Existence of Two Heme B Centers in Cytochrome $b_{561}$ from Bovine Adrenal Chromaffin Vesicles as Revealed by a New Purification Procedure and EPR Spectroscopy*

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We have established a new purification procedure of cytochrome $b_{561}$ from bovine adrenomedullary chromaffin vesicles. The heme content analysis of the purified sample indicated the presence of 1.7 molecules of heme B/cytochrome $b_{561}$ molecule. EPR spectroscopy of the purified enzyme in oxidized state showed that there were three types of low spin heme species. Two of them showed usual EPR signals at $g_z = 3.14$ and $g_z = 2.84$ arising from the same heme and were interchangeable depending on pH. The other species showed a highly anisotropic low spin signal at $g_z = 3.70$, with a lower redox potential than the others, and a temperature-sensitive character. These properties are very similar to those of the mitochondrial low potential cytochrome $b$ ($b_5$ or $b_{566}$) of the mitochondrial complex III, indicating that the $g_z = 3.70$ species is derived from a heme component different from the one that shows the usual low spin EPR signals. Based on our new structural model, these two heme B prosthetic groups are likely to be located on both sides of the membranes in close contact with the ascorbic acid- and semidehydroascorbic acid-binding sites, respectively, to facilitate the electron transfer across the membranes. This molecular architecture may provide a structural basis for the transmembrane electron transfer catalyzed by this hemoprotein.

Cytochrome $b_{561}$ is believed to play a key role in electron transfer across the chromaffin vesicle membranes required for noradrenaline biosynthesis inside these specialized organelles of the secretory cells (1). The mechanism of this process remains still unclear. The cytochrome is a highly hydrophobic hemoprotein with a molecular mass of ~28 kDa and contains six or five transmembrane $\alpha$-helices (2, 3). Its amino acid sequence shows no apparent homology with any other membrane-bound cytochromes so far known (2). The cytochrome is characterized by a rather high redox midpoint potential (4) and an asymmetric absorption peak in the $\alpha$-band with a maximum at 561 nm and a shoulder at approximately 558 nm (5). Apps et al. (6) revealed the presence of two potentiometrically different forms (midpoint potentials, 170 and 70 mV, respectively) of cytochrome $b_{561}$ and reported the high and low potential components to have identical absorption spectra. Burbaev et al. (7) reported that the intact chromaffin vesicle membranes from bovine adrenal medullae showed three different EPR signals of ferric cytochrome $b_{561}$. A typical $g_z$ signal of a low spin cytochrome observed at $g_z = 3$ comprised a high potential component with $g_z = 3.14$ and a low potential one with $g_z = 3.11$. In addition, a highly temperature-sensitive heme signal at $g_z = 3.7$ was observed. The latter signal was fully retained in the preparation of vesicle membranes with cytochrome $b_{561}$ reduced by 50% but disappeared upon full reduction of the cytochrome by ascorbic acid. The properties of the signal were strikingly similar to those of the mitochondrial low potential cytochrome $b$ heme ($b_5$ or $b_{566}$) (8, 9).

Despite these pieces of evidence, it is widely accepted that purified cytochrome $b_{561}$ contains only one heme B per molecule (10–12). Apps et al. used the pyridine hemochrome method and Western blotting for quantitation of heme and apoprotein, respectively. They found a heme B/cytochrome stoichiometry of 0.92 (6). The sigmoid shape in the Nernst plot of redox titration was, therefore, explained by negative cooperativity in oligomeric cytochrome $b_{561}$ (6).

In the present study, we have established a new purification procedure for cytochrome $b_{561}$ from bovine adrenal chromaffin vesicles. Heme content analysis of the purified sample showed 1.7 heme B molecules/cytochrome $b_{561}$ molecule. We found further that the purified cytochrome in oxidized state showed three types of low spin EPR signals originating from two distinct heme components.

MATERIALS AND METHODS

Purification of Cytochrome $b_{561}$—Chromaffin vesicle membranes were isolated from bovine adrenal glands according to the procedure of Bartlett and Smith (13). The washed chromaffin vesicle membranes (~500 mg of protein with a concentration of ~5 mg of protein/ml) were solubilized with 1.0% (w/v) $\beta$-octyl glucoside in 20 mM Tris-HCl (pH 8.0) buffer containing 20% (v/v) glycerol and 1.0 mM sodium ascorbate with stirring at 4 °C for 1 h. The solubilized extract was centrifuged for 20 min at 19,000 rpm in a model 7800 centrifuge (Kubota, Tokyo, Japan) equipped with an RA-300 rotor. The extract was loaded onto a column of a-aminooxyacety-Sephrose 4B (2.5 cm diameter) × 14 cm) previously equilibrated with 20 mM Tris-HCl (pH 8.0) buffer containing 20% (v/v) glycerol, 1.0% (w/v) $\beta$-octyl glucoside, and 1.0 mM sodium ascorbate (buffer A). After the loading, the column was washed with buffer A extensively, until turbid fractions and reddish fractions were eluted out. The column was then treated with buffer A containing 50 mM KCl. During this wash, cytochrome $b_{561}$ was eluted as a sharp red band. Fractions containing cytochrome $b_{561}$ were pooled and concentrated to
Table I

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein content mg</th>
<th>Total cytochrome b&lt;sub&gt;561&lt;/sub&gt; nmol</th>
<th>Specific content nmol/mg</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified chromaffin vesicles</td>
<td>57.6</td>
<td>901.1</td>
<td>1.39</td>
<td>100</td>
</tr>
<tr>
<td>β-Octyl glucoside extract</td>
<td>508.0</td>
<td>557.0</td>
<td>1.10</td>
<td>92.8</td>
</tr>
<tr>
<td>First ω-aminooctyl-Sepharose column</td>
<td>116.5</td>
<td>404.1</td>
<td>3.47</td>
<td>67.3</td>
</tr>
<tr>
<td>Concanavalin A-Sepharose column</td>
<td>93.2</td>
<td>371.7</td>
<td>3.99</td>
<td>61.9</td>
</tr>
<tr>
<td>Second ω-aminooctyl-Sepharose column</td>
<td>6.88</td>
<td>232.4</td>
<td>33.78</td>
<td>38.7</td>
</tr>
</tbody>
</table>

Table II

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>Millimolar extinction coefficient (μM&lt;sup&gt;-1&lt;/sup&gt; cm&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>561</td>
<td>46.1</td>
</tr>
<tr>
<td>530</td>
<td>24.0</td>
</tr>
<tr>
<td>427</td>
<td>267.9</td>
</tr>
<tr>
<td>415</td>
<td>203.7</td>
</tr>
<tr>
<td>561–575</td>
<td>37.3</td>
</tr>
<tr>
<td>429–411</td>
<td>246.7</td>
</tr>
</tbody>
</table>

RESULTS

Purification of Cytochrome b<sub>561</sub>—Table I shows a summary of a typical purification of the cytochrome using 50 bovine adrenal chromaffin vesicles as starting materials. The procedure as described in detail under “Materials and Methods” was shown to be reproducible. Just after turbid fractions were eluted out, a part of cytochrome b<sub>561</sub> was also eluted from the 1st ω-aminooctyl-Sepharose 4B column during a wash with buffer A. SDS-PAGE analysis showed that the fraction contained many protein bands with higher molecular weights, in addition to cytochrome b<sub>561</sub>, indicating that cytochrome b<sub>561</sub> was not fully dissipated even in 1% β-octyl glucoside or cytochrome b<sub>561</sub> might interact strongly with other membrane proteins in chromaffin vesicles. Before loading onto the second ω-aminooctyl-Sepharose 4B column, the crude cytochrome b<sub>561</sub> sample was passed through a concanavalin A-Sepharose column to remove contaminating dopamine β-monooxygenase. Upon loading on the second ω-aminooctyl-Sepharose 4B column, cytochrome b<sub>561</sub> was adsorbed in the column as a red band. Washing the column with the equilibrating buffer caused a gradual elution of cytochrome b<sub>561</sub>. The sample at this stage showed a single protein band on SDS-PAGE with an apparent molecular weight of 27,700 and was considered as pure cytochrome b<sub>561</sub>.

Heme Content Analysis—The purified cytochrome b<sub>561</sub> was analyzed with visible absorption spectroscopy and on SDS-PAGE. The pyridine hemochrome analysis of the purified sample in the aqueous alkaline condition showed α, β, and Soret absorption maxima at 556.5, 524.5, and 418.5 nm, respectively, confirming presence of heme B as the prosthetic group (5). The purified cytochrome b<sub>561</sub> contained 61.4 nmol of heme B/mg protein (the mean value of 6 measurements with S.D. = 2.14). This corresponds to 1.70 molecules of heme B/cytochrome b<sub>561</sub> molecule, assuming the molecular weight as 27,700. It was essential to oxidize cytochrome b<sub>561</sub> before the pyridine hemochrome analysis; otherwise significant destruction of heme B was observed upon treatment of the cytochrome in the aqueous alkaline solution.

EPR Analyses—Visible absorption spectra of the purified cytochrome b<sub>561</sub> in various redox levels are shown in Fig. 1 (upper panel). In almost (93%) oxidized state at pH 8.0 (Fig. 1, upper panel, trace C), three types of low spin species were observed in the EPR spectra at 15 K (Fig. 1, center panel, trace...
C): a highly anisotropic species with $g_z$ value at 3.70 and two typical low spin species (one with $g_z = 3.14$ and the other with $g_z = 2.84$, $g_y = 2.24$, and $g_x = 1.66$).

The two typical low spin signals ($g_z = 3.14$ and 2.84) showed a significant pH dependence and were found to be interconvertible. Upon lowering the pH to 6.8, the $g_z = 2.84$ signal was completely gone in the EPR spectrum at 15 K (Fig. 2A). On the other hand, at pH 8.8, the intensity of the $g_z = 2.84$ signal became much stronger than that of the $g_z = 3.14$ signal (spectra not shown). The apparent $pK_a$ of this transition was estimated at approximately 8.2. The $g_y$ and $g_x$ components of the $g_z = 3.14$ species could be clearly seen at $\approx 2.1$ and 1.49, respectively (Fig. 2A), as reported previously for the oxidized chromaffin vesicles (pH 7.2). Although Burbaev et al. reported that the $g_z = 3.12$ species comprised a high potential component with $g_z = 3.14$ and a low potential one with $g_z = 3.11$ (7), our present data showed only one species. The $g$ values of both the $g_z = 3.14$ and $g_z = 2.84$ species are very similar to those of microsomal cytochrome $b_5$ ($g_z = 3.05$, $g_y = 2.22$, and $g_x = 1.41$) (16), chloroplast cytochrome $b_{590}$ ($g_z = 2.84$, $g_y = 2.27$, and $g_x = 1.54$) (17), and cytochrome $b$ of bo-type ubiquinol oxidase ($g_z = 2.98$, $g_y = 2.26$, and $g_x = 1.45$) (18), all of which are known to have bimidazole ligands.

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2 M. Tsubaki, M. Nakayama, and H. Hori, unpublished result.
In the present study we have established a new purification procedure that enabled us to prepare a large quantity of highly purified cytochrome b<sub>561</sub> from bovine chromaffin vesicles. The heme content analysis of the purified cytochrome b<sub>561</sub> showed 1.7 molecules of heme B/molecule. This number is in marked contrast to the previously reported value of 1.0 molecule of heme B/cytochrome b<sub>561</sub> molecule (6, 10–12). It is very likely that previous purification procedures suffered a significant loss of heme B prosthetic group from the cytochrome. We noticed that previous purification procedures suffered a significant loss of heme B/cytochrome (1.7 molecules of heme B/molecule). This number is in marked contrast to the previous observation on oxidized chromaffin vesicle preparations (7).

The nature of this species is not clear at this stage. There was another type of EPR signal at g<sub>z</sub> = 3.70 in the partially (43%) oxidized spectrum. These observations indicate that the redox potential of the g<sub>z</sub> = 3.70 species is lower than those of the usual low spin species (g<sub>z</sub> = 3.14 and 2.84 species).

The high spin heme signal (g ~ 6.1) represents merely a minor population of cytochrome b<sub>561</sub> based on spin contents. The nature of this species is not clear at this stage. There was another type of EPR signal at g = 4.3. It is likely a product of the heme decomposition.

**DISCUSSION**

In the present study we have established a new purification procedure that enabled us to prepare a large quantity of highly purified cytochrome b<sub>561</sub> from bovine chromaffin vesicles. The heme content analysis of the purified cytochrome b<sub>561</sub> showed 1.7 molecules of heme B/molecule. This number is in marked contrast to the previously reported value of 1.0 molecule of heme B/cytochrome b<sub>561</sub> molecule (6, 10–12). It is very likely that previous purification procedures suffered a significant loss of heme B prosthetic group from the cytochrome. We noticed that a part of heme B prosthetic group is particularly labile and is easily lost during the purification. Even with our new purification procedure, the heme content values of as low as 1.4 is easily lost during the purification. Even with our new purification procedure, the heme content values of as low as 1.4 is easily lost during the purification. Even with our new purification procedure, the heme content values of as low as 1.4 is easily lost during the purification.

The presence of several EPR species of cytochrome b<sub>561</sub> in chromaffin vesicle membranes had been reported (7) and was confirmed in the present study for the purified sample. There were three types of low spin species; two of them showed usual low spin EPR signals (g<sub>z</sub> = 3.14 and g<sub>z</sub> = 2.84) arising from the same heme component and were interconvertible depending on pH. A similar transition of EPR signals upon elevation of pH was reported for cytochrome b<sub>5</sub>, in which a neutral form (g<sub>z</sub> = 3.05, g<sub>x</sub> = 2.22, g<sub>y</sub> = 1.41 at pH 6.2) was converted to an alkaline form (g<sub>z</sub> = 2.76, g<sub>x</sub> = 2.26, g<sub>y</sub> = 1.67 at pH 12.0) (16).

The cause of this transition is likely either due to deprotonation of one of the axial bisimidazole ligands to form an imidazolate ligation or the imidazole ligand to become strongly hydrogen-bonded from a nearby amino acid residue (19). Since the pH within chromaffin vesicles is around 5.7 (20), the g<sub>z</sub> = 2.84 species may not participate in the physiological electron transfer reaction.

The other EPR species showed a highly anisotropic low spin signal (g<sub>z</sub> = 3.70), a lower redox potential than the others, and a temperature-sensitive character, being very similar to cytochromes b (b<sub>566</sub>, g<sub>z</sub> = 3.75; b<sub>562</sub>, g<sub>z</sub> = 3.45) of the mitochondrial complex III (8, 9) and to chloroplast cytochrome b<sub>5</sub> (b<sub>563</sub>; g<sub>z</sub> = 3.5) (21). The g<sub>z</sub> = 3.70 species showed only a slight pH-dependent spectral change. These properties indicate that the g<sub>z</sub> = 3.70 species is derived from a heme component different from the one that shows the usual low spin EPR signals. Presence of two independent heme B centers is consistent with the observation of two different forms (midpoint potentials, 170 and 70 mV, respectively) of cytochrome b<sub>561</sub> determined by an optical potentiometric technique (6).

Recently we have proposed a plausible structural model of cytochrome b<sub>561</sub> on the basis of comparison of the deduced amino acid sequences of seven species. In the model, a polypeptide spans the vesicle membranes six times. There are two fully conserved regions in the sequences; the first conserved sequence (69ALLVYRVFR77) is located on the extravesicular side of an α-helical segment, and the second one (120SLHSW124) is located in an intravesicular loop connecting two α-helical segments, respectively. The first and second conserved sequences are likely to form the binding sites for extravesicular ascorbic acid and intravesicular semidehydroascorbic acid, respectively (1). In addition, there are six totally conserved histidyl residues (His<sup>54</sup>, His<sup>88</sup>, His<sup>92</sup>, His<sup>110</sup>, His<sup>122</sup>, and His<sup>161</sup>) in the cytochrome. Thus, we have proposed that one of the two heme ions of cytochrome b<sub>561</sub> (extravesicular side) is coordinated with the pair, either His<sup>54</sup> (helix 3)-His<sup>122</sup> (helix 5) or His<sup>88</sup> (helix 3)-His<sup>161</sup> (helix 5). The pair, His<sup>54</sup> (helix 2)-His<sup>122</sup> (end of helix-4) is likely to coordinate with another heme ion (intravesicular side).

Consideration on the EPR properties of the two heme B species suggests further that a lower redox potential of the g<sub>z</sub> = 3.70 species is favorable for an electron acceptor from extravesicular ascorbic acid, whereas the usual low spin heme species has a higher redox potential suitable for donating an electron to intravesicular semidehydroascorbic acid. Indeed, the EPR signal of cytochrome b<sub>561</sub> is derived from the heme at the extravesicular side and that the usual low spin EPR signals (g<sub>z</sub> = 3.70) of cytochrome b<sub>561</sub> is derived from the heme at the intravesicular side. Therefore, the two heme B centers are located on both sides of the vesicle membrane.

**Fig. 2.** EPR spectra of the purified cytochrome b<sub>561</sub> in fully oxidized state at 20 mM sodium phosphate buffer (pH 6.8) containing 20% (v/v) glycerol, 1.0% (w/v) β-octyl glucoside measured at 15 K (trace A) and 5 K (trace B) and in 20 mM Tris-HCl buffer (pH 8.0) containing 20% (v/v) glycerol, 1.0% (w/v) β-octyl glucoside measured at 5 K (trace C). Other conditions are the same as in Fig. 1.

At 5 K, the g<sub>z</sub> = 3.70 species dominated in the low spin signal region of the fully oxidized spectra (both pH 6.8 and 8.0) (Fig. 2, B and C), being consistent with the previous observation on the oxidized chromaffin vesicle preparation (7). There was a slight pH-dependent change in the g<sub>z</sub> value (Fig. 2, B and C). We noticed further that, at 5 K, intensity of the g<sub>z</sub> = 3.70 signal in the partially oxidized spectrum was almost fully retained compared with the one in the 93% oxidized spectrum (Fig. 1, lower panel, traces B versus C). At 15 K, the g<sub>z</sub> = 3.70 signal intensity in the 43% oxidized spectrum was almost fully retained as well (Fig. 1, center panel, traces B versus C). These observations indicate that the redox potential of the g<sub>z</sub> = 3.70 species is lower than those of the usual low spin species (g<sub>z</sub> = 3.14 and 2.84 species).

The high spin heme signal (g ≈ 6.1) represents merely a minor population of cytochrome b<sub>561</sub> based on spin contents. The nature of this species is not clear at this stage. There was another type of EPR signal at g = 4.3. It is likely a product of the heme decomposition.

### Structure of Cytochrome b<sub>561</sub>

the membranes in close contact with the ascorbic acid- and semidehydroascorbic acid-binding sites, respectively, to facilitate the electron transfer across the membranes. The well allocated molecular architecture provides a structural basis for the efficient transmembrane electron transfer catalyzed by this hemoprotein. Further studies are in progress to reveal the mechanism of the ascorbic acid-regenerating system in the secretory vesicles.

REFERENCES
Existence of Two Heme B Centers in Cytochrome \textit{b} 561 from Bovine Adrenal Chromaffin Vesicles as Revealed by a New Purification Procedure and EPR Spectroscopy

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