (see Refs. [12,42]). At lower pH, this process is followed by a second spectroscopic transition with $pK_{a2} = 3.8$, which may reflect the cleavage, or the severe weakening, of the proximal HisF8-Fe bond of β-chains. These data indicate that in tetrameric HbNO the proximal HisF8-Fe bond energy of α- and β-chains, and thus the structural arrangement of the proximal side of the heme pocket, is markedly different from that of ferrous nitrosylated monomeric α- and β-chains. Although no crystal structures that can provide the structural bases for pK_a differences between the ferrous nitrosylated monomeric α- and β-chains and the tetramer are available, these results probably reflect a 'quaternary effect', i.e., structural changes in both subunits upon tetrameric assembly, which is associated to a relevant variation of functional properties (i.e., proton affinity). In particular, we are referring to possible alterations of the stereochemistry of the proximal side of the heme pocket as a consequence of the involvement of the G and H α-helices of one chain with the C and D α-helices of the partner chain. Thus, this portion is structurally altered upon quaternary conformational changes in tetrameric Hb [5,9,10,12,19] and it is likely to undergo a change in the structural arrangement between the tetrameric assembly (in Hb) and the monomeric species (in α and β monomeric subunits), thus affecting the stereochemistry (and the energy) of the proximal HisF8-Fe bond.

Table 2
pK_a values of the HisF8 residue in tetrameric HbNO and in the ferrous nitrosylated monomeric α- and β-chains

	Tetrameric Hb	Monomeric chain
Ferrous nitrosylated α-chains	$pK_{a1} = 5.8$	$pK_a = 4.8$
Ferrous nitrosylated β-chains	$pK_{a2} = 3.8$	$pK_a = 4.7$

In conclusion, data here reported reflect the consequence of a 'quaternary effect' linked to the assembly of the different α- and β-subunits. It underlies that upon assembly, even in the high-affinity R-quaternary-state, subunits undergo a structural change, which affects to a large extent the heme pocket, leading to a different stereochemistry between the heme and the protein moiety. This event is not completely unexpected in Hb, in view of the different functional behavior between the R-state tetramer and the isolated chains, such as for the Bohr effect, which is observed, though to a limited extent, in the tetramer but not in the isolated subunits [45].

Notation

EPR	electron paramagnetic resonance
Hb	human adult hemoglobin
HbNO	ferrous nitrosylated Hb

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