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Characterization of neutrophil *b*-type cytochrome in situ by electron paramagnetic resonance spectroscopy

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Electron paramagnetic resonance spectroscopy at 4.2 K was successfully used to characterize neutrophil *b*-type cytochrome in situ. The spectra of resting neutrophils taken under aerobic conditions gave a set of characteristic signals in a high magnetic field (g = 2.85, 2.21 and 1.67) beside signals for myeloperoxidase and others. From the g values, shapes and the results of other experiments, these signals were attributed to those of cytochrome b_{558}. The results indicate that cytochrome b_{558} in resting neutrophils is a hexa-coordinated ferric hemoprotein in a low-spin state. The obtained g_z and g_x values for the hemichrome were consistent with that of bis(imidazole)-coordinated hemoprotein.

Cytochrome b_{558}; Neutrophil; EPR spectroscopy; O_2^--generation; Crystal field splitting parameter

1. INTRODUCTION

A unique *b*-type cytochrome, cytochrome b_{558}, found in the plasma membrane of phagocytes [1–3] and B lymphocytes [4,5] is considered to be involved in superoxide (O_2^-) generation in these cells as a constituent of the electron transport system from NADPH to molecular oxygen. However, the role of the cytochrome in the reaction has not yet been elucidated nor its molecular properties such as electronic and coordination structures. This is because isolation of the cytochrome in intact form is difficult by its extremely fragile nature. To avoid denaturation of the cytochrome during isolation process and to know its physicochemical properties in situ, we measured electron paramagnetic resonance (EPR) spectra of 'intact' porcine neutrophils at 4.2 K and obtained a set of signals for a hemichrome in a high magnetic field. Based on the findings, we show in this paper that cytochrome b_{558} in resting neutrophils is a hexa-coordinated ferric hemoprotein in a low-spin state and probably ligated in its fifth and sixth coordinated positions by histidine nitrogens.

2. MATERIALS AND METHODS

Porcine neutrophils were isolated from fresh blood as described previously [6] with minor modifications. The thick neutrophil suspension was transferred into the EPR cuvette (3.5 mm inner diameter, 300 mm total length) and frozen in liquid nitrogen. The cuvette was fixed in a cavity of JES FE-3x ESR spectrometer equipped with LTR Heli-Tran liquid-helium transfer refrigerator (Air Products and Chemicals Inc.). The conditions for measurements were as follows: microwave power, 1 mW; modulation amplitude, 6.3 Gauss at 100 kHz; magnetic field, 2500 + 2500 Gauss; response, 0.3 s; sweep time, 4 min; amplitude, 1.25, 1.6 or 4 × 1000; and temperature, 4.2 K.

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Abbreviations: EPR, electron paramagnetic resonance; O_2, superoxide anion; MPO, myeloperoxidase; SOD, superoxide dismutase

3. RESULTS

Fig. 1a shows a wide field EPR spectrum of porcine neutrophils measured at 4.2 K under aerobic conditions. Two sets of characteristic signals are recognized in the spectrum, which are g = 6.80 and 5.28 in a low magnetic field region and g = 2.85, 2.17 and 1.67 in a high magnetic field region. The former two g values are in good agreement with those of myeloperoxidase (MPO) in ferric high spin state [7] and the latter three are consistent with those reported for the ferric low spin compounds [8]. Other signals observed appear to be those of non-heme iron (g = 4.23), cupric ion of superoxide dismutase (SOD, $g_\perp = 2.07$), flavin free radical (g = 2.00) and unidentified substance (g = 1.92). Slight raise at g = 6.0 may be due to slightly contaminating methemoglobin.

Under the conditions where cytochrome b_{558} was completely reduced but MPO was only partially reduced by sodium dithionite [8], the signals in the high magnetic field except that for flavin free radical disappeared but those for MPO in the low magnetic field partially remained (Fig. 1b).

When neutrophils were exposed to sodium cyanide, which is known to bind to the heme iron of certain chromophores such as MPO or mitochondrial

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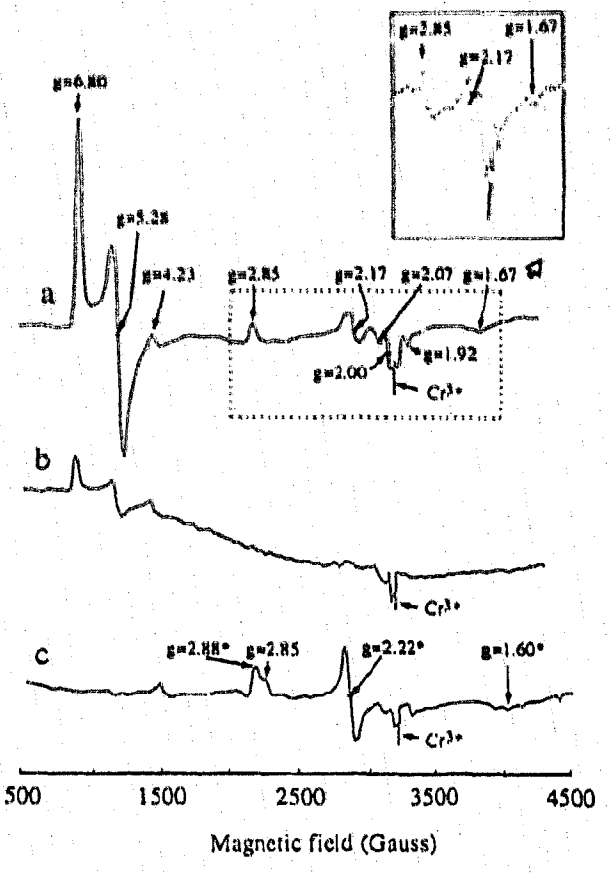

Fig. 1. EPR spectra of neutrophils at 4.2 K. Neutrophil suspensions (about 4.8×10^9 cells/ml) were transferred to the empty EPR cuvettes directly (a) or after addition of 1 mM sodium cyanide (c) or to the cuvette containing 100 mg of sodium dithionite in a small volume of HBSS (b). These cuvettes were frozen and EPR spectra were recorded at 4.2 K. A fine spectrum from 2000 to 4000 Gauss of (a) taken at a higher amplitude (4×1000) was shown in the Inset. In (c), the newly-appeared signals for MPO-CN were marked by asterisks.

cytochrome *c* oxidase but not to that of cytochrome b_{558} in neutrophils, the ferric low spin signal of hemichrome at g = 2.85 was not affected by the treatment but the ferric high spin signals of MPO (g = 6.80 and 5.28) disappeared (Fig. 1c). Instead, a new set of signals at g = 2.88, 2.22 and 1.60 appeared in the high magnetic field, which obscured other signals for the hemichrome (g = 2.17 and 1.67). Signals for non-heme iron, cupric ion of SOD, flavin free radicals were not influenced by the cyanide treatment.

4. DISCUSSION

The EPR spectrum shown in this paper clearly demonstrated a set of ferric low spin signals for a hemichrome in neutrophils. As shown previously [9], only hemoprotein spectrophotometrically detected in intact neutrophils beside MPO was cytochrome b_{558}. Other hemoproteins such as mitochondrial cytochromes are present in much less amounts in neutrophils [9]. In addition, we showed in this paper that the set of the low spin signals disappeared when the cells were reduced. The disappearance of the low spin signals of hemichrome took place before the signals for MPO disappeared, indicating that the hemichrome was reduced at a faster rate than MPO. This agreed well with our spectroscopic observations reported previously [9]. Furthermore, sodium cyanide, which is known to bind to ferric high spin hemoprotein such as MPO and mitochondrial cytochrome *c* oxidase, but not to cytochrome b_{558}, affected signals for MPO but not those for the hemichrome. Based on these findings, we concluded that the set of signals, whose g values were 2.85, 2.17 and 1.67, was ascribable to that of cytochrome b_{558}.

As a consequence, cytochrome b_{558} in resting neutrophils is in a ferric low spin state. To infer the two axial ligands at the fifth and sixth positions of the heme iron in cytochrome b_{558}, we compared g values determined in this study with those of the hemoproteins listed by Walker et al. [10] and Sakurai et al. [11]. For this comparison, we took maximum (2.85), intermediate (2.17) and minimum values (1.67) as g_x, g_y and g_z, respectively. These values except that of g_y were within the range of variation of those corresponding to most of *b*-type cytochromes, related bis(imidazole)-coordinated proteins and low spin hemin complexes [10]. Values of g_x for axial ligands other than bis(imidazole) (2.69–3.03) were either larger or smaller than the value observed in this study; larger values are methionine-imidazole (3.2–3.21), aliphatic amine-imidazole (3.33–3.41) and bis(aliphatic amide) (3.57–3.63), and smaller values are thiolato-imidazole or imidazolate (2.34–2.46), bis(thiolato) (2.28–2.36) and bis(thiol) (2.30–2.39). It is likely, therefore, that the heme iron of cytochrome b_{558} is ligated in its fifth and sixth coordination positions by two imidazole nitrogens of histidine residues. Cytochrome b_{558} is known to consist of two types of subunits and the small subunit is predicted as the heme-bearing component based on the common structural motifs with other heme-containing proteins, a stretch containing a histidine (His-94) that resembles a region of the heme-bearing subunit of the mitochondrial cytochrome *c* oxidase [12] and the results of radiation-inactivation target analysis [13]. The present results suggest that the heme group resides between large and small subunits, since only one histidine was found in the small subunit [14].

According to the equations described by Walker et al. [10], we calculated tetragonal ($\blacktriangle/\lambda$) and rhombic (V/λ) crystal field splitting parameters and rhombicity ($V/\blacktriangle$) of cytochrome b_{558} from the g values of the components. The rhombic splitting of $V/\lambda = 2.17$ and rhombicity of $V/\blacktriangle = 0.44$ were within the range of the variation of the values of bis(imidazole) coordinated hemoproteins listed by Walker et al. [10]. However, the tetragonal splitting of $\blacktriangle/\lambda = 4.96$ was very large as com-

pared with the listed values (3.11–4.25) and dit not correspond to any of the types of the low-spin ferric hemoproteins so far reported. The large Δ/λ value exhibits stronger ligand electric field, i.e. shorter bond-length between the heme iron and the coordinated imidazole nitrogens than those in the other bis(imidazole) coordinated *b*-type cytochromes. This results in the lifting of the highest occupied *d*-orbital to the high energy side, which may explain the extremely low redox potential of this cytochrome [15]. It may be possible, therefore, that the unique feature of axial coordination by two imidazoles of cytochrome b_{559} enables the electron transfer from Fe^{2+} in the heme to molecular oxygen without the formation of oxygen adduct.

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