Distinct Roles of Two Heme Centers for Transmembrane Electron Transfer in Cytochrome b\textsubscript{561} from Bovine Adrenal Chromaffin Vesicles as Revealed by Pulse Radiolysis*

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The reaction of monodehydroascorbate (MDA) radical with purified cytochrome b\textsubscript{561} from bovine adrenal chromaffin vesicles was investigated by the technique of pulse radiolysis. Radiolytically generated MDA radical oxidized rapidly the reduced form of cytochrome b\textsubscript{561} to yield the oxidized form. Subsequently the oxidized form of cytochrome b\textsubscript{561} was re-reduced by ascorbate in the medium. The second-order rate constants of the reaction of MDA radical were increased with decreasing pH, whereas a maximum of the second-order rate constant for the reaction with ascorbate was obtained around pH 6.8. At excess MDA radical to cytochrome b\textsubscript{561} concentration, only half of the heme in cytochrome b\textsubscript{561} was oxidized, indicating that only one of the two heme centers can react with MDA radical. On the other hand, when the reactions were examined using cytochrome b\textsubscript{561} pretreated in a mild alkaline condition in the oxidized state, the cytochrome b\textsubscript{561} could not be oxidized with MDA radical, suggesting that the heme center specific for the electron donation to MDA radical is selectively modified upon the alkaline treatment. These results suggest that the two heme b centers have distinct roles for the electron donation to MDA radical and the electron acceptance from ascorbate, respectively.

In neurosecretory vesicles, such as adrenal chromaffin vesicles and pituitary neupeptide secretory vesicles, intravesicular ascorbate (AsA\textsuperscript{−}) functions as the electron donor for copper-containing monooxygenases such as dopamine β-monooxygenase and peptidyl-glycine α-amidating monooxygenase (1). Upon these monooxygenase reactions, monodehydroascorbate (MDA) radical is produced by univalent oxidation of AsA\textsuperscript{−} (2, 3). It is believed that the intravesicular MDA radical is reduced back to AsA\textsuperscript{−} by membrane-bound cytochrome b\textsubscript{561}, and subsequently cytochrome b\textsubscript{561} is reduced by extravesicular AsA\textsuperscript{−} (4–7). Thus, cytochrome b\textsubscript{561} is likely to serve as an electron shuttle, maintaining the AsA\textsuperscript{−} concentration inside the vesicles.

Cytochrome b\textsubscript{561} is a highly hydrophobic hemoprotein with a molecular mass of ~28 kDa and contains five or six transmembrane α-helices (8, 9). It had been widely accepted that cytochrome b\textsubscript{561} contains only one b-type heme per molecule, by analyses with pyridine hemochrome and Western blotting methods for quantitation of heme and apoprotein, respectively (10–13). However, very recently, we have established a new purification procedure of cytochrome b\textsubscript{561} from bovine adrenal chromaffin vesicles (14). We found that the purified cytochrome b\textsubscript{561} contained two b-type hemes per molecule. In addition, each heme b center exhibited independent EPR signals in the oxidized state. Based on these results and comparison of the amino acid sequences of cytochrome b\textsubscript{561} from various species, we have proposed that the two heme prosthetic groups are located on both sides of the membrane in close contact with AsA\textsuperscript{−} and MDA binding sites, respectively, to facilitate the electron transfer across the membranes (14, 15). However, the role of each of the two heme b centers has not been elucidated.

Because of the instability of the MDA radical, which disproportionate rapidly to dehydroascorbate and AsA\textsuperscript{−}, the electron transfer reaction with cytochrome b\textsubscript{561} was measured indirectly in the presence of AsA\textsuperscript{−} and ascorbate oxidase (5, 6). However, it is possible to investigate directly the reaction of MDA radical using a pulse radiolysis technique (16–20). We could observe electron donations to MDA radical from hepatic NADH-cytochrome b\textsubscript{5} reductase (19) and from MDA reductase purified from cucumber chloroplasts (20). In particular, MDA reductase was shown to be a good electron donor for MDA radical (20). The present study describes a successful application of the pulse radiolysis technique to investigate the reaction of MDA radical with cytochrome b\textsubscript{561} purified from bovine adrenal chromaffin vesicles. We obtained clear evidence that the two heme b centers in cytochrome b\textsubscript{561} have distinct roles in the reaction with MDA radical and AsA\textsuperscript{−}, respectively.

MATERIALS AND METHODS

Purification of Cytochrome b\textsubscript{561}—Cytochrome b\textsubscript{561} was purified to a homogeneous state, as reported previously (14). The purity of cytochrome b\textsubscript{561} was analyzed with visible absorption spectra and SDS-polyacrylamide gel electrophoresis. Before use, cytochrome b\textsubscript{561} was passed through a Sephadex G-25 column equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 1.0% (w/v) β-ocetyl glucoside and 1.0 mM AsA\textsuperscript{−}. The passed fractions of cytochrome b\textsubscript{561} were concentrated in the Amicon concentrator. All other reagents were commercially obtained as the analytical grade. The concentration of cytochrome b\textsubscript{561} was determined using a millimolar extinction coefficient of 267.9 mm\textsuperscript{−1} cm\textsuperscript{−1} at 427 nm in the reduced state (14).

Pulse Radiolysis—Samples of cytochrome b\textsubscript{561} for pulse radiolysis were prepared as follows. Solutions containing 10 mM potassium phosphate buffer (pH 5–8), 1% β-ocetyl glucoside, and 5 mM AsA\textsuperscript{−} were bubbled with N\textsubscript{2}O gas for 5 min. Then, a concentrated solution of cytochrome b\textsubscript{561} was added to the solution to make an appropriate final concentration as indicated in the figure legends.

Pulse radiolysis experiments were performed with an electron linear accelerator at the Institute of Scientific and Industrial Research, Osaka.
University (19–23). The pulse width and energy were 8 ns and 27 MeV, respectively. The sample was placed in a quartz cell with an optical path length of 1 cm. The temperature of the sample was maintained at 20 °C. The light source for a spectrophotometer was a 150-watt halogen lamp. After passing through an optical path, the transmitted light intensities were analyzed and monitored by a fast spectrophotometric system composed of a Nikon monochromator, an R-928 photomultiplier, and a Unisoku data analyzing system. For each measurement, a fresh sample was used even though pulse radiolysis did not cause any damage to the sample as judged by its visible absorption spectrum.

The concentration of MDA radical generated by pulse radiolysis was estimated from the absorbance at 360 nm, using a molar extinction coefficient of 3300 M⁻¹ cm⁻¹ (18). This concentration could be adjusted by varying the dose of electron beams.

**Treatment in Alkaline pH in the Oxidized Form—** Purified cytochrome b₅₆₁ (100 μM) in 10 mM potassium phosphate buffer (pH 7.0 and 8.4) containing 1.0% β-octyl glucoside was oxidized with stepwise additions of potassium ferricyanide (100 mM) solution. The fully oxidized cytochrome b₅₆₁ samples were kept in the dark overnight on ice. The samples thus obtained were passed through Sephadex G-25 equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 1.0% (w/v) β-octyl glucoside and 1.0 mM AsA⁻. Optical absorption spectra were recorded with a UVIKON 922 (Kontron), a UV-2200 A (Shimadzu), or a Hitachi U-3000.

**RESULTS**

A transient spectrum of MDA radical with an absorbance maximum at 360 nm was observed 100 ns after pulse radiolysis of N₂O-saturated aqueous solutions in the presence of 5 mM AsA⁻ and 1.0% β-octyl glucoside. Under the conditions employed here, the primary species (hydrated electron (e⁻aq), OH⁻, H⁺) generated by pulse radiolysis of aqueous solutions were efficiently converted to MDA radical at an approximate concentration of 20–30 μM (17–20). The MDA radicals thus formed reacted rapidly with the reduced form of cytochrome b₅₆₁. A decrease at 430 nm and an increase at 405 nm reflected this reaction (Fig. 1A). The kinetic difference spectrum obtained 20 ms after the pulse is similar to that of the difference spectrum of the oxidized minus reduced forms of cytochrome b₅₆₁ (Fig. 1B). It is therefore concluded that MDA radical reacts with the reduced form of cytochrome b₅₆₁ to produce the oxidized form, as shown in Reaction 1.

\[
\text{MDA}^- + \text{Fe}^{2+} + \text{H}^+ \rightarrow \text{AsA}^- + \text{Fe}^{3+} \rightarrow \text{AsA}^+ + \text{Fe}^{2+} + \text{H}^+ 
\]

**REACTION 1**

For determination of the rate constant of Reaction 1, the concentration of MDA radical was lowered to 1–2 μM MDA radical, and the cytochrome b₅₆₁ concentration was varied between 46 and 100 μM. Fig. 2 shows the concentration dependence of cytochrome b₅₆₁ on the apparent rate constants. From the slope of Fig. 2, the second-order rate constant of the reaction was calculated to be \(2.6 \times 10^6 \text{M}^{-1} \text{s}^{-1}\) at pH 7.0. This value is in good agreement with the one reported previously (1.2 \(\times 10^6 \text{M}^{-1} \text{s}^{-1}\) at pH 7.0) in which a steady-state kinetic method was used employing chromaffin vesicle membranes and an AsA⁻-ascorbate oxidase system (7).

Subsequently, the initial changes in absorbance reversed in the time range of seconds (Fig. 3), indicating that re-reduction of cytochrome b₅₆₁ occurred. The rate constant of this process increased with increases in the concentration of AsA⁻ (data not shown). This indicates that the reduction process is a consequence of a bimolecular reaction of AsA⁻ with the oxidized form of cytochrome b₅₆₁, as shown in Reaction 2.

\[
\text{AsA}^- + \text{Fe}^{3+} \rightarrow \text{MDA}^- + \text{Fe}^{2+} + \text{H}^+ 
\]

**REACTION 2**

The second-order rate constant of the reaction was calculated to be \(8 \times 10^2 \text{M}^{-1} \text{s}^{-1}\) at pH 7.0. The value is also good agreement with the value reported previously (4.5 \(\times 10^2 \text{M}^{-1} \text{s}^{-1}\)) in which a stopped-flow method was employed to analyze the electron transfer reaction from AsA⁻ to oxidized cytochrome b₅₆₁ in chromaffin vesicle membranes (7, 24).

The effects of pH on the oxidation rates of cytochrome b₅₆₁ with MDA radical and for reduction with AsA⁻ were examined. It is evident that pH profiles for the oxidation and the reduction reactions are different, as shown in Fig. 4. The rate constant for the oxidation of cytochrome b₅₆₁ increased with decreasing pH. The maximum reaction rate of 4.3 \(\times 10^2 \text{M}^{-1} \text{s}^{-1}\) was obtained at pH 5.5. The pH-dependent change could be fitted to a single deprotonation process with a \(pK_a\) value of 6.7. In contrast, the rate constants of the reduction of cytochrome b₅₆₁ increased with increasing pH in the range of 5 to 6.5 and then decreased with increasing pH in the range of 7 to 8. The maximum reaction rate constant of 8 \(\times 10^2 \text{M}^{-1} \text{s}^{-1}\) was obtained around pH 6.8.

To elucidate the contribution of two heme b centers in the electron transfer reaction, MDA radical concentration dependence was examined. Under the experimental conditions employed, the concentration of MDA radical could be varied between 2 and 20 μM by attenuating the dose of the electron beam whereas the concentration of the reduced cytochrome b₅₆₁ was maintained constant at 12 μM. As shown in Fig. 5, the oxidized...
cytochrome \( b_{561} \) by the reaction (at 20 ms after the pulse) increased with increasing the concentration of MDA radical up to 5 \( \mu \)M, and it reached a plateau in the range 8 to 20 \( \mu \)M. A distinct inflection was observed at a point of \( 0.5 \) eq of MDA radical to cytochrome \( b_{561} \). It is noteworthy that only half of the heme center could be oxidized with an excess concentration of MDA radical.

In a previous report, we showed that incubation of oxidized cytochrome \( b_{561} \) in a mild alkaline condition specifically depletes the electron-accepting ability from AsA\(^2\) for about one-half of the heme centers (15). Fig. 6A shows the visible spectra of cytochrome \( b_{561} \) (pre-treated at pH 8.2 and 7.0, respectively, in the oxidized state) in the presence of AsA\(^-\) and \( \text{N}_2\text{O} \) at pH 7.0. Experimental conditions were the same as in Fig. 1.

Fig. 2. Concentration dependence of the apparent rate constants of the reaction of the reduced form of cytochrome \( b_{561} \) with MDA radical. The rates were determined by the absorption change at 561 nm. The reaction medium contained 5 mM AsA\(^2\), 1% \( \beta \)-octyl glucoside, 10 mM potassium phosphate buffer at pH 7.0, and 1–2 \( \mu \)M MDA radical.

Fig. 3. Absorbance changes after pulse radiolysis of the reduced form of cytochrome \( b_{561} \) measured at 430 and 405 nm in the presence of AsA\(^-\) and \( \text{N}_2\text{O} \) at pH 7.0. Experimental conditions were the same as in Fig. 1.

In a previous report, we showed that incubation of oxidized cytochrome \( b_{561} \) in a mild alkaline condition specifically depletes the electron-accepting ability from AsA\(^-\) for about one-half of the heme centers (15). Fig. 6A shows the visible spectra of cytochrome \( b_{561} \) (pre-treated at pH 8.2 and 7.0, respectively, in the oxidized state) in the presence of AsA\(^-\). Addition of 5 mM AsA\(^-\) to the alkaline treatment of cytochrome \( b_{561} \) caused a reduction of only about half of the heme centers, whereas all the heme centers of cytochrome \( b_{561} \) treated at pH 7.0 (control sample) could be reduced. Fig. 6, B and C, shows the absorbance changes at 430 nm after pulse radiolysis of these samples. The absorbance changes of the control sample after the pulse were not affected. For the alkaline-treated sample, on the other hand, the decrease in absorbance at 430 nm after the pulse was very small, although about half of the heme \( b \) centers were in

Fig. 4. pH dependence of the rate constants of the reaction of MDA radical with the reduced form of cytochrome \( b_{561} \) (A) and the reaction of AsA\(^-\) with the oxidized form of cytochrome \( b_{561} \) (B). Experimental conditions were the same as in Fig. 2.

Fig. 5. MDA radical concentration dependence on the oxidation of cytochrome \( b_{561} \) observed after pulse radiolysis. The reaction mixture contained 12 \( \mu \)M cytochrome \( b_{561} \), 5 mM AsA\(^2\), 1% \( \beta \)-octyl glucoside, 10 mM potassium phosphate buffer at pH 7.0.
Transmembrane Electron Transfer in Cytochrome \(b_{561}\)

**DISCUSSION**

The present study clearly shows that one of the heme \(b\) centers in cytochrome \(b_{561}\) specifically reacts with MDA radical, whereas the other does not. This is verified by the MDA radical concentration dependence on the oxidation of heme \(b\) after the pulse as shown in Fig. 5. Almost stoichiometric oxidation of heme \(b\) was observed under the condition of MDA radical \(< [1/2 \text{ of cytochrome } b_{561}]\), whereas only one-half of heme \(b\) was oxidized at excess MDA radical concentrations. In addition, the distinct functions of two heme \(b\) centers were revealed by pulse radiolysis experiments on the alkaline-treated sample. Our previous report showed that one of the heme centers is very labile to the alkaline treatment, whereas the other heme retains the ability to accept electrons from AsA\(^-\) (15). It is likely that one of the two heme centers, which is very labile to the alkaline treatment, participates in the electron donation to MDA radical, because very slight oxidation of the heme center was observed after pulse radiolysis of the alkaline-treated sample.

EPR spectra of the purified cytochrome \(b_{561}\) in the oxidized state showed the presence of two distinct heme \(b\) species (14). One of them shows usual low spin signals and is very similar to those of microsomal cytochrome \(b_2\) (25), chloroplast cytochrome \(b_{559}\) (26), and cytochrome \(b\) of \(bo\)-type ubiquinol oxidase (27), all of which are known to have bisimidazole ligands. The other species shows a highly anisotropic low spin signal (\(g_z = 3.70\)) with a lower redox potential and is very similar to those of cytochrome \(b\) of the mitochondrial complex III (28, 29) and chloroplast cytochrome \(b_5\) (30). The presence of two independent heme centers was supported by the observation of two potentiometrically different forms of cytochrome \(b_{561}\) determined by an optical potentiometric technique (31). We could not identify definitely which heme center is responsible for the electron donation to MDA radical, but it is very likely that the heme center with the lower redox potential (the \(g_z = 3.70\) species) participates in the electron-accepting reaction from the extravesicular AsA\(^-\). On the other hand, the usual low spin heme species (the \(g_z = 3.14\) species) with a higher redox potential is responsible for the electron donation to MDA radical. This assumption is reasonable since the intramolecular electron transfer of cytochrome \(b_{561}\) occurs from extravesicular to intravesicular to sides. This is also consistent with the result of the alkaline treatment experiment. Indeed, the heme center having the \(g_z = 3.14\) signal can be converted to another form (the \(g_z = 2.84\) species) upon elevation of pH, whereas the other heme center (the \(g_z = 3.70\) species) showed only a slight pH-dependent spectral change (14).

Following the electron donation to MDA radical from the heme with a higher redox potential, an intramolecular electron transfer to the oxidized heme from the other heme with a higher redox potential should take place. The expected intramolecular electron transfer, however, could not be followed directly by the present method, because the two heme centers have indistinguishable visible spectra (14, 31). If the rate of the intramolecular electron transfer (\(k_i\)) is much faster and the rate-determining step is the oxidation of the heme center with MDA radical (\(k_i > k_j\), [MDA radical]), the two hemes would be oxidized simultaneously. In the present work, however, only half of the heme in cytochrome \(b_{561}\) was oxidized under excess MDA radical concentration. This fact indicates that the intramolecular electron transfer must occur later than 1 ms region. In our previous studies, the intramolecular electron transfer in the region of milliseconds was observed for copper-containing nitrite reductase (21, 22) and cytochrome \(cd_1\) nitrite reductase (23). However, the two redox centers in these proteins are only 10 to 20 Å apart (32, 33). In cytochrome \(b_{561}\), the two heme centers are expected to be located on both sides of vesicular membranes (15), and therefore the distance between these two redox centers might be 40 to 50 Å. Thus, an intramolecular electron transfer in the range of seconds may be quite reasonable.

The pH dependence of the rate constants of the electron transfer reactions (Fig. 4) provides further insight into the function of cytochrome \(b_{561}\). The optimal pH of the oxidation (pH = 5.5) and the reduction (pH 6.8) of the heme centers of cytochrome \(b_{561}\) correspond to the physiological pH at the intra- and extravesicular sides, respectively. Recently, we have proposed a plausible structural model of cytochrome \(b_{561}\) on the basis of a comparison of the deduced amino acid sequences of seven species (15). In the model, there are two fully conserved regions in the sequences; the first conserved sequence (\(^{69}\text{ALLVYRVFR}^{77}\)) is located on the extravesicular side of \(\alpha\)-helical segment, and the second one (\(^{120}\text{SLHSW}^{124}\)) is located...

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**Fig. 6. Effects of treatment at pH 7.0 (---) and pH 8.2 (-----) on the visible spectra of cytochrome \(b_{561}\) (A) and absorbance changes at 430 nm after pulse radiolysis of the treated cytochrome \(b_{561}\), at pH 7.0 (B) and pH 8.2 (C) in the presence of AsA\(^-\) and \(N_2O\) at pH 7.0. Alkaline-treated (at pH 8.2 for 20 h and then restored to pH 7.0) and control (incubated at pH 7.0) samples of cytochrome \(b_{561}\) in the oxidized state were prepared as described in the text. Solid sodium ascorbate was added (5 mM), and the spectra were recorded immediately. Other conditions were the same as in Fig. 1.**
in an intravesicular loop connecting two α-helical segments. Since these conserved sequences are likely to form the binding sites for extravesicular AsA− and intravesicular MDA, respectively, the present results support our proposal that the two heme b centers are located on both sides of the vesicular membranes in close contact with the AsA− and MDA-binding sites (15).

It is important to note that this is the first direct observation of electron transfer between a ferrous hemoprotein and an MDA radical generated by pulse radiolysis. In the previous report, we could not observe such electron transfer reactions with MDA radical for several ferrous hemoproteins (19), although the reactions are expected to be energetically favorable on the basis of redox potential differences between MDA7AsA− ($E_{m,7} = 330 \text{ mV}$) (34) and hemoproteins (35). It is evident that, for biological molecules, the occurrence of electron transfer with MDA radical cannot be predicted simply in terms of the redox potentials (19). The highest rate constant for the reaction of MDA radical with biological molecules was obtained for MDA reductase (2.6 × $10^8 \text{ m}^{-1} \text{s}^{-1}$) (20). We have proposed previously that the high rate constant of the enzymatic reaction might be governed by both the specific geometry of substrate within the active site and the redox potential difference. The facilitated electron transfer reaction in MDA reductase is likely to be provided by several cationic amino acid residues near the active site, which may have a role in electrostatic guidance of the anionic MDA radical substrate to the active center (20). It is noteworthy that, in the intravesicular side of cytochrome $b_{561}$, there are several conserved positively charged residues located in the loop connecting helices 3 and 4, and the fully conserved sequence (120SLHSW124) follows this region immediately (15).

In conclusion, the following unique properties of cytochrome $b_{561}$ have now become evident. First, an efficient transmembrane electron transfer is catalyzed by this hemoprotein, using AsA− as a physiological electron supply. Second, the two heme b centers have distinct roles for the electron donation to MDA radical and the electron acceptance from AsA−. Further studies are in progress to reveal the structural properties of this cytochrome.

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