Altered Heme Environments in Opossum and Rabbit Methemoglobins

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Z. Naturforsch. **36 c**, 964–967 (1981); received May 12, 1981

Methemoglobin, ESR, Heme Environment, Inositol Hexaphosphate, Spin State

The effect of pH and inositol hexaphosphate (IHP) on the symmetry of the heme environments in opossum (*Didelphius marsupialis*) and New Zealand White rabbit hemoglobins has been studied using electron spin resonance (ESR). Each methemoglobin is found to contain two different heme environments as detected by the rhombicity observed in the ESR spectrum. In both cases the rhombic nature of the ESR spectrum is influenced by pH and IHP binding, although in the case of rabbit methemoglobin the high spin ESR signal disappears above pH 8.0. In both hemoglobins, amino acid alterations in the α-chains are known to affect the properties of the ferrous derivatives. It is concluded that these alterations also provide the basis for the ESR spectral properties observed with the methemoglobins.

Introduction

In the ferric state of normal hemoglobins both α and β heme environments have symmetrical (tetragonal) arrangements [1–3]. Thus, the ferric hemoglobins in the frozen state can be characterized by their high spin absorption maxima at $g = 6.0$ using electron spin resonance (ESR) spectroscopy. A change in the geometry of the hemes (α or β) as in the case of heme pocket mutations will be reflected in the high spin ESR signal ($g = 6.0$) as a splitting (rhombicity) or broadening of this signal [1–3]. Certain animal hemoglobins have amino acid substitutions in their heme pockets which could result in an altered heme environment. In this study, we have used ESR techniques to study the rhombicity of the heme plane in two animal hemoglobins, opossum and rabbit. Opossum hemoglobin differs from hemoglobin A by 83 amino acid residues. These include substitutions in the α-heme pockets, the most important of which is the replacement of the α-distal histidine by glutamine [4]. Rabbit hemoglobin also has extensive alterations in the α-heme environment when compared to hemoglobin A, including (B 10 α29) Leu → Val; (CD 6 α48) Leu → Phe and (CD 7 α49) Ser → Thr [5].

Materials and Methods

Blood samples from opossum (*Didelphius marsupialis*) and rabbit (New Zealand White) were obtained by cardiac puncture. Hemoglobin M Milwaukee was obtained from a patient heterozygous for the M hemoglobin. Cells were washed three times in cold 0.9% saline and lysed with cold water. Hemoglobin M Milwaukee was isolated by ion exchange chromatography (Bio-Rex 70; 200–400 mesh, Bio-Rad Lab). Hemoglobins were stripped of organic phosphates by the method of Berman, *et al.* [6]. Methemoglobin samples were prepared by addition of $K_3Fe(CN)_6$. Inositol hexaphosphate (Sigma Chemicals) was dissolved in 0.1 M bis-Tris pH 7.0 and titrated to the pH of the protein solution. Concentrations of all samples were measured at 419 nm following reduction with $Na_2S_2O_4$ and bubbling with CO ($\varepsilon = 194\ mM^{-1}\ cm^{-1}$) [7]. ESR studies of the heme environments were carried out at liquid nitrogen temperature using a Varian E-4 spectrometer with a microwave power of 50 mW, a microwave frequency of around 9.15 GHz and modulation amplitude of 12.5 G.

Results and Discussion

Fig. 1. shows the effect of pH and inositol hexaphosphate (IHP) on the high spin ESR spectrum of opossum methemoglobin. As the pH is increased from 6.0 to 9.5 it is seen that a splitting of g-value occurs ($g = 6.0$ and $g = 6.12$). Addition of IHP to samples a neutral or alkaline pH also results in the

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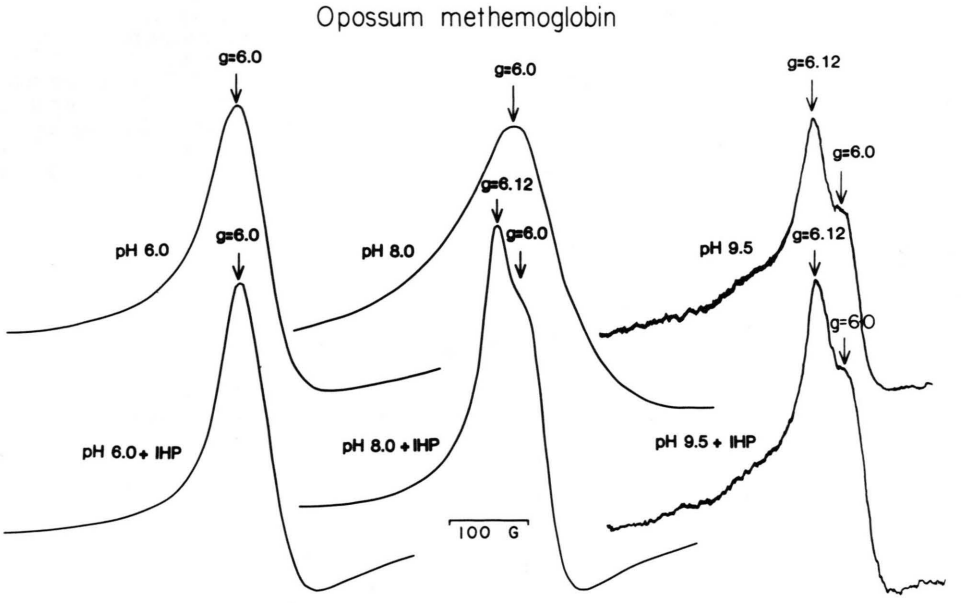

Fig. 1. Effect of variation in pH and inositol hexaphosphate binding on the high spin ESR spectrum of opossum methemoglobin at 77 °K. Heme concentration was 1.6 mM. The molar ratio of inositol hexaphosphate to hemoglobin tetramer was 10:1. Buffers used were 0.1 M citrate, pH 6.0 and 0.1 M glycine/NaOH, pH 8.0 and pH 9.5.

development of rhombicity of the heme plane as detected by a doublet in the ESR spectrum (Fig. 1). In a previous study, it has also been observed that this splitting is temperature dependent [8]. The ESR signals in high spin ferric hemoglobins arise from the transitions of electron within the sub-levels of the lowest energy level of the ferric heme [3]. These unpaired electrons are sensitive to only tetragonal and rhombic symmetry elements in their environment and the ESR absorption is spread between $g = 6.0$ and $g = 2.0$ due to the large value of the tetragonal field [2, 3]. Hence departure from tetragonal symmetry as indicated by splitting of the $g = 6.0$ signal indicates a change in the heme ligand environment in methemoglobins.

Methemoglobin opossum and methemoglobin A differ in both magnetic and optical properties. In opossum methemoglobin, the pH-dependent transition from the aquomet form to the hydroxymet form is inhibited leading to the retention of high spin at alkaline pH [9]. Also, in opossum methemoglobin, the IHP-dependent spectra arise primarily from its α-chains [9]. As with normal methemoglobin A, the ESR spectrum of opossum methemoglobin at pH 6.0 is nearly symmetrical (Fig. 1). However as a function of increasing pH the line shape and the positions in the magnetic field of the ESR absorption band changes. Above pH 9.0 the low field absorption is split into $g = 6.12$ and $g = 6.0$ (Fig. 1). At or above pH 7.5, IHP produces similar splitting of the $g = 6.0$ signal. Rabbit methemoglobin also shows development of rhombicity ($g = 6.0$ and $g = 6.1$) upon addition of IHP at alkaline pH (Fig. 2). However, unlike opossum methemoglobin, rabbit methemoglobin does not retain enough high spin character to show a $g = 6.0$ signal at pH 9.5. In opossum methemoglobin, the role of IHP seems to be only to enhance the spin state of the hydroxymet forms thereby sharpening the broad ESR spectrum (Fig. 1).

In opossum methemoglobin it is likely that a water molecule takes part in a linkage between an amino acid and the heme iron in one of the subunit types. Such a mechanism can explain the rhombicity as well as the magnetic and spectral characteristics of opossum methemoglobin. The critical substitution of (E7 α58) His → Gln [4] makes the α-chains of opossum methemoglobin the most likely source of heme symmetry alterations. However, it is unlikely that in opossum methemoglobin an amino acid is directly bonded to the heme at the sixth coordination position.

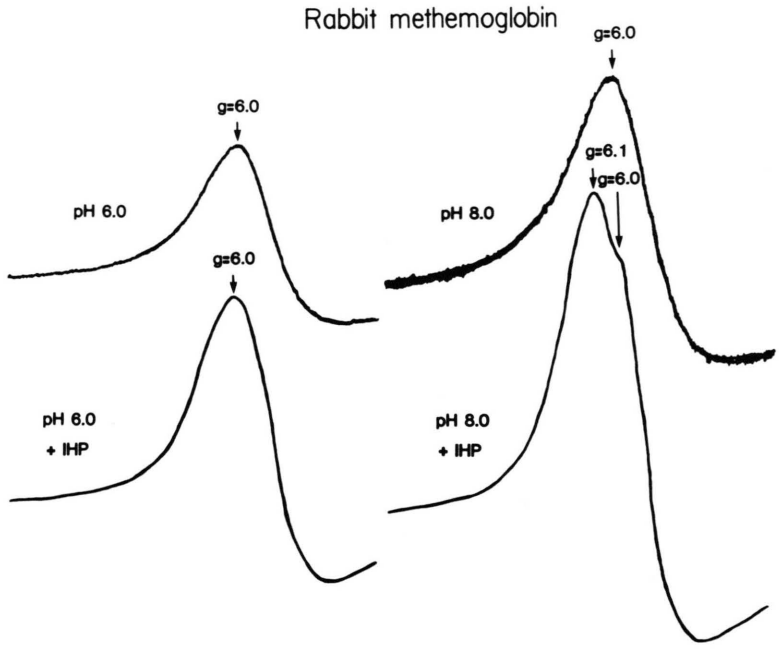

Fig. 2. Effect of variation in pH and inositol hexaphosphate binding on the high spin ESR spectrum of rabbit methemoglobin at 77 °K. Heme concentration was 1.8 mM. The molar ratio of inositol hexaphosphate to hemoglobin tetramer was 10:1. Buffers used were 0.1 M citrate, pH 6.0 and 0.1 M glycine/NaOH, pH 8.0.

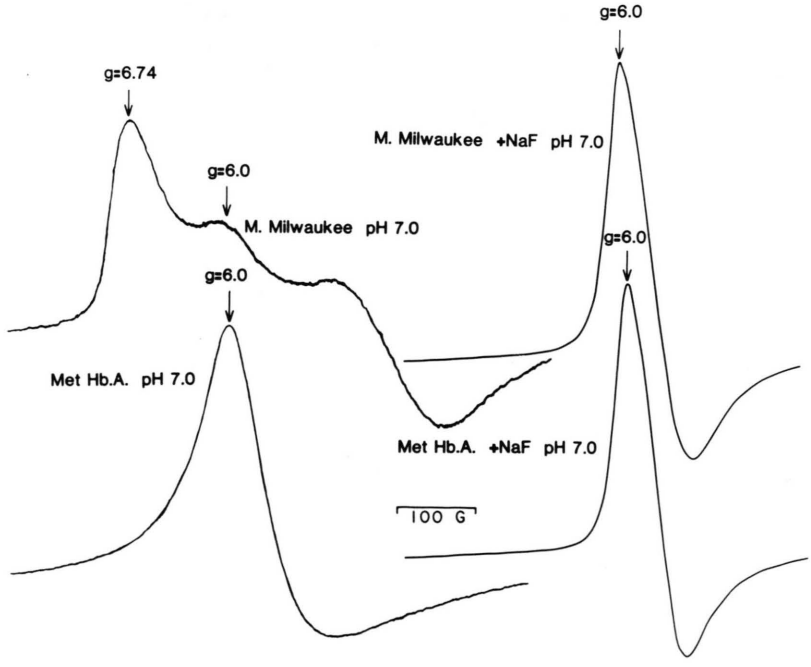

Fig. 3. Disappearance of rhombicity in the high spin ESR spectrum of hemoglobin M Milwaukee as a result of fluoride binding. Heme concentration was 1.6 mM and spectra measured at 77 °K. Buffer used was 0.1 M bis-Tris, pH 7.0.

Cytochrome c is another heme protein that is known to show rhombicity in the ESR spectrum as a function of pH [3]. In this instance a decrease of pH from 4.0 results in rhombicity. In cytochrome c it is assumed that the axial ligands (imidazole and sulfur) dissociate from the iron and are probably replaced by the oxygen atom of a water molecule [3]. In rabbit hemoglobin the distal histidine is not replaced. However, it is known that the substitutions in the α-heme pocket result in altered ligand binding properties [5, 10]. In rabbit methemoglobin the rhombicity becomes apparent only after addition of IHP (Fig. 2). Thus, it is possible that the development of rhombicity in rabbit methemoglobin is due to IHP mediated changes in its heme stereochemistry.

Since rhombicity of the heme plane appears to be due to constraints on the heme symmetry, it is possible to remove the rhombicity by removing the constraints. This is demonstrated with the human mutant M Milwaukee ($\alpha_2\beta_2{}^{67\,\mathrm{Val}\rightarrow\mathrm{Glu}}$) in Fig. 3. In this case the departure from the tetragonal heme symmetry is caused by the bond which exists between β67 glutamic acid and the β heme iron. When the bond is broken and an external ligand such as fluoride binds at the sixth coordination postition the ESR absorbance at $g = 6.7$ disappears and normal fluromet spectrum ($g = 6.0$) develops (Fig. 3). Similarly addition of fluoride to opossum and rabbit methemoglobins also abolishes the rhombicity in their ESR spectra suggesting that the water molecule is now replaced by fluoride ion as the sixth ligand (data not shown).

In summary, it is shown that two types of heme environments exist in opossum and rabbit methemoglobins. The rhombicity that appears in the ESR spectrum is pH dependent and amino acid substitutions on the distal side of the α-heme pockets of both opossum and rabbit hemoglobins could be responsible for this observation. There is no evidence of a direct bond between the heme iron and an amino acid as in the M-type hemoglobins and in the case of these animal hemoglobins a water molecule may form a linkage between the ferric heme iron and an amino acid.

Acknowledgement

This work was supported by Research Grant I-624 from The Robert A. Welch Foundation.

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