STRUCTURAL CHARACTERISTICS OF NITROSYL HEMOGLOBINS AND THEIR RELATION TO ESR SPECTRA

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1. Introduction

Nitric oxide forms stable paramagnetic complexes with hemoglobin which can be studied using electron spin resonance spectroscopy (ESR). The ESR spectra of nitrosyl hemoglobins (NO–Hb) are sensitive to the conformational states of the complex and the *R* and *T* quaternary states can be identified by their characteristic spectra [1–3]. Binding of allosteric effectors such as inositol hexaphosphate (IHP) or 2,3-diphosphoglyceric acid (DPG) converts NO–Hb from the *R* to the *T* quaternary state [3,4]. Thus, the rhombic ESR spectrum with weak hyperfine splitting in the g_z region represents the *R* state while 3 strong hyperfine lines in the g_z region indicates the *T* quaternary state. In the *T* state the bond between the proximal histidine and iron has been shown ruptured in the α-chains of NO–Hb [3,5,6]. Since conformational characteristics of NO–Hb are reflected in the ESR spectra we have studied a number of hemoglobins having modified heme environments and have found that in the *T* state, changes in the g_x region of the ESR spectrum can be unambiguously identified with the changes in the α-58 residue. In addition using spin labels attached to the β-93 sulfhydryl groups it is shown that the tertiary conformation of the β-chains in NO–Hb in the *T* state is not the same as that of the β-chains in deoxyhemoglobin, but rather the teritary conformation appears to be more similar to that of the β-chains in oxyhemoglobin which has an *R* quaternary structure.

2. Materials and methods

Blood samples were collected in heparinized tubes. Hemoglobins M Milwaukee and M Saskatoon were isolated from blood samples of two different individuals heterozygous for these M hemoglobins by using Bio-Rex 70 [7]. Hemoglobins of oppossum (*Didelphius marsupialis*), New Zealand White rabbits and Sprague-Dawley rats were isolated directly from their respective blood samples. All hemoglobins were stripped of DPG and other organic phosphates by the method in [8]. Nitric oxide derivatives were prepared as in [9]. For spin label studies, a nitroxide derivative of iodoacetamide (3-(2-iodoacetamide 2,2,5,5, tetramethyl-l-pyrrolidinyl oxyl) (Syva Inc.) was used as in [10]. Following reaction of the spin label with hemoglobin and the excess reagent was removed by gel filtration. The sample was then reacted with NO for 2 min in an ice bath and ESR measurements taken immediately. All ESR measurements were carried out using a Varian E-4 spectrometer. The NO–Hb spectra were measured at 77 K while the spin label spectra were recorded at room temperature.

3. Results and discussion

Figure 1 shows the ESR spectra of nitrosyl complexes of hemoglobins A, M Saskatoon, M Wilwaukee, opossum, rabbit and rat in the presence of IHP at pH 6.8. The strong hyperfine splitting

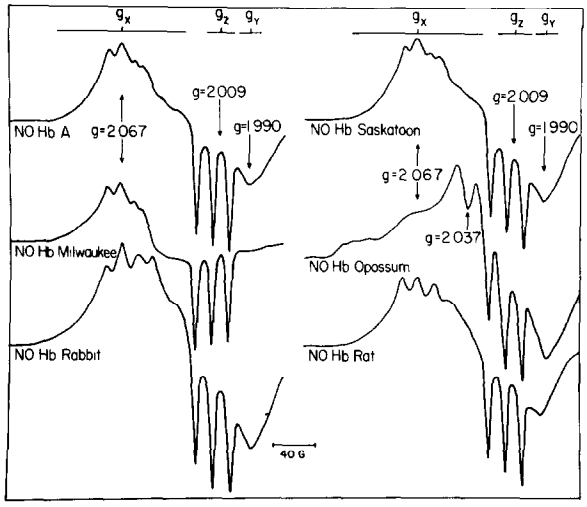

Fig.1. ESR spectra of nitrosyl hemoglobins in 0.1 M bis–Tris (pH 6.8) in the presence of IHP. Heme was 1.3–1.5 mM and the IHP: hemoglobin tetramer molar ratio was 1:1. Spectra were measured at 77 K using 10 mW microwave power; 9.16 GHz microwave frequency; 2.0 G modulation amplitude.

centered at $g = 2.009$ in the g_z region is indicative of the *T* quaternary structure in each of the NO–Hb samples (fig.1). Notice the trough in the g_y region ($g = 1.990$) is absent in the NO–Hb M Milwaukee ESR spectrum. This is due to two factors:

(i) The β-chains of M Milwaukee are not reduced by NO remaining in the high spin ferric form
(ii) Most of the α-hemes in the presence of IHP become pentacoordinate.

Beyond this, the most profound differences in the 6 ESR spectra are found in the g_x region (fig.1). The modifications in the α and β heme environments of these hemoglobins and their ESR spectral characteristics are summarized in table 1. Nitrosyl hemoglobin A, M Milwaukee and M Saskatoon have virtually identical spectral characteristics in the g_x region. This spectral region is modified in rabbit and rat NO–Hb while in opossum NO–Hb the spectral characteristics seen in the g_x region of NO–Hb A are absent. In [11] NO–Hb M Boston was shown to have an ESR spectrum very similar to that shown in fig.1. for opossum NO–Hb. Since hemoglobins M Boston

Table 1
Important amino acid differences and ESR characteristics in nitrosyl hemoglobins under investigation

Species	Structural modifications	ESR spectral characteristics in comparison to NO–Hb A + IHP (pH 6.8)
NO–M Saskatoon + IHP	β-63[E7]His → Tyr: β-distal histidine absent, but β-chain in ferrous form	Normal
NO–M Milwaukee + IHP	β-67[E11]Val → Glu: β-chain in the ferric form	g_y trough absent
NO–Opossum[a] ± IHP	α-58[E7]His → Gln: α-distal histidine absent, but α-chains in ferrous form	g_x region modified Small hyperfine at $g = 2.037$
NO–M Boston ± IHP [11]	α-58[E7]His → Tyr: α-distal histidine absent, but α-chains in ferrous form	g_x region modified Spectrum similar to that of opossum
NO–Rabbit[a] + IHP	α-48[CD6]Leu → Phe: may affect the position of distal histidine	g_x region modified

[a] Opossum and rabbit hemoglobins differ at many residue positions when compared to Hb A

Only modifications which affect the distal side of the heme are listed in this table

and A differ only at position α-58 it is concluded that the differences between the ESR spectrum of opossum NO–Hb and that of NO–Hb A arise solely from the substitution of glutamine for the distal histidine at position α-58 in opossum hemoglobin. From the fact that NO–Hb M Saskatoon has an identical ESR spectrum with NO–Hb A it can be concluded that the β-63 distal histidine does not contribute to the g_x region in the *T* state. This is further illustrated by the fact that in NO–Hb M Milwaukee where the β-chains do not bind NO, the g_x region is also identical with that of NO–Hb A. The g_x region appears to be sensitive to any substitution which affects the α-58 distal histidine. In rabbit hemoglobin the substitution at α-48 is thought to affect the position of the α-distal histidine [12–14]. Thus, the small changes in the g_x region of ESR spectrum of rabbit NO–Hb (fig.1) arise due to this alteration which in turn affects the α-distal histidine. The rat NO–Hb ESR spectrum also shows small changes in the g_x region. While the structural basis for these changes are not understood, it is considered likely that they arise from the α-distal side.

These findings further strengthen the postulate that the distal histidine takes part in an interaction with the sixth ligand [5,12,15]. Studies with hemoglobins M Boston [11], opossum [16] and chironomous [17] have shown that these hemoglobins are in the *T* quaternary state upon NO ligation in the absence of organic phosphates at pH < 7.0. While M Boston and opossum hemoglobins lack α-distal histidine residues, in chironomous hemoglobin the E helix and distal histidine are directed away from the ligand binding site [18,19]. It seems clear that for the stabilization of the *R* state in nitrosyl hemoglobins an interaction between the α-distal histidine and liganded NO is necessary and in its absence the proximal histidine–iron bonds are ruptured upon NO ligation triggering a transition to *T* state. Furthermore, upon IHP binding to NO–Hb A (*T* state) an interaction between the α-distal histidine and the NO ligand still exists as reflected by the g_x region of the ESR spectrum (fig.1). In opossum and M Boston NO–Hb this interaction is absent as indicated by the absence of absorption in the g_x region. The ESR spectrum of these two hemoglobins around $g = 2.067$ resembles that of the nitrosyl derivative of isolated β-chains from hemoglobin A [20]. In rabbit and rat NO–Hb the interaction between the α-distal histidine and NO ligand is present in the *T* state but it is somewhat different from that observed with NO–Hb A based on the g_x regions of the ESR spectra.

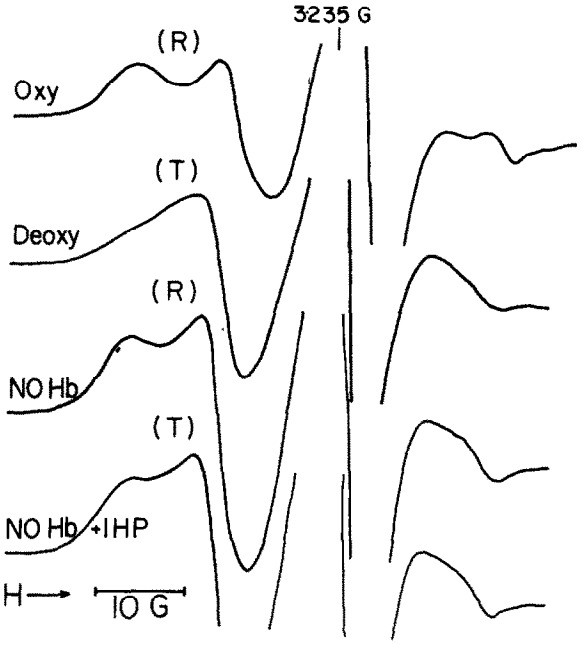

Fig.2. ESR spectra of spin-labeled oxy and deoxy Hb A in the absence of IHP and spin-labeled nitrosyl Hb A in the presence and absence of IHP. All samples were in 0.1 M bis–Tris (pH 6.8). Spectra were measured at room temperature using: 10 mW microwave power 9.16 GHz microwave frequency; 5.0 G modulation amplitude.

Among the ferrous low spin derivates of hemoglobin only nitrosyl hemoglobins undergo an *R–T* transition upon IHP binding. However, the tertiary conformation of this liganded *T* form has not been investigated to date. Spin labels attached to the β-93 sulfhydryl groups indicate a weakly immobilized ESR spectra in both the *R* and *T* states of NO–Hb A (fig.2). Thus the tertiary conformation of β-chains in a liganded *T* structure (NO–Hb + IHP) resembles more closely that of β-chains in a liganded *R* structure (oxy) rather than that of β-chains in an unliganded *T* structure (deoxy).

In summary, it has been shown that the g_x region of the ESR spectra of nitrosyl hemoglobins in the *T* state is directly related to the interaction between the α-58 distal histidine and the liganded NO molecules.

Such an interaction is necessary to stabilize the R quaternary structure in nitrosyl hemoglobins. The β-distal histidine has no effect on the g_x region of the ESR spectrum or on the maintenance of R quaternary structure in nitrosyl hemoglobins. Furthermore, it is seen that the tertiary conformation of the β-chains in nitrosyl hemoglobin in the T state is more like that in oxyhemoglobin than in deoxyhemoglobin.

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