ELECTRON SPIN RESONANCE SPECTRA OF FELINE NO-HEMOGLOBINS

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1. Introduction

Organic phosphate and pH-dependent hyperfine structures (hfs) at $g = 2.009$ have recently been reported for the ESR spectrum of human adult nitric oxide hemoglobin (HHbA) [1–3]. The hfs consists primarily of the development of a three line spectrum (triplet) with spacing and intensity indicating the localization of the unpaired electron on the nitrogen nucleus of nitric oxide. Since both protons and organic phosphates are known to interact with deoxy-hemoglobin at sites that are far removed from the heme-ligand binding site [4, 5], the development of hfs in HHbA-NO has been interpreted as resulting from an altered spatial environment of the heme-bound NO caused by conformational transitions in the protein [1]. The intensity of the induced hyperfine triplet is maximal under conditions that lower the oxygen affinity of the protein, such as low pH and/or saturating concentrations of phosphate effectors. Hence, it is possible that hfs may be characateristic of a low affinity conformation and may be observed in a hemoglobin with a low oxygen affinity even in the absence of organic phosphates or at neutrality.

The major hemoglobin components of the domestic cat (CHbA) and (CHbB), provide a system that permits examination of the relationships between oxygen affinity, hfs intensity and protein structure. Both cat hemoglobins exhibit lower intrinsic oxygen affinities than other mammalian hemoglobins [7]. Furthermore, since CHbA is functionally regulated by organic phosphates while CHbB is not, it is of interest to see what influence phosphate effectors might have on the ESR spectra of the NO derivatives of these proteins. Hence, X-band and Q-band ESR spectra of CHbA-NO and CHbB-NO have been obtained and are compared with the corresponding spectra of HHbA-NO.

2. Materials and methods

Cat blood was obtained and hemoglobin components prepared as described previously [8]. Human blood was obtained from the Milwaukee Blood Center, Inc. Hemolysates were prepared and hemoglobin concentrations determined by conventional procedures. Hemoglobin solutions were 'stripped' free of phosphates [9], concentrated to about 10% by ultrafiltration (Amicon, PM-10 membrane), and used within one week of drawing.

NO-Hb was prepared from deoxy-Hb by flushing anaerobically with NO (Matheson Gas Products) which was passed over a column (1 × 20 cm) of solid NaOH. The NO-Hb solutions were then transferred to a nitrogen-filled glove box where they were frozen in liquid nitrogen for ESR measurements.

All ESR spectra were obtained with a Varian E-9 spectrometer equipped with an X-band bridge, X-band dual cavity, X-band liquid nitrogen finger dewar, Q-band bridge and Q-band low temperature accessory.

3. Results

The first and second derivative X-band ESR spectra of CHbA-NO and CHbB-NO are compared with that of HHbA-NO in fig. 1. The second derivative display of the NO-hemoglobin spectra makes it easier to compare the nine shoulders in the $g = 2.02$ to $g = 1.99$ regions. At neutral pH and in the absence of effectors, the spectra of the NO derivatives of both cat hemoglobins are very similar to the spectrum of HHbA-NO. The line shape and g-values previously characterized by Kon [2] and later by Rein et al. [1] and Trittelvitz et al. [3] for HHbA-NO are superimposable with those of the cat hemoglobins.

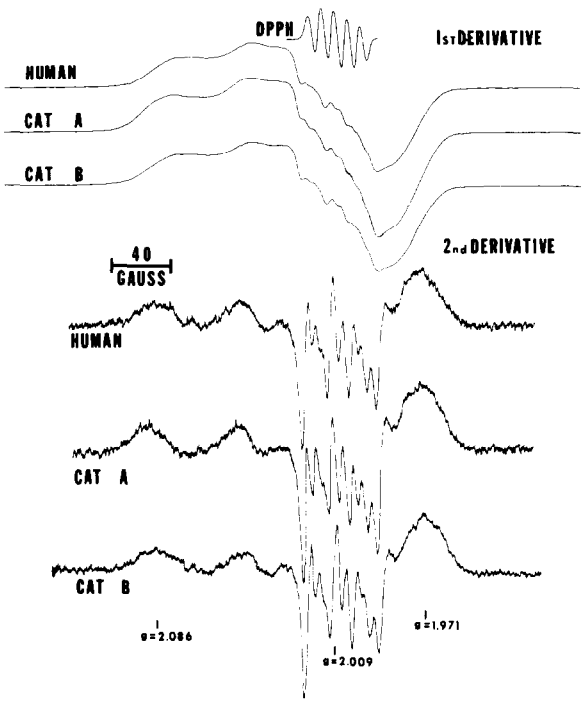

Fig. 1. First and second derivative, X-band ESR spectra of HHb-NO, CHbA-NO, and CHbB-NO at pH 7, 0.05 M bis-Tris, and 0.4 mM as heme.

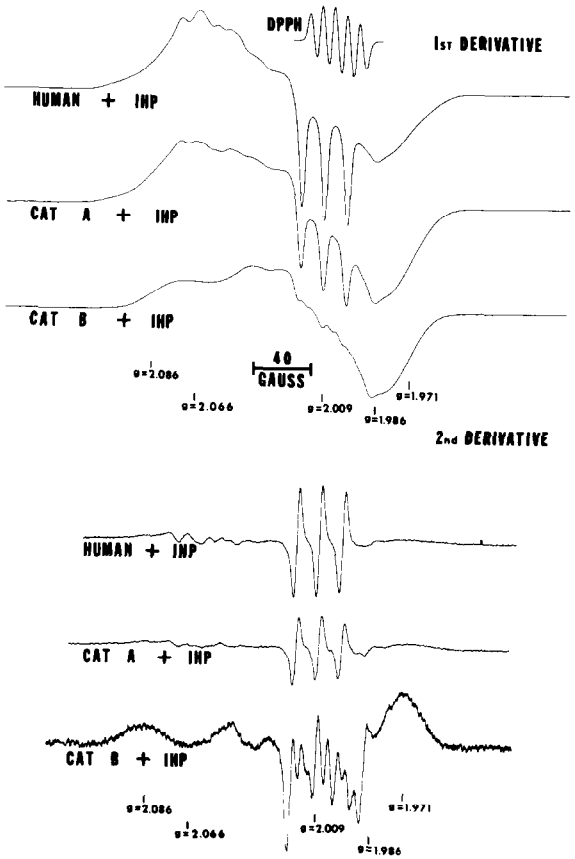

Fig. 2. First and second derivative, X-band ESR spectra of HHb-NO, CHbA-NO, and CHbB-NO with conditions as in fig. 1 except for addition of a saturating concentration of IHP.

Addition of inositol hexaphosphate (IHP) in saturating concentrations of HHbA-NO and CHbA-NO causes changes in the spectra consisting of the appearance of triplet hyperfine lines at $g = 2.009$, and increased spectra intensity at $g = 2.06$ (fig. 2). In contrast, addition of IHP to CHbB-NO caused no change in the spectrum. The spectrum for HHbA-NO + IHP is identical to that previously reported [1], and although the spectrum for CHbA-NO is similar, the magnitude of the hyperfine structure is less. In addition, the line shape in the $g = 2.06$ region is not identical. Second derivative-displays allowed more precise peak-to-peak measurements of the triplet hyperfine structures as shown in the same figure.

Fig. 3 shows the dependence of hfs on IHP concentration for HHbA-NO, CHbA-NO and CHbB-NO. As indicated above, the maximum hfs observed for CHbA-NO is considerably less than that observed for human hemoglobin. There was no evidence for an increase in hfs for CHb-NO with addition of IHP.

To determine whether the difference in ESR spectra of CHbA-NO and HHbA-NO in the presence of excess IHP is due to i) differences in spatial geometry of the NO as indicated by a shift in g-value or ii) differences in amounts of two or more conformational states as indicated by the magnitude of peak intensity [1, 3, 10], NO-Hb spectra were resolved at Q-band frequency (fig. 4). In the absence of IHP, the spectra of HHbA-NO and CHbA-NO resemble those reported by Yonetani for NO-myoglobin and other heme proteins [11]. The g values for $g_y = 1.986$, $g_x = 2.070$, $g_? = 2.034$ and $g_z = 2.007$ are clearly seen. Changes in spectra of the NO-hemoglobins induced by IHP are also more clearly resolved at this frequency, because the lines no longer overlap extensively. The

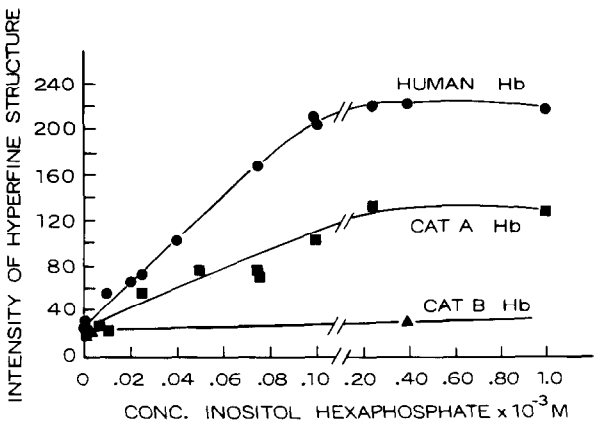

Fig. 3. Dependence of intensity of hfs from second derivative ESR spectra of HHb-NO, CHbA-NO, and CHbB-NO on the concentration of IHP.

resolution and separation of $g_y = 1.991$, $g_x = 2.065$, $g_? = 2.037$ and $g_z = 2.011$ indicate that there are no differences in g values between the two hemoglobins. Only the magnitude of relative peak intensities differ.

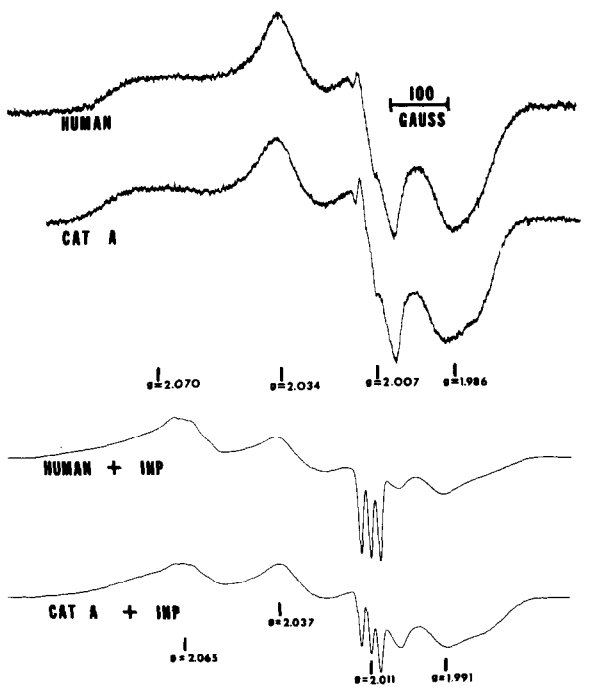

Fig. 4. First derivative Q-band ESR spectra of HHb-NO and CHbA-NO at pH 7, 0.05 M bis-Tris, and approximately 2.8 mM as heme without IHP and with a saturating concentration of IHP.

Associated with the increased triplet intensity at $g = 2.011$ in HHbA-NO, additional structure that is probably due to another principal hyperfine value for nitrogen as reported by Trittelvitz et al. [3] can be observed at $g = 2.065$.

4. Discussion

The similarity of the ESR spectra of HHbA-NO, CHbA-NO, and CHbB-NO indicates that the heme iron environment in each is the same. Since the titration of HHbA-NO [12] and the UV difference spectrum of HHbA-NO vs. deoxy-Hb [13] indicate that the conformation of HHbA-NO is very similar if not identical to that of HHbA-O_2, it is reasonable to conclude that the heme iron environment of the oxygenated form of each hemoglobin is also identical. From this conclusion, it seems clear that the low oxygen affinity of cat hemoglobin does not result from any structural peculiarity of the heme pocket in the liganded state.

The fact that no greater intensity of hfs is observed in ESR spectra of CHbA-NO and CHbB-NO than in the spectrum of HHbA-NO shows that hfs is not directly related to low oxygen affinity. Hence the suggestion that hfs results from a conformational change triggered by IHP binding seems to be an adequate explanation [1, 3, 10]. In other words, there is no absolute correlation between hfs intensity and P_{50} (partial pressure of O_2 for half saturation).

One major site of phosphate binding to deoxyhemoglobin is the β-chain amino terminal residue. The amino group of this residue is acetylated in CHbB [8], and as a result organic phosphates have no effect on the oxygen binding properties of this hemoglobin [7]. The failure of IHP to increase hfs in the ESR spectrum of CHbB-NO indicates that the phosphate binding site of Hb-NO shares certain features with that of deoxy-Hb. However, the conformations of all liganded hemoglobins are presumably very similar to each other and distinct from that of unliganded hemoglobin. Furthermore, liganded hemoglobins are supposed to bind organic phosphates weakly if at all. The development of hfs nevertheless indicates distinct interactions between Hb-NO and organic phosphates that may be correlated with conformational transitions between low and high ligand affinity states of the protein.

There does not appear to be a simple obvious explan-

ation for the observation that the maximal hfs of CHbA-NO is about 60% of the maximal hfs of HHbA-NO. Since the P_{50} of HHbA and CHbA are identical in the presence of excess IHP [7] it had originally been expected that their hfs intensities would also be the same. One possible explanation for the difference in maximal hfs intensity is that IHP binding to CHbA-NO is weaker than IHP binding to HHbA-NO. While direct binding measurements are not available, examination of fig. 4 indicates that this is probably not true since the concentrations of IHP necessary for half-maximal hfs intensity for both HHbA-NO and CHbA-NO are the same. It is unlikely that instability of CHbA-NO caused the differences in hfs intensity since its ESR and optical spectra were identical to those of HHbA-NO. However, at pH below neutrality CHbA-NO readily oxidizes so that the effect of pH on hfs could not be examined. A more involved explanation is suggested by the observations of Banerjee and Henry on the ESR spectra of NO-Hb valence hybrids which demonstrate that HHbA-NO hfs arises exclusively from the α-chain NO [10]. Since organic phosphates bind to β-chain residues, hfs must be generated through conformational changes originating at the β-chain phosphate binding site that are transmitted through the β-chain and α-chain to the α-chain heme. Therefore, any difference in primary structure that affects these transitions may also affect the hfs intensity.

Acknowledgements

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