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Low-lying electronic states of the ferrous high-spin ($S=2$) heme in deoxy-Mb and deoxy-Hb studied by highly-sensitive multi-frequency EPR

Hiroshi Hori [a,*], Haruhiko Yashiro [a,b], Kenta Ninomiya [a], Masaki Horitani [c], Takanori Kida [a], Masayuki Hagiwara [a]

[a] Center for Quantum Science and Technology under Extreme Conditions (KYOKUGEN), Osaka University, Toyonaka, Osaka 560-8531, Japan
[b] PRESTO, Japan Science and Technology Agency, Japan
[c] RIKEN SPring-8 Center, Harima Institute, Sayo-cho, Hyogo 679-5148, Japan

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ABSTRACT

The low-lying electronic states of the ferrous high-spin heme in deoxy-myoglobin (deoxy-Mb) and deoxy-hemoglobin (deoxy-Hb) were probed by multi-frequency electron paramagnetic resonance (MFEPR) spectroscopy. An unexpected broad EPR signal was measured at the zero magnetic field using cavity resonators at 34–122 GHz that could not be simulated using any parameter sets for the $S=2$ spin Hamiltonian assuming spin quintet states in the $^5B_2$ ground state. Furthermore, we have observed novel, broad EPR signals measured at 70–220 GHz and 1.5 K using a single pass transmission probe. These signals are attributed to the ferrous high-spin heme in deoxy-Mb and deoxy-Hb. The resonant peaks shifted to a higher magnetic field with increasing frequency. The energy level separation between the ground singlet and the first excited state at the zero magnetic field was directly estimated to be 3.5 cm^{-1} for deoxy-Hb. For deoxy-Mb, the first two excited singlet states are separated by 3.3 cm^{-1} and 6.5 cm^{-1}, respectively, from the ground state. The energy gap at the zero magnetic field is directly derived from our MFEPR for deoxy-Mb and deoxy-Hb and strongly supports the theoretical analyses based on the Mössbauer and magnetic circular dichroism experiments.

1. Introduction

Iron ions are ubiquitous in biological systems. One class of iron-containing proteins known as hemoproteins contains the iron-porphyrin moiety (heme) as a prosthetic group in the active center. Among the many hemoproteins, myoglobin (Mb) and hemoglobin (Hb) are among the best understood both in terms of their atomic structures and their biological functions. The spin state of the deoxy-heme in Mb or Hb is a high-spin ferrous species with the spin quantum number $S=2$ [1]. The ferrous form of the deoxy-heme in these hemoproteins binds molecular oxygen (O_2) reversibly. Upon binding of triplet O_2 ($S=1$) to this site, the spin state of the ferrous heme changes to a low-spin diamagnetic species ($S=0$) and is magnetically inactive [2]. The nature of reversible oxygen binding to the heme with spin inversion has attracted biophysical researchers' interest for many years. This understanding requires elucidation of the electronic structures of the ferrous heme in Mb and Hb. However, despite extensive experimental and theoretical efforts, the electronic states of the ferrous high-spin heme in these hemoproteins have not been well understood. The readers who want to know general paramagnetic properties and electronic structures of the heme in hemoproteins in more detail are referred to a review article written by Kotani [3]. Electron paramagnetic resonance (EPR) spectroscopy has been used to observe the electronic states of metal centers with a half-integer spin state. However, for paramagnetic metal centers with integer spin, a large zero-field splitting (ZFS) will often render them EPR-inactive or EPR-silent under conventional EPR conditions. Thus, EPR might not be considered appropriate for study of ferrous high-spin ($S=2$) hemoproteins. However, other techniques, such as magnetic susceptibility have been used and we will briefly survey previously reported work on the electronic structures of the ferrous high-spin hemoproteins.

Pauling and Coryell reported the static magnetic susceptibility of deoxy-Hb at room temperature [1]. The effective number of the Bohr magneton was 5.4, which was somewhat larger than the spin-only value of 4.9. The increase from 4.9 to 5.4 is due to the incomplete quenching of the orbital magnetic moment. Assuming that the spin quintet $^5B_2$ state describes the ground state and the energy separation between the ground $^5B_2$ state and the nearest excited quintet ^5E state is much larger than the spin-orbit interaction and the Zeeman energies, an effective spin Hamiltonian could be used to describe the ferrous high-spin system. The conventional spin Hamiltonian for the $S=2$ spin state is then given by

$$H = \mu_B B \cdot \tilde{g} \cdot S + D\left(S_z^2 - 2\right) + E\left(S_x^2 - S_y^2\right) \quad (1)$$

* Corresponding author. Tel.: +81 6 6850 6687; fax: +81 6 6850 6662.
E-mail address: hori@mag.cqst.osaka-u.ac.jp (H. Hori).

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where μ_B is the electron Bohr magneton; $\boldsymbol{B}$ is the external magnetic field; $\tilde{g}$ is the g tensor for the Fe^{2+} spin system; $\boldsymbol{S}$ is the spin operator for $S=2$; and D and E are the axial and rhombic zero-field splitting parameters, respectively [4]. Using the spin Hamiltonian formalism given by Eq. 1, Nakano et al. analyzed their magnetic susceptibility data for deoxy-Mb and deoxy-Hb measured at cryogenic temperatures [5,6]. They proposed spin Hamiltonian parameter values of $D=5.3\ cm^{-1}$ and $E=0.9\ cm^{-1}$, assuming $g_z=2.0$ for deoxy-Hb [6]. If these parameters are applied to the low-lying electronic states of the ferrous high-spin heme, an allowed EPR transition between $M_S=-1$ and $M_S=0$ levels is predicted near the level crossing at high magnetic fields. We attempted to measure EPR using a K-band (24 GHz) EPR spectrometer equipped with a superconducting (SC) magnet in magnetic fields up to 6 T, but detected no signal from deoxy-Mb (Hori, unpublished results). In 1980, Champion and Sievers observed very broad absorptions at $3.5\ cm^{-1}$ from deoxy-Mb and deoxy-Hb using far-infrared magnetic resonance spectroscopy [7]. However, they failed to detect any additional signals up to $13\ cm^{-1}$. These results contradict the spin Hamiltonian parameters calculated by Nakano et al., which predict the two lowest exited levels at $3.05\ cm^{-1}$ and $8.45\ cm^{-1}$ above the ground state at the zero applied magnetic field. In the late 1980s, Hendrich and Debrunner observed a broad EPR absorption from deoxy-Mb near the zero magnetic field at X-band (9 GHz) for both parallel and perpendicular oscillating modes [8,9]. They quantitatively analyzed the low-lying electronic states of the ferrous high-spin heme in deoxy-Mb assuming the spin quintet $^5B_2$ ground state discussed above. This broad EPR absorption with very low signal intensity was assigned to the transition between the closely spaced ($\sim 0.3\ cm^{-1}$) non-Kramers doublet, $M_S=\pm 2$ energy levels ($\Delta M_S=4$). Although the ZFS parameters, $D=4.85\ cm^{-1}$ and $E=0.90\ cm^{-1}$, were compatible with those of the magnetic susceptibility measurements [6], the far-infrared spectroscopy measurements for deoxy-Mb could not be consistently explained [7].

Early Mössbauer measurements of deoxy-Mb and deoxy-Hb were similarly analyzed for a spin quintet $^5B_2$ ground state [10]. In the 1970s, extensive Mössbauer measurements and theoretical calculations were carried out on deoxy-Mb and deoxy-Hb to elucidate the low-lying electronic states of the ferrous high-spin heme [11-18]. The temperature dependent quadrupole splitting, ΔE_Q, of the ferrous high-spin heme in deoxy-Mb suggested the presence of an excited state nearby. The Mössbauer results further suggested that the low-lying ^5E, ^3E, and $^1A_1$ states were mixed with the $^5B_2$ ground state owing to the low symmetry of the crystalline field and the spin-orbit interaction. Eicher et al. concluded that the ground state in the ground multiplets must be one of the components of ^5E state ($^5E_\eta$) and that the energy separation between the ground singlet and the first excited state is only $\sim 2\ cm^{-1}$ for both deoxy-Mb and deoxy-Hb [15].

Seno et al. gave a theoretical interpretation of the magnetic circular dichroism (MCD) spectra of deoxy-Mb and deoxy-Hb in the Soret, the visible, and the near-infrared regions. In their analysis, the ^5E state was taken as the ground state, and the usual spin-orbit interaction of the 3d electrons was taken into account for the three quintet states $^5E_\eta$, $^5E_\xi$, and $^5B_2$ [19,20]. Following the electronic level scheme adopted by Eicher and Trautwein [10], Oganesyan and Sharonov [21] proposed a mixed four-term energy level scheme ($^5B_2$, ^5E, ^3E, and $^1A_1$ states) for the ferrous high-spin hemoproteins. The low-lying excited levels were reported to be 4.2, 7.5, 16.1, and $26.0\ cm^{-1}$ from the ground state for deoxy-Mb. The level separation between the ground singlet and the first excited state is well compatible with the value of $3.5\ cm^{-1}$ that derived from the far-infrared experiments [7]. The calculated transition probability for the far-infrared experiments, attributed to the transition from the ground singlet to the second excited state, was five times lower than that to the first excited state [21]. Therefore, they concluded that the absence of an absorption feature at $\sim 7.5\ cm^{-1}$ might be due to this low transition probability.

EPR is a direct probe of the magnetic field dependent low-lying electronic levels of the ferrous high-spin heme in deoxy-Mb or deoxy-Hb. In order to observe a direct transition between energy levels separated by more than $3\ cm^{-1}$, it is necessary to increase the microwave frequency and the applied magnetic field to significantly higher values than those used in conventional EPR apparatuses. Recently developed multi-frequency EPR (MFEPR) spectrometers have enabled us to investigate a variety of spin systems in transition metal complexes [22-25]. We measured EPR signals in the manganese (III) proto-porphyrin IX reconstituted Mb, Mn(III)Mb, by our MFEPR system and precisely determined the ZFS parameters using a spin Hamiltonian formalism for $S=2$ [26]. Mn(III) porphyrin and the ferrous high-spin heme are both $S=2$ integer spin systems, but with $3d^4$ and $3d^6$ electronic configurations, respectively. We initially established the experimental methodology for an $S=2$ integer spin system using Mn(III)Mb [25]. The aim of this study is now to detect the EPR signals attributed to the ferrous high-spin heme in deoxy-Mb and deoxy-Hb and to their low-lying electronic states. We report the measurement of novel EPR signals from the deoxy-Mb and deoxy-Hb by our newly developed highly-sensitive multi-frequency and high-field EPR techniques. We then discuss which analytical method is suitable for interpreting the observed EPR absorptions, namely the spin Hamiltonian formalism assuming spin quintet states in the $^5B_2$ ground state or the four-term energy level formalism.

2. Experimental procedures

2.1. Sample preparation

The Mb used in this study was extracted from sperm whale meat. A lump of the meat had been stored in a deep freezer at −80 °C. Oxygenated Mb (oxy-Mb) was prepared as described by Yamazaki et al. [27]. Purified oxy-Mb was concentrated up to ca. 25 mM (heme base) with an Amicon Ultra-4 Centrifugal Filter Concentrator (Millipore Co., USA), and the buffer (50 mM Tris–HCl, pH 8.4) was exchanged with 0.1 M potassium phosphate at pH 7.0. A portion of the oxy-Mb was crystallized at room temperature from a 75% saturated ammonium sulfate–phosphate buffer at pH 6.0 at room temperature [28]. However, during the single crystal growth process, the oxy-Mb was auto-oxidized to form the aquamet-Mb crystal. The single crystal of the oxidized Mb (aquamet-Mb) was then reduced to fully deoxy-Mb crystal by dissolving a few grains of sodium dithionite ($Na_2S_2O_4$) in the mother liquid under anaerobic conditions just before EPR measurements.

Fresh human adult blood hemolysate was prepared according to the method described by Kilmartin and Rossi-Bernardi [29]. The carbon monoxide (CO)-form hemolysate was prepared first to avoid partial oxidation of the Hb during the preparation process [30]. The CO-form hemolysate was subsequently de-salted by passage through an Amberlite column. The minor components, HbA_1 and HbA_2, were removed using the method described by Shibayama et al. [31], and the HbA was purified. The heme bound CO of the salt-free CO-HbA was exchanged to the oxygen (O_2)-form (oxy-Hb; in 0.1 M Tris buffer, pH 8.1) with a modified version of the technique described by Kilmartin and Rossi-Bernardi [29]. The oxy-Hb solution was concentrated up to 20–27 mM (heme base) by the same procedure used for the oxy-Mb solution. These concentrated oxy-Mb and oxy-Hb solutions were frozen in liquid nitrogen and stored at −80 °C before use.

Due to the high oxygen affinity of Mb and Hb (at pH 8.1), it was difficult to obtain completely deoxygenated samples by only the de-aeration procedure. A few grains of sodium dithionite were used to prepare the fully deoxygenated sample solution. The sample solution was transferred into the EPR sample container and frozen immediately.