THE INFLUENCE OF QUATERNARY STRUCTURE ON THE EPR SPECTRA OF FERRIC HAEMOGLOBIN

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1. Introduction

Haem–haem interaction is known to arise from the reversible transition between the high affinity form (*R*-state) and the low affinity form (*T*-state) [1,2]. If the free energy of cooperation is localized at the haem, the switch of the quaternary structure is expected to alter the electronic state of the haem iron. The influence of the quaternary structure on the electronic state of the haem is most pronounced in nitrosyl haemoglobin and cobalt-substituted haemoglobin [3–9]. In the present study, we have examined the electron paramagnetic resonance (EPR) spectra of ferric haemoglobin and valency hybrid haemoglobins in which either the α- or β-subunit is in the ferric form [10]. Since valency hybrids show highly cooperative oxygen equilibrium curves under certain conditions, these hybrids can take up two alternative quaternary structures, namely the *T*- and *R*-states, depending on whether or not the ferrous subunit is oxygenated [11]. In the present study, an attempt was made to observe the influence of quaternary structure on the EPR spectra of valency hybrid haemoglobins at liquid helium temperature where small changes in the EPR spectra can be detected.

2. Materials and methods

Human oxyhaemoglobin was prepared without using any organic solvent. The isolated α- and β-chains were prepared in the carbon-monoxy form as in [12]. Aquomet hybrid haemoglobins were prepared as in [11] except that DE-52 (Whatman) column chromatography was used instead of isoelectric focusing [11]. Methaemoglobin was obtained by addition of ferricyanide (1.05 equiv./haem) to oxyhaemoglobin at 37°C. All haemoglobin solutions were stripped of phosphate by passage through a column of mixed-bed resin. Deoxygenation of valency hybrid haemoglobin was achieved by repeated evacuation and flushing with nitrogen under gentle shaking. EPR spectra were recorded at 4.2 K using a dual finger dewar with a JEOL ME-2X spectrometer.

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3. Results and discussion

Figure 1 (A) and (B) show the EPR spectra of oxygenated and deoxygenated forms of $\alpha_2^+\beta_2$ and $\alpha_2\beta_2^+$ measured at liquid helium temperature (4.2 K) ($^+$ denotes ferric form). The EPR spectra of deoxygenated hybrids are different from those of oxygenated hybrids. Reoxygenation of the hybrids restored the EPR spectra of the oxygenated hybrids implying that the changes in the EPR spectra observed here are reversible. Both $\alpha_2^+\beta_2$ and $\alpha_2\beta_2^+$ exhibited highly cooperative oxygen equilibrium curves in the presence of IHP at pH 7.4 [11]. Hill's co-efficient n was found to be 1.44 and 1.75 for $\alpha_2^+\beta_2$ and $\alpha_2\beta_2^+$, respectively. Under this condition, the quaternary structure of the hybrid must change from the *T*- to the *R*-state when the ferrous subunit is oxygenated. Therefore, $\alpha_2^+\beta_2^{deoxy}$ and $\alpha_2^{deoxy}\beta_2^+$ are found to be in the *T*-state and $\alpha_2^+\beta_2^{oxy}$ and $\alpha_2^{oxy}\beta_2^+$ are in the *R*-state.

On going from oxy (R-state) to deoxy (T-state), broadening of the signal around g 6 was observed for both hybrids and the decrease in the intensity of the low spin signals was observed for $\alpha_2\beta_2^+$. Figure 1 (C) shows the EPR spectra of aquomet haemoglobin measured in the presence and absence of IHP. Addition of IHP to aquomet haemoglobin produced the

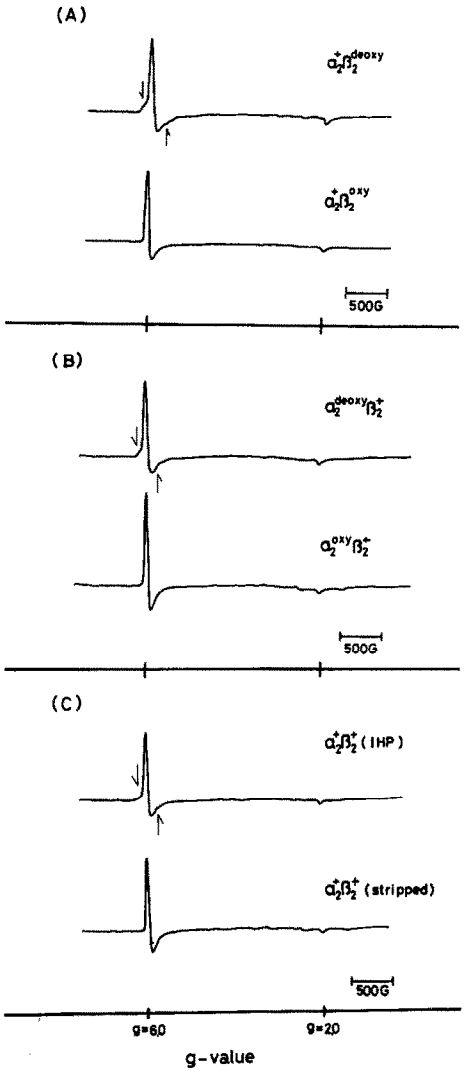

Fig.1. The EPR spectra of valency hybrids and methaemoglobin measured at 4.2 K. (A) $\alpha_2^+\beta_2^{deoxy}$ and $\alpha_2^+\beta_2^{oxy}$ in 0.05 M bis–Tris 0.1 M Cl^- 2 mM IHP, pH 7.4; (B) $\alpha_2^{deoxy}\beta_2^+$ and $\alpha_2^{oxy}\beta_2^+$. Experimental conditions are as in (A); (C) $\alpha_2^+\beta_2^+$ measured in the presence and absence of 2 mM IHP in 0.05 M bis–Tris 0.1 M Cl^-, pH 7.4.

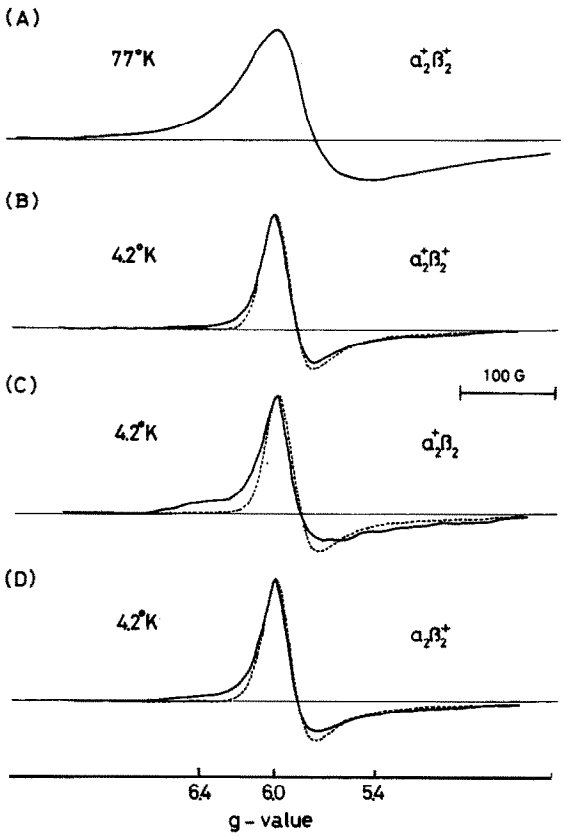

Fig.2. Low field portion of the EPR spectra in the R-state (- - -) and the T-state (——). (A) $\alpha_2^+\beta_2^+$ measured at 77 K; (B) $\alpha_2^+\beta_2^+$ measured at 4.2 K in the presence (——) and absence (- - -) of 2 mM IHP; (C) $\alpha_2^+\beta_2^{deoxy}$ (——) and $\alpha_2^+\beta_2^{oxy}$ (- - -) at 4.2 K; (D) $\alpha_2^{deoxy}\beta_2^+$ (——) and $\alpha_2^{oxy}\beta_2^+$ (- - -) at 4.2 K. Experimental conditions are as in fig.1. Note that the small changes in the EPR spectra accompanying the $R-T$ transition cannot be detected at 77 K.

broad EPR signal similar to those of $\alpha_2^+\beta_2^{deoxy}$ and $\alpha_2^{deoxy}\beta_2^+$ which are known to be in the T-state [13,14].

Figure 2 shows the low field portion of the EPR spectra of aquomet haemoglobin and aquomet hybrids. Figure 2 also includes the EPR spectrum of aquomet haemoglobin measured at 77 K. The signal is much broader at 77 K and thus the small changes in the EPR spectra accompanying the $R-T$ transition could not be observed [10]. The EPR spectra in the R-state (dotted line) do not show significant in-plane g anisotropy whilst those of the T-state (full line) are composed of at least two paramagnetic species: one

has symmetry similar to that of the R-state and the other shows large in-plane g anisotropy ($g_x \neq g_y$). Such rhombic distortion of the EPR spectrum is a manifestation of the constraint imposed on the haem. We also performed the experiment at pH 6.0 where the T-state is even more stabilized and the fraction of the rhombic signal would be increased. However, the fraction of the rhombic signal was unexpectedly decreased by lowering to pH 6.0. At pH 7.4, methaemoglobin takes up 4 different states:

(i) Aquomet (high spin);
(ii) Aquomet (low spin);
(iii) Hydroxymet (high spin);
(iv) Hydroxymet (low spin).

Among these 4 states, only two high spin states can exhibit an EPR signal at g 6. It might be considered that the EPR signal with large g anisotropy might derive from high spin hydroxymet form. If the R–T transition causes merely a shift in the equilibrium between the aquomet and hydroxymet forms without a primary change in the electronic state of the haem iron, high spin hydroxymet form must be responsible for the rhombic EPR signal observed for the valency hybrid in the T-state at pH 7.4. If this is the case, the broad rhombic EPR signal should become dominant at high pH. However, the shape of the EPR signal of methaemoglobin at g 6 remained unchanged with raising the pH in the absence of IHP. Therefore, it can be concluded that the change in the EPR spectra observed on the R–T transition is due to the primary change in the electronic state of the haem iron. It may be that the haem pocket may undergo a structural change between pH 6.0 and 7.4 and the difference in the electronic state of the haem iron between the R- and T-states becomes distinct only around pH 7.4. The haem-linked water molecule is hydrogen-bonded to distal histidine (E7) and may play a role in this structural change.

We have also examined the EPR spectra of cyanomet, azidemet and fluoromet hybrid haemoglobins but failed to observe any change in the EPR spectra accompanying the R–T transition. Haemoglobin M Hyde Park, which is a naturally occurring valency hybrid, undergoes a change in the EPR spectrum when the normal α-subunit is deoxygenated [15]. In contrast, the EPR spectra of other haemoglobin Ms were reported to remain unchanged when the normal subunits are deoxygenated [15]. However, our reinvestigation at 4.2 K over a wider range of conditions revealed the changes in the EPR spectra under some conditions (H. H. and K. N., unpublished results).

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