Electron paramagnetic resonance properties of liver fluke (*Dicrocoelium dendriticum*) nitrosyl hemoglobin

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The electron paramagnetic resonance properties of the nitric oxide derivative of liver fluke (*Dicrocoelium dendriticum*) hemoglobin (DD-Hb) have been investigated in the pH range from 4.8 to 7.8. In the neutral and alkaline regions the spectra have a rhombic shape, with $g_x = 2.09$, $g_y = 1.99$ and $g_z = 2.009$, and a triplet hyperfine structure of 2.2 mT, due to the nitrogen of the bound NO molecule, in the center resonance. No superhyperfine lines in the g_z region, related to the interaction of the iron with the proximal histidine, are detected, suggesting a large distance between the metal and the N_ϵ of the imidazole. By lowering the pH the EPR spectrum undergoes a reversible change showing a 3-line pattern in the high-field region. Such a spectrum is fully formed at pH 4.8 and is interpreted in terms of a dissociation of the proximal histidine from the heme iron.

EPR *Hemoglobin* Dicrocoelium dendriticum *Liver fluke* *Distal histidine*

1. INTRODUCTION

The EPR properties of nitrosyl hemoglobins are strongly affected by the iron ligand *trans* to the NO molecule [1]. Therefore, they provide valuable information on the interactions between the metal and the proximal histidine. Differences in the heme environment between the α and the β subunits in the deoxyhemoglobin tetramer have been elucidated by this method [1].

Two types of EPR spectra are usually observed for nitrosyl hemoglobins. The first type is characterized by a 9-line superhyperfine pattern in the g_z region of the spectrum. This results from the concomitant contributions to the resonance from the N_ϵ atom of the proximal histidine and the nitrogen of the NO molecule. Thus, variations in the length of the N_ϵ–Fe bond will be reflected in variations in the resolution of the superhyperfine structure of the spectrum [2]. The second type of EPR line pattern observed for Hb-NO is characterized by a triplet in the high-field region and the total absence of superhyperfine structure in the g_z region. This type of spectrum has been attributed to the NO–heme complex in which the iron is pentacoordinated [3]. A transition from the first to the second type of spectrum has been observed in nitrosyl human adult hemoglobin as well as in some mutants [2]. This is induced by pH changes and addition of allosteric effectors, such as ATP, 2,3-diphosphoglycerate and inositol hexaphosphate, and has thus been correlated to the T-to-R transition. However, an analogous EPR spectral change has recently been shown to occur also in *Aplysia* myoglobin [4], demonstrating that this type of phenomenon must not necessarily be

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ascribed to quaternary structure conformational equilibria.

In *Dicrocoelium dendriticum* hemoglobin (DD-Hb) the replacement of the distal histidine by a glycine is accompanied by very high affinity for oxygen [5]. The protein also displays an acid Bohr effect [6,7]. The kinetics of ligand binding to DD-Hb are characterized by very fast and pH-independent association rates. In contrast, progressive lowering of the ligand dissociation velocities is observed if the pH is brought below 7 [8]. An analysis of the EPR properties of nitrosyl DD-Hb seemed most pertinent in order to gain more insights into the molecular basis of the functional behavior of this protein.

2. MATERIALS AND METHODS

Liver fluke hemoglobin was purified as in [8]. The NO derivative was obtained by addition of sodium nitrite and ascorbic acid to the protein solution, under anaerobic conditions [2]. X-band EPR spectra were recorded at 110 K on a Varian E-9 instrument. The pH of the solutions was determined at 20°C.

3. RESULTS AND DISCUSSION

The EPR spectra of nitrosyl-DD-Hb as a function of pH are reported in fig.1. The high pH form has a rhombic shape, $g_x = 2.09$, $g_y = 1.99$, $g_z = 2.009$, but lacks the characteristic triplets of the triplet pattern in the g_z region of the spectrum. The existence of superhyperfine splitting in the g_z EPR signal of the ferrous NO derivative of several hemoproteins [9,10] has been taken as evidence for the presence of a nitrogenous ligand coordinated *trans* to the heme-bound NO. In fact it arises from the electron-spin nuclear-spin interaction of the unpaired electron of the bound NO with the N_ϵ atom of the proximal histidine. For the absence of superhyperfine splitting in the g_z region in the EPR spectra of a number of NO-heme derivatives [9] several explanations are given, such as a very short electron spin relaxation or the absence of a nuclear spin possessing ligand bound *trans* to the NO molecule. Another possible explanation, given for the NO derivative of the mutant hemoglobins Zurich and Iwate [2], is a weak bonding interaction between the *trans* nitrogen atom and the heme

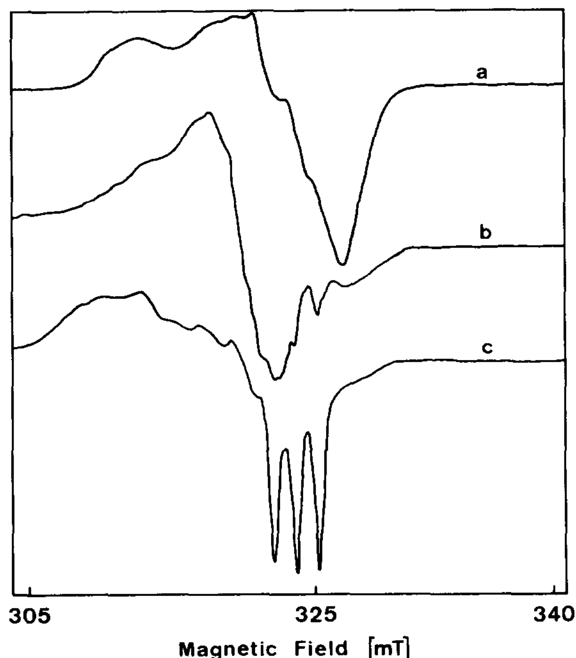

Fig.1. EPR spectra of nitrosyl DD-Hb. (a) 0.3 mM Hb in 0.1 M Bistris-phosphate buffer (pH 7.8), (b) 0.3 mM Hb in 0.1 M citrate buffer (pH 5.2), (c) 0.4 mM Hb in 0.1 M citrate buffer (pH 4.8). Setting conditions: 9.13 GHz microwave frequency; 20 mW microwave power and 0.2 mT modulation amplitude.

iron, with a concomitant strengthening of the NO-iron bond. This latter hypothesis could fit the present case as DD-Hb is known to have the proximal histidine [11,12]. Moreover, shorter iron-NO distance should cause the unpaired electron of NO to behave more as a d-electron, with an increased spin orbit coupling, thus explaining the large shift of the $g_z = 2.009$ from the spin-only value (2.0023), greater than that usually found (2.005) in the nitrosyl derivatives of several heme-proteins [2,13].

Lowering the pH produces a reversible transition towards a species characterized by an EPR spectrum with 3-line splitting in the high-field region. Such a pattern, which is taken as diagnostic for a pentacoordinated nitrosyl-heme complex [3], is completely formed at pH 4.8 (fig.1c). Its EPR parameters are reported in table 1 together with those of the high pH species. A similar type of transition has previously been reported for the nitrosyl derivative of normal human adult

Table 1

EPR parameters of NO DD-Hb

pH	g_x	g_y	g_z	A_x NO	A_y NO	A_z NO
7.8	2.09	1.99	2.009	–	2.2 mT	–
4.8	2.1	2.06	2.01	–	1.8 mT	1.64 mT

hemoglobin and in some variants [2]. In this case the species with 3-line high-field hyperfine splitting has been associated by some authors [14,15] to a low reactivity state. In fact, on lowering the pH, parallel to the appearance of the 3-line splitting of the EPR spectrum, an increase in the ligand dissociation rates is observed both in adult and fetal human hemoglobin. In DD-Hb the trend is opposite, i.e. the ligand dissociation rates decrease by lowering the pH, the association rates being unaffected [8]. In the nitrosyl derivative of *Aplysia* myoglobin the 3-line hyperfine spectrum is completely formed only at very low pH and is associated with an increase of the ligand binding rates [16]. In conclusion, in different proteins, similar EPR spectral transitions seem to be associated with different ligand binding properties, thus indicating that information on the local structure around the heme is not sufficient to explain the functional behavior of the macromolecule. However, the results on DD-Hb support the idea that changes at the proximal side of the heme may play a very important role in the regulation of the ligand dissociation kinetics.

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