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Reversely-Oriented Cytochrome b_{561} in Reconstituted Vesicles Catalyzes Transmembrane Electron Transfer and Supports Extravesicular Dopamine β-Hydroxylase Activity

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Cytochrome b_{561} from bovine adrenal chromaffin vesicles contains two heme B prosthetic groups. We verified that purified cytochrome b_{561} can donate electron equivalents directly to cytochrome *c*. The purified cytochrome b_{561} was successfully reconstituted into cholesterol-phosphatidylcholine-phosphatidylglycerol vesicles by a detergent-dialysis and extrusion method. When ascorbate-loaded vesicles with cytochrome b_{561} were mixed with ferricytochrome *c*, the intravesicular ascorbate was able to reduce external thiazole blue or cytochrome *c*. The reduction of thiazole blue or cytochrome *c* was dependent on the presence of cytochrome b_{561} in the vesicle membranes. Pre-treatment of cytochrome b_{561} with diethylpyrocarbonate suppressed the reduction of extravesicular cytochrome *c* significantly, confirming that the reduction was not due to leakage of ascorbate from the vesicles. The topology of the reconstituted cytochrome b_{561} in the vesicle membranes was examined by treatment with trypsin followed by SDS-PAGE and MALDI-TOF-MS analyses. Only one major cleavage site at Lys191 was identified, indicating that cytochrome b_{561} was reconstituted into the membranes in an inside-out orientation irrespective of the modification with diethylpyrocarbonate. The addition of a soluble form of dopamine β-hydroxylase to the external medium resulted in the successful reconstitution of the hydroxylation activity towards tyramine, an analogue of dopamine, suggesting that a direct electron transfer *via* complex formation occurred. This activity was enhanced significantly upon the addition of ferricyanide as a mediator between cytochrome b_{561} and dopamine β-hydroxylase.

Key words: ascorbate, cytochrome b_{561}, dopamine β-hydroxylase, reconstituted vesicle, transmembrane electron transfer.

Abbreviations: AsA, ascorbate; DBH, dopamine β-hydroxylase; MDA, monodehydroascorbate; DEPC, diethyl pyrocarbonate; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MTT, 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2*H*-tetrazolium bromide; thiazole blue.

In neurosecretory vesicles, such as adrenomedullary chromaffin vesicles and pituitary neuropeptide secretory vesicles, intravesicular ascorbate (AsA)[2] functions as the electron donor for copper-containing monooxygenases including dopamine β-hydroxylase (DBH) and peptidylglycine α-amidating monooxygenase (*1*, *2*). In these monooxygenase reactions, the monodehydroascorbate (MDA) radical is formed within the vesicles by the univalent oxidation of AsA (*3*). Since neither AsA nor the MDA radical can pass through the vesicle membrane (*4–6*), it is believed that the intravesicular MDA radical is reduced back to AsA by membrane-spanning cytochrome b_{561}, and that, subsequently, the oxidized cytochrome b_{561} is reduced by AsA on the extravesicular side (*i.e.*, the cytosolic side). The cytosolic AsA is subsequently regenerated by reduction of the MDA radical with NADH *via* NADH-cytochrome b_5 reductase (*7*). It has been demonstrated that cytochrome b_{561} in chromaffin vesicles can transfer electrons across the vesicle membrane in either the inward (*8*) or outward direction (*9*). It is also known that cytochrome b_{561} in chromaffin vesicles is reduced by AsA and oxidized by the MDA radical at its outer surface (*10*).

The purified cytochrome b_{561} was found to contain two distinct heme *b* centers (*11*). In the oxidized state, one heme *b* shows a usual low spin EPR signal (g_z = 3.14), and the other a highly anisotropic low spin EPR signal (g_z = 3.70) (*11*, *12*). It must be noted, however, that several research groups have different views regarding the heme content and EPR species (*13–15*). Based on the deduced amino acid sequence, we proposed a structural model of cytochrome b_{561} (*16*) in which there are two well-conserved regions and fully-conserved His residues in the sequences. The conserved sequences are likely to form a part of the binding sites for the extravesicular AsA and the intravesicular MDA radical (*16*), whereas pairs of His residues might bind two hemes *b* in close contact with these binding sites (*16*). Our pulse radiolysis analysis

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showed that, indeed, the two heme *b* centers play distinct roles in electron donation to the MDA radical and the electron acceptance from AsA (*17*). We found further that the electron accepting ability from AsA is selectively destroyed by the treatment of oxidized cytochrome b_{561} with diethylpyrocarbonate (DEPC) (*18*, *19*). However, the electron donating activity from the reduced heme *b* center to the MDA radical was retained after the treatment (*18*). Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometric analyses revealed that two fully conserved histidyl residues (His88 and His161), possible heme ligands, and one well-conserved lysyl residue (Lys85) on the extravesicular side, are the modification sites (*18*). Further, the electron accepting ability from AsA is protected by the presence of AsA during the DEPC treatment, suggesting that the AsA-binding site is on the extravesicular side (*19*, *20*).

It has been believed that, among various cytochromes, cytochrome b_{561} is unique in its localization to neuroendocrine cells in the adrenal medulla, gut, pituitary and different regions of brain (*21*, *22*). However, the situation is now changing drastically. As whole genome projects for various organisms have proceeded, we have come to realize that there are many akin and distant members of the cytochrome b_{561} family (*23–25*). Therefore, elucidation of the precise mechanism of electron transfer to/from cytochrome b_{561} is increasingly important and will be very valuable for clarifying the physiological roles of these transmembrane electron carriers.

In this study, we show that our highly purified cytochrome b_{561} in reconstituted phospholipid-cholesterol vesicle membranes catalyzes transmembrane electron transfer. Further, we show that cytochrome b_{561} in AsA-loaded vesicle membranes can supply electron equivalents to support extravesicular DBH activity without the addition of any mediator, but that this activity is enhanced significantly by the addition of ferricyanide.

MATERIALS AND METHODS

Purification of Cytochrome b_{561}—Cytochrome b_{561} was purified from bovine adrenal medullary chromaffin vesicles to a homogeneous state as described previously (*11*). The purity of the cytochrome b_{561} was analyzed by visible absorption spectrophotometry, heme content analysis, and SDS-polyacrylamide gel electrophoresis (*11*). All other reagents were commercially obtained in analytical grade. The concentration of cytochrome b_{561} was determined using a millimolar extinction coefficient of 267.9 mM^{-1} cm^{-1} at 427 nm in the reduced state (*11*).

Modification of Cytochrome b_{561} with DEPC—The concentrated cytochrome b_{561} solution was acidified to pH 6.5 by adding 0.5 M potassium phosphate buffer (pH 6.0) and oxidized by the step-wise addition of potassium ferricyanide solution (100 mM). Complete oxidation was confirmed by visible absorption spectroscopy. The oxidized cytochrome b_{561} was gel-filtered through a PD-10 column (Amersham Pharmacia Biotech) equilibrated with 50 mM potassium phosphate buffer (pH 6.0) containing 1.0% (w/v) octyl β-glucoside, and then diluted with the same buffer to an appropriate concentration. The diluted sample was treated with DEPC (final 0.5 mM) for 30min, as previously described (*18*). The DEPC treatment of cytochrome b_{561} in the presence of 20 mM of sodium ascorbate was done similarly. The DEPC-treated samples were gel-filtered through a PD-10 column equilibrated with 50 mM potassium phosphate buffer (pH 6.0) containing 1.0% (w/v) octyl β-glucoside to remove unreacted DEPC. The DEPC-treated cytochrome b_{561} sample was then analyzed for reactivity with AsA with a Shimadzu UV-2400PC spectrophotometer (Kyoto). Finally, the sample was fully reduced with sodium dithionite and its absorption spectrum was recorded to check the integrity of the heme moiety.

Purification of the Soluble Form of Dopamine β-Hydroxylase—A soluble form of dopamine β-hydroxylase (DBH) was purified from bovine adrenal medulla according to the procedure of Ljones *et al*. (*26*) with several minor modifications (Seike *et al.*, unpublished). The purified DBH was analyzed by SDS-PAGE to confirm homogeneity and stored at –80°C until use.

Direct Electron Transfer Assay from Cytochrome b_{561} to Ferricytochrome c—The ability to donate an electron directly from reduced cytochrome b_{561} to ferricytochrome *c* was assayed as follows. The purified cytochrome b_{561} in 50 mM potassium phosphate buffer (pH 6.0) containing 1.0% octyl β-glucoside was fully-reduced with sodium dithionite followed by rapid gel-filtration through a PD-10 column (Amersham Pharmacia Biotech) equilibrated with the same buffer. The reduced cytochrome b_{561} (2.0 μM, 0.5 ml) was then mixed with an equal amount of ferricytochrome *c* (2.0 μM, 0.5 ml) (on a heme basis) and the spectral changes were recorded with a Shimadzu UV-2400PC spectrophotometer with a scan interval of 2 min for at least 30 min at room temperature. The direct electron transfer from the DEPC-treated cytochrome b_{561} to ferricytochrome *c* was done similarly, except for the temperature. Due to the increased auto-oxidizabilty of the reduced heme *b* of cytochrome b_{561} after the DEPC treatment, the gel-filtration and following spectroscopic monitoring were done at 13°C instead.

Preparation of AsA-Loaded Vesicles without Cytochrome b_{561}—To prepare AsA-loaded vesicles without cytochrome b_{561}, 15 mg of a mixture of dipalmitoyl-L-α-phosphatidyl-DL-glycerol, dipalmitoyl-L-α-phosphatidylcholine, and cholesterol (0.2:1:1; molar ratio) (Presome PPG1, Nippon Fine Chemical, Tokyo) was combined with 1.0 ml of 100 mM sodium ascorbate in 20 mM potassium phosphate buffer (pH 7.0) containing 0.15 M NaCl. Then, the solution was treated with 10 cycles of freeze (in liquid nitrogen) and thaw to ensure solute equilibration between the trapped and bulk solutions. The multilamellar vesicles were extruded through a polycarbonate filter (pore size, 200 nm; Nuclepore, Pleasanton, CA, USA) mounted in the mini-extruder (LiposoFast Basic, Avestin, Ottawa, Canada), as previously described (*27*). The sample was subjected to 31 passes through the filter to obtain single-lamellar vesicles and to avoid contamination of the samples by residual large vesicles, which might not have passed through the filter. The single-lamellar vesicles thus obtained were gel-filtered through an Ampure SA column (Amersham Life Science) equilibrated with 20 mM potassium phosphate buffer (pH 7.0) containing 0.15 M NaCl at room temperature.

Preparation of AsA-Loaded Vesicles wih Cytochrome b_{561}—Reconstitution of cytochrome b_{561} into the AsA-loaded vesicle membranes was done as follows. The purified cytochrome b_{561} (typically, 7.5 nmoles) in 50 mM potassium phosphate buffer (pH 6.0) containing 1.0% octyl β-glucoside was mixed rapidly with the multi-lamellar vesicles prepared in 20 mM potassium phosphate buffer (pH 7.0) containing 0.15 M NaCl and 100 mM sodium ascorbate. The mixture was then dialyzed extensively against 500 ml of 20 mM potassium phosphate buffer (pH 7.0) containing 0.15 M NaCl and 100 mM of sodium ascorbate at 4°C overnight with several changes. After dialysis, the multi-lamellar vesicles were extruded through a polycarbonate filter (typically, 200 nm pore size) similarly to the AsA-loaded vesicles without cytochrome b_{561} to obtain single-lamellar vesicles. The AsA-loaded vesicles without cytochrome b_{561} were gel-filtered through an Ampure SA column (Amersham Life Science) equilibrated with 20 mM potassium phosphate buffer (pH 7.0) containing 0.15 M NaCl at room temperature. The opalescent, pale pink vesicles were diluted appropriately with the same buffer. The reconstitutions of cytochrome b_{561} pretreated with DEPC either in the presence or absence of 20 mM AsA into AsA-loaded vesicle membranes were done similarly. The concentration of cytochrome b_{561} in the vesicle membranes was determined spectrophotometrically from the reduced *minus* oxidized difference spectra.

Transmembrane MTT Reductase Assay—Electron transfer from AsA trapped inside the vesicles (AsA; 100 mM) to the external medium was assayed by the reduction of extravesicular MTT [3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2*H*-tetrazolium bromide; thiazole blue]. The assay is based on the reduction of a yellow, water-soluble monotetrazolium salt, MTT, to an insoluble red-purple formazan (*28*). Twenty microliters of MTT solution (5 mg/ml in 20 mM potassium phosphate buffer (pH 7.0) containing 0.15 M NaCl) was mixed with 1.0 ml of AsA-loaded vesicles with or without cytochrome b_{561} (diluted 20-fold with the same buffer) followed by incubation at 20°C for varying times. The medium and MTT solutions were then removed by centrifugation, and 1.0 ml of dimethyl sulfoxide was added to the precipitate to solubilize the formazan. Absorbance was read at 540 nm using a Shimadzu UV-2400PC spectrophotometer.

Transmembrane Electron Transfer to Extravesicular Cytochrome c—Transmembrane electron transfer from AsA trapped inside the vesicles (AsA; 100 mM) was also examined by observing the spectral change of ferricytochrome *c* added to the external medium. Typically, 10 μl of ferricytochrome *c* (horse heart type IV; 10 μM) was mixed with a 40-fold-diluted vesicle solution (1.0 ml) in a cuvette and the spectral change was monitored using a Shimadzu UV-2400PC spectrophotometer in automatic scanning mode for 30 min at 20°C. Then, the vesicles were lysed by the addition of Triton X-100 (final 1.0%) and full reduction of cytochrome *c* was confirmed to assess the intactness of the vesicles.

Extravesicular Dopamine β-Hydroxylase Activity Supported by AsA-Loaded Vesicles with Cytochrome b_{561}—To demonstrate the ability of cytochrome b_{561} to transfer electrons to the soluble form of DBH, 300 μl of vesicles containing approximately 0.18 nmol of cytochrome b_{561} in the membranes and 100 mM AsA in the lumen were suspended in 100 μl of 20 mM potassium phosphate buffer (pH 7.0) containing 0.15 M NaCl. All samples contained 10 mM tyramine, 2000 units of catalase, 10 μM of ferricyanide, and 10 units of ascorbate oxidase. After the addition of 10 μl of the soluble form of DBH (0.414 mg/ml = 5.45 μM of DBH protein in the same buffer), the samples were incubated in 1.5-ml Eppendorf microtubes at 4°C for 4.5 h using a rotary mixer (TAITEC, model RT-50). At appropriate intervals, the tube was centrifuged at 15,000 rpm for 10 min and the supernatant was analyzed by a Shimadzu LC-VP HPLC-system (Shimadzu Corp, Kyoto) equipped with an ODS column (IRICA QC-Pack C18; ϕ4.6 mm × 250 mm). The HPLC-system was operated in isocratic mode using a mobile phase composed of 17%(v/v) methanol in H_2O, containing 10 mM acetic acid, 10 mM 1-heptanesulfonic acid (the ion-pairing reagent), and 12 mM tetrabutylammonium phosphate at a flow rate of 0.8 ml/min. Peaks were detected with an SPD-10A*VP* UV/Vis detector (Shimadzu Corp.) at 280 nm.

Trypsin Treatment of Cytochrome b_{561} inVesicle Membranes—The topology of cytochrome b_{561} in the reconstituted vesicle membranes was assayed by partial digestion with trypsin followed by SDS-PAGE analysis. To the AsA-loaded vesicles containing cytochrome b_{561}, TPCK-treated trypsin (bovine pancreas, Sigma Chemical, St. Louis, MO) was added at a molar ratio of 4:1, and the mixture was incubated at 4°C overnight or more. Aliquots of the vesicles were withdrawn at appropriate intervals and washed extensively with 20 mM potassium phosphate buffer (pH 7.0) containing 0.15 M NaCl by a repeated (5 cycles) centrifugation (18,000 rpm × 20 min; Kubota) and homogenization to remove the trypsin. The vesicles were finally treated with 20 mM potassium phosphate buffer (pH 7.0) containing 2.0% octyl β-glucoside for 2h on ice to solubilize the vesicle membranes, followed by centrifugation at 18,000 rpm × 40 min. A part of the reddish supernatant was subjected to SDS-PAGE analysis. Residuals were concentrated by centrifugation through Amicon Microcon YM-3 filters (MWCO=3,000) (Millipore Corporation, Bedford, MA, USA) for MALDI-TOF-MS analysis.

MALDI-TOF Mass Spectrometry—Mass spectrometric analyses were carried out on a Voyager RP mass spectrometer (Perseptive Biosystems, Farmingham, MA) using a 20 kV accelerating voltage as previously described (*20*). The positions of the protease cleavage sites in the cytochrome b_{561} amino acid sequence were identified based on the molecular masses of the polypeptide fragments detected by MALDI-TOF mass spectrometry and the specificity of the proteases used. The search for corresponding fragments in the amino acid sequence of cytochrome b_{561} was carried out using the program GPMAW (v 3.15) (Lighthouse Data, Odense M, Denmark). The molecular masses of all polypeptides measured matched the theoretical values obtained from the bovine cytochrome b_{561} amino acid sequence (*29*), except for the posttranslational acetylation of the NH_2-terminal Met residue (*30*).

RESULTS

The reconstitution of cytochrome b_{561} into vesicle membranes was achieved by a combined method consisting a classical detergent dialysis method followed by a mem-

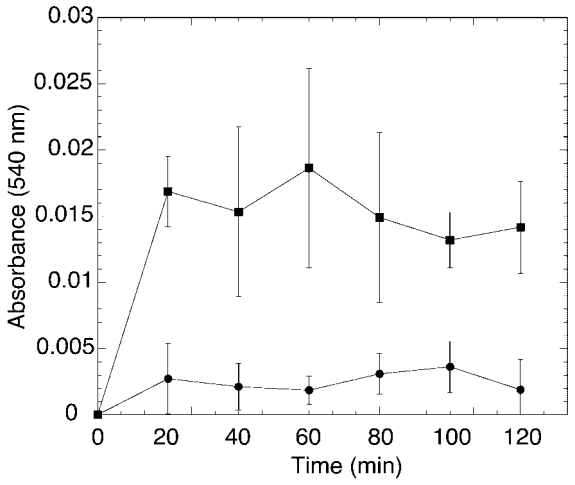

Fig. 1. **Transmembrane electron transfer to extravesicular MTT from AsA-loaded vesicles with cytochrome b_{561}.** The AsA-loaded vesicles (AsA; 100 mM) were prepared either with (solid squares) or without cytochrome b_{561} (solid circles). The assay is based on the reduction of a water-soluble MTT to an insoluble red-purple formazan on the extravesicular side. One milliliter of dimethyl sulfoxide was added to the precipitate to solubilize the formazan and the absorption at 540 nm was measured. Experimental details are described in the text. Error bars indicate the standard deviation (for 3 independent measurements).

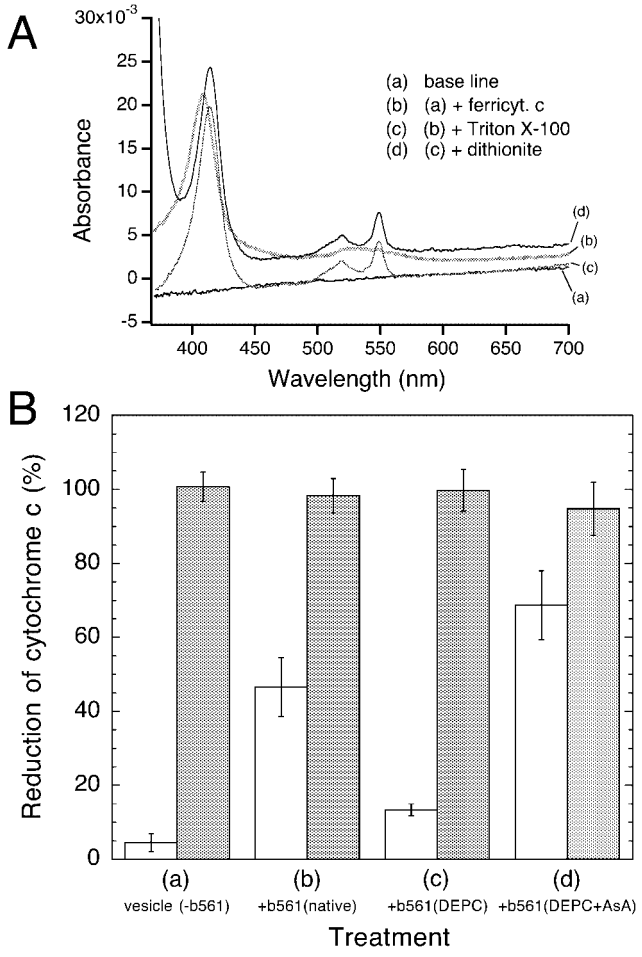

Fig. 2. **Transmembrane electron transfer to extravesicular cytochrome *c* from AsA-loaded vesicles with or without cytochrome b_{561}.** Panel A shows the absence of transmembrane electron transfer from AsA-loaded vesicles (AsA; 100 mM) without cytochrome b_{561}: trace (a), base line; trace (b), spectrum measured 30 min after the addition of ferricytochrome *c* to the vesicle; trace (c), spectrum measured 20 min after the addition of Triton X-100 (final 1%) to the above sample; and trace (d), fully-reduced spectrum of the above sample measured after the addition of sodium dithionite. Panel B shows the final percent reduction of extravesicular cytochrome *c* upon addition to various AsA-loaded vesicles (AsA; 100 mM). (a) AsA-loaded vesicles without cytochrome b_{561}, (b) AsA-loaded vesicles with native cytochrome b_{561}, (c) AsA-loaded vesicles with cytochrome b_{561} pretreated with DEPC in the oxidized state, (d) AsA-loaded vesicles with cytochrome b_{561} pretreated with DEPC in the presence of 20 mM AsA. Open columns indicate the percent reduction of cytochrome *c* 30 min after addition to vesicles; shaded columns indicate the percent reduction 20 min after the further addition of Triton X-100 (final 1%) to the above samples. All values were normalized to the dithionite-reduced state based on the absorbance difference between 550.0 and 565.0 nm. Error bars indicate the standard deviation (for 3 independent measurements).

brane extrusion technique. Since the purified cytochrome b_{561} solution contained 1% octyl β-glucoside, the detergent dialysis method was required for successful incorporation into vesicle membranes. Extensive dialysis of the mixture containing cyotchrome b_{561}, dipalmitoyl-L-α-phosphatidyl-DL-glycerol (DPPG), dipalmitoyl-L-α-phosphatidylcholine (DPPC), cholesterol and 100 mM AsA against the AsA-containing buffer, followed by cycles of freezing and thawing, resulted in the insertion of cytochrome b_{561} into multi-lamellar vesicles. The extrusion technique was then employed. This method is particularly powerful for the preparation of single-lamellar vesicles, as demonstrated in the present and other studies (*27*, *31*, *32*). AsA-loaded vesicles without cytochrome b_{561} could be prepared in a similar manner within a few hours. The AsA-loaded vesicles without cytochrome b_{561} were found to be very stable. Additions to the external medium did not cause any appreciable reduction of MTT (Fig. 1) or ferricytochrome *c* [Fig. 2, A and B-(a)] after long periods (up to 2 h) of incubation at an ambient temperature. Inclusion of AsA in the vesicle lumen was confirmed by the full reduction of ferricytochrome *c* (Fig. 2A) upon the addition of 1% Triton-X100 leading to the spontaneous rapture of the single-lamellar membranes.

The AsA-loaded vesicles with cytochrome b_{561} could supply electron equivalents to external MTT (Fig. 1) and external ferricytochrome *c* [Fig. 2B-(b)]. To demonstrate that the transmembrane electron transfer from intravesicular AsA was dependent on cytochrome b_{561} in the membranes, the DEPC-pretreated cytochrome b_{561} was reconstituted into the AsA-loaded vesicle membranes in a similar way. The reduction of external ferricytochrome *c* was inhibited significantly as shown in Fig. 2B-(c). The inclusion of AsA within the vesicle was confirmed by the full reduction of cytochrome *c* upon the addition of 1% (final) Triton X-100 in both cases. Thus, the inhibition of electron transfer to cytochrome *c* was due to the pretreatment of cytochrome b_{561} with DEPC, which causes a specific inability to accept electron equivalents from AsA (*18*, *20*). As a control, cytochrome b_{561} was similarly treated with DEPC but in the presence of AsA (20 mM), followed by reconstitution into AsA-loaded vesicle mem-

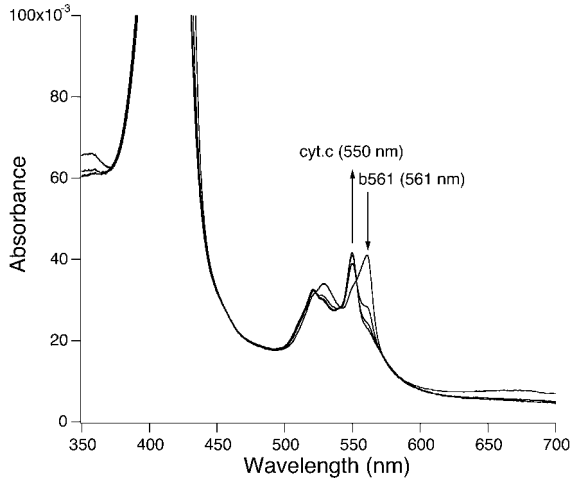

Fig. 3. **Direct electron transfer from reduced cytochrome b_{561} to ferricytochrome *c* in the detergent-solubilized state.** Equal amounts of reduced cytochrome b_{561} (2.0 μM, 0.5 ml) in the detergent-solubilized state and ferricytochrome *c* (2.0 μM, 0.5 ml) were mixed in a cuvette and the spectral changes were recorded with a scan interval of 2 min. Experimental details are described in the text.

branes. In this case, the transmembrane electron transfer was fully recovered [Fig. 2B-(d)], confirming that the inhibition of electron transfer by the DEPC treatment could be prevented by the inclusion of AsA during the treatment to suppress the *N*-carbethoxylation of the conserved His residues that might participate in the acceptance of electron equivalents from AsA (*18*, *20*). The electron transfer rate from intravesicular AsA *via* cytochrome b_{561} to ferricytochrome *c* was estimated based on the increase in the concentration of ferrocytochrome *c* (Fig. 2B). For AsA-loaded vesicles without cytochrome b_{561}, external ferricytochrome *c* was reduced at a rate of 0.187 nM/min, whereas for AsA-loaded vesicles with cytochrome b_{561}, external cytochrome *c* was reduced at a rate of 1.68 nM/min. Pretreatment of cytochrome b_{561} with DEPC in the oxidized state caused a reduction rate as low as 0.373 nM/min. Inclusion of AsA (20 mM) during the DEPC treatment caused an increase in the reduction rate of external cytochrome *c* to as high as 2.24 nM/min.

The direct electron transfer from the detergent-solubilized form of reduced cytochrome b_{561} to ferricytochrome *c* was confirmed by a direct mixing method as shown in Fig. 3. The dithionite-reduced form of DEPC-treated cytochrome b_{561} (although it auto-oxidized rather quickly) could also donate electron equivalents directly to ferricytochrome *c* in the detergent-solubilized state (data not shown). These results suggest that the reduced form of cytochrome b_{561}, irrespective of the DEPC treatment, can form a complex with ferricytochrome *c* to donate electron equivalents. These observations support the notion that the reduction of external ferricytochrome *c* is fully due to transmembrane electron transfer *via* cytochrome b_{561} in the membranes.

To investigate the topology of cytochrome b_{561} in the reconstituted vesicle membranes, the AsA-loaded vesicles with cytochrome b_{561} were treated with TPCK-treated trypsin at 4°C for a longer time. After removal of trypsin in the external medium by extensive washings with buffer (by repeated cycles of homogenization and centrifugation), followed by solubilization with octyl β-glucoside, the digested product was analyzed by SDS-PAGE. As shown in Fig. 4, only one major proteolytic product with an apparent molecular mass of 22 kDa was accumulated (lanes d, f, h, and j). This form did not undergo further degradation, although a very faint proteolytic product with an apparent molecular mass of 18 kDa was observed after a longer period of incubation (lanes h and j). To clarify the nature of these proteolytic products, the solubilized digest was concentrated and analyzed by a MALDI-TOF-MS technique (Fig. 5). The major proteolytic product corresponding to the 22 kDa band was found to have an M/z ~ 21,228. Although the theoretical molecular weight of peptide 1–191 is 21,185.2, we found recently that the amino-terminus of cytochrome b_{561} is acetylated (*30*), leading to an increase of 42.0 in molecular weight. Thus, we identified the 22 kDa band as a peptide comprising the N-terminus to Lys191 (peptide NAc-191). Other proteolytic products with M/z values of ~12,258, ~12,381, and ~12,515 were also observed. These products were assigned as peptides produced upon cleavage at the cluster of Lys and Arg residues (Arg111-Lys112-Lys113) in the intravesicular loop connecting helices 3 and 4; *i.e.*, peptide NAc-111, peptide NAc-112, and peptide NAc-113, respectively. Further, peptides corresponding to 112–191 (M/z ~ 9,008), 113–191 (M/z ~ 8,887), and 114–191 (M/z ~ 8,756) were identified. These results suggest that a majority of cytochrome b_{561} resided in the vesicular membranes in an inside-out orientation; *i.e.*, in the reverse direction from that observed in the

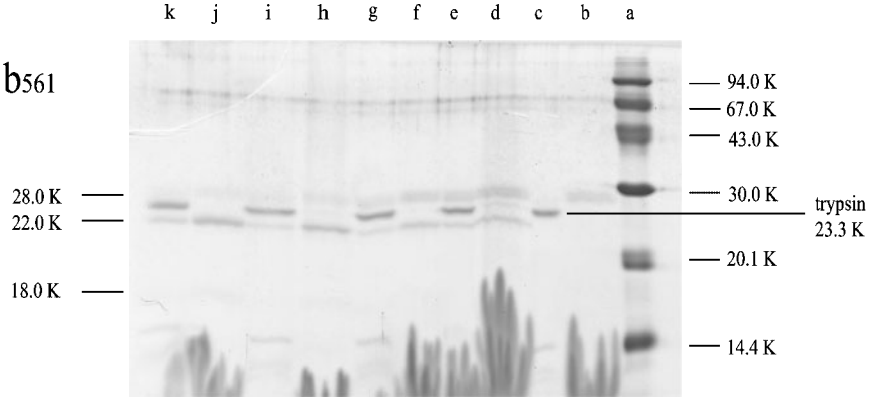

Fig. 4. **SDS–polyacrylamide gel analysis of tryptic peptides from cytochrome b_{561}–reconstituted vesicles.** The topology of cytochrome b_{561} in the reconstituted vesicle membranes was assayed by partial digestion with TPCK-treated trypsin followed by SDS-PAGE analysis. (a), LMW markers; (b), native b_{561}; (c), trypsin only (1.0 μg); (d), trypsin-treated b_{561} (for 4 h); (e), (d) + trypsin (1.0 μg); (f), trypsin-treated b_{561} (for 8 h); (g), (f) + trypsin (1.0 μg); (h), trypsin-treated b_{561} (for 24 h); (i), (h) + trypsin (1.0 μg); (j), trypsin-treated b_{561} (for 72 h); (k), (j) + trypsin (1.0 μg). Other experimental conditions are described in the text.

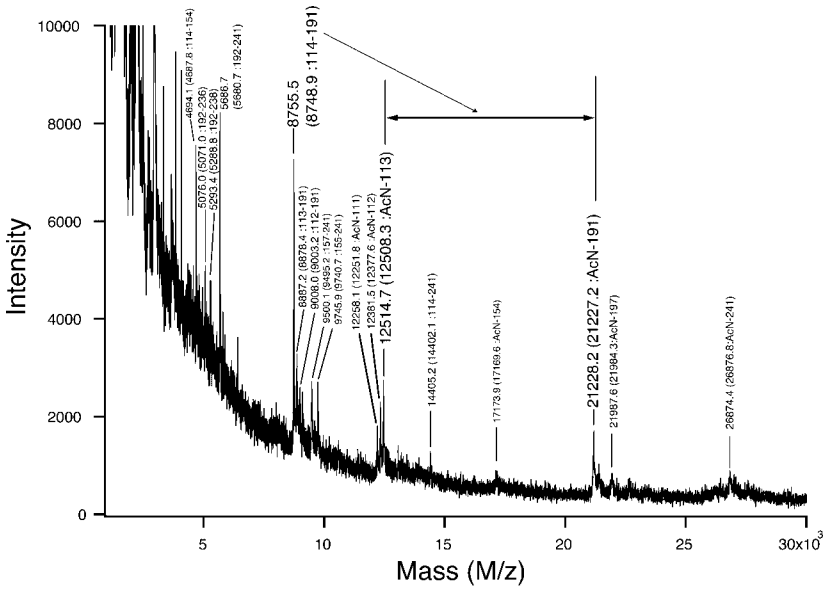

Fig. 5. **MALDI-TOF mass spectrum of tryptic peptides from cytochrome b_{561}–reconstituted vesicles.** Experimental conditions are described in the text. Major peaks are denoted by the observed mass (M/z). Assignment and theoretical mass based on the amino sequence of Perin *et al.* (*29*) are indicated in parentheses for each peak. Theoretical masses were calculated including the posttranslational acetylation at the N-terminus (*30*).

chromaffin vesicle membranes (Fig. 6). A minor proteolytic product corresponding to 18 kDa was detected at an M/z ~ 17,174 and identified as peptide NAc-154. Other peptides with a cleavage sites at Lys154 or Lys241 were found at ~26,874 (NAc-241), ~14,405 (114–241), ~9,746 (155–241), ~9,500 (157–241), and ~4,694 (114–154). Among these minor peptides, peptides 114–241 and 114–154 were likely derived from aggregates of cytochrome b_{561} not inserted in the single-lamellar membranes, since cleavage occurred on both the intravesicular and extravesicular sides.

DEPC-treated cytochrome b_{561}, either in the absence or presence of AsA, was also reconstituted into vesicle membranes, and its topology was analyzed in a similar manner. The results were the same as for the native species (data not shown). Thus, a major part of cytochrome b_{561} was reconstituted into vesicle membranes in an inside-out orientation irrespective of the DEPC treatment. Similar results were also obtained for vesicle preparations using membranes with other pore sizes ranging from 50 to 400 nm (data not shown).

The reversed orientation of cytochrome b_{561} in the AsA-loaded vesicle membranes suggests that the addition of the soluble form of DBH to the extravesicular medium will provide an opportunity to test transmembrane electron transfer from intravesicular AsA to extravesicular DBH via cytochrome b_{561}. It must be noted that the direction of electron flow is topologically a reversal of that in chromaffin vesicles. To exclude the possibility of the slow leakage of AsA from the intravesicular side during longer incubation times, ascorbate oxidase which scavenges AsA by converting it to dehydroascorbate, was added. The

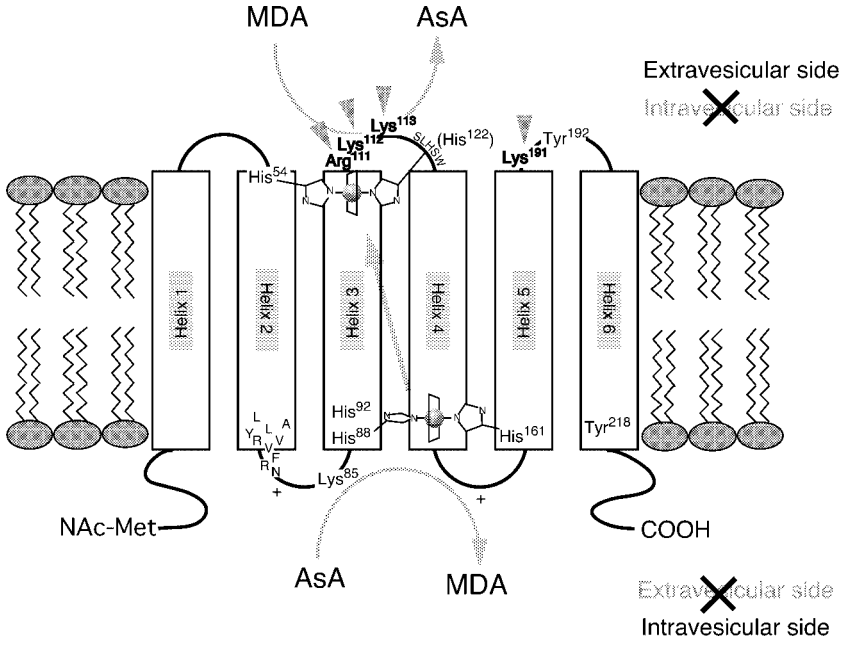

Fig. 6. **Transmembrane structural model of bovine cytochrome b_{561} and the cleavage sites upon treatment with TPCK-treated trypsin in reconstituted vesicles.** Two well-conserved sequences (^{69}ALLVYRVFR^{77} and ^{120}SLHSW^{124}) and five conserved histidyl residues (His54, His88, His92, His110, His122, and His161) are indicated based on the model in reference (*16*). His54 and His122 are likely to be the heme axial ligands on the intravesicular side, whereas His161 and His88 are considered to be the heme axial ligands on the extravesicular side. The heme *b* on the extravesicular side functions as an electron acceptor from AsA; the heme *b* on the intravesicular side may donate an electron equivalent to the MDA radical to reproduce AsA. One major and three minor cleavage sites of cytochrome b_{561} in the AsA-loaded vesicle membranes upon treatment with TPCK-treated trypsin are indicated by inverted solid triangles.

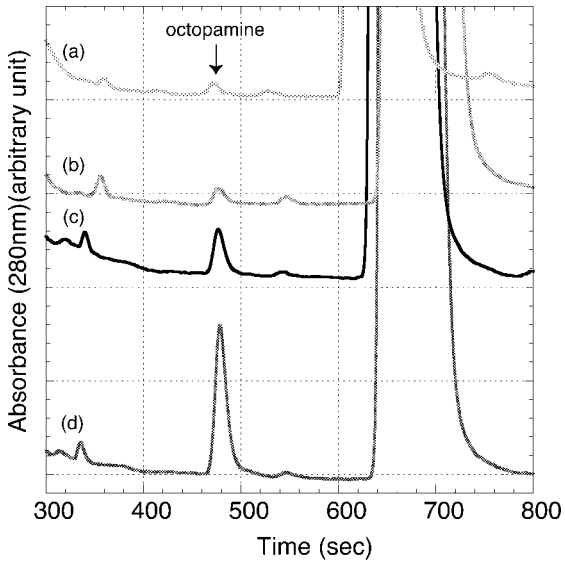

Fig. 7. **HPLC analysis of dopamine β-hydroxylase activity supported by transmembrane electron transfer from AsA-loaded vesicles with cytochrome b_{561}.** Traces (a) and (b) represent the activities upon mixing with the AsA-loaded vesicles without cytochrome b_{561} in the absence and presence of ferricyanide (10 μM), respectively. Traces (c) and (d) represent the activities upon mixing with the AsA-loaded vesicles with cytochrome b_{561} in the absence and presence of ferricyanide (10 μM), respectively. Other experimental conditions are described in the text. The peak around 480 s is due to the product, octopamine, whereas the huge broad peak around 660 s is due to the substrate, tyramine.

addition of the soluble form of DBH to the extravesicular medium of AsA-loaded vesicles without cytochrome b_{561} did not cause any appreciable formation of octopamine, an analogue of noradrenaline (Fig. 7a and Table 1). On the other hand, cytochrome b_{561}–containing AsA-loaded vesicles could supply electron equivalents to the external monooxygenase reactions (Fig. 7c and Table 1). The addition of 10 μM ferricyanide to the external medium enhanced the monooxygenase reaction significantly (5.4-folds) (Fig. 7d and Table 1). However, the addition of 10 μM ferricyanide to the external medium of the AsA-loaded vesicles without cytochrome b_{561} did not cause any appreciable formation of octopamine (Fig. 7b and Table 1). It is well established that ferrocyanide, the reduced form of ferricyanide, instead of AsA, can support DBH activity. These results suggest that direct electron transfer from cytochrome b_{561} to the soluble form of DBH did occur, probably *via* the formation of a bimolecular complex on the external surface of the membranes. Ferricyanide worked apparently as the redox mediator between the membranous cytochrome b_{561} and the soluble form of the monooxygenase in the absence of bimolecular complex formation and, thereby, enhanced the monooxygenase activity.

DISCUSSION

In the present study we demonstrate that purified cytochrome b_{561}, when reconstituted into an artificial phospholipid bilayer, is capable of transferring electron equivalents across the membrane (Figs. 1 and 2B) to reduce extravesicular MTT or ferricytochrome *c*. Further, this transmembrane electron transfer can support the monooxygenase activity of the soluble form of DBH (Fig. 7 and Table 1), even in the absence of a small redox mediator. The addition of a small redox mediator, ferricyanide, caused a significant increase in the monoxygenase activity (Fig. 7 and Table 1).

A similar experimental design was first reported by Njus *et al*. (*4*). They showed that AsA-loaded chromaffin vesicle "ghosts" are able to reduce an external electron acceptor *via* a transmembrane electron transfer. They suggested that cytochrome b_{561} was the logical vesicle membrane protein to catalyze this electron transfer reaction. Later, Srivastava *et al*. (*33*) reported that purified cytochrome b_{561}, when reconstituted into AsA-loaded liposome membranes, could catalyze transmembrane electron transfer to ferricytochrome *c*. Kent and Fleming (*2*) further demonstrated that purified cytochrome b_{561}, when reconstituted into AsA-loaded phosphatidylcholine vesicles, could supply transmembrane electrons for both the soluble and the membranous forms of DBH, as well as for peptidylglycine α-amidating monooxygenase activity.

Most importantly, we found that the electron transfer from cytochrome b_{561} to DBH does not necessarily require a redox mediator, which is different from the conclusion of Kent and Fleming (*2*). However, as shown in our present study and in previous studies (*33*, *34*), ferricytochrome *c* can receive electron equivalents directly from reduced cytochrome b_{561} in the detergent-solubilized state or in vesicular membranes without the addition of any redox mediator. The exposed surface of cytochrome b_{561} on the vesicular membranes can make enough contact with its proteineous electron acceptor, cytochrome *c*, to form a transient bimolecular complex for the electron

Table 1. **Reconstitution of transmembrane electron transport to the soluble form of dopamine β-hydroxylase.**

Assay conditions[a]	pmoles of octopamine formed/ pmolesof DBH/h
DBH + AsA-loaded vesicles without b_{561}	1.39 (±1.08)[b]
DBH + AsA-loaded vesicles without b_{561} + ferricyanide (10 μM)	3.53 (±0.78)[b]
DBH + AsA-loaded vesicles with b_{561}	6.52 (±2.23)[b]
DBH + AsA-loaded vesicles with b_{561} + ferricyanide (10 μM)	35.25 (±5.68)[b]
DBH + ferrocyanide (2.0 mM)	80.6 (±24.5)[b]
DBH + AsA (2.0 mM)	2,790 (±360)[b]
DBH (without AsA)	0.0

[a]Details are described in the text. Lower three assays were performed in the absence of the AsA-loaded vesicles.
[b]Three determinations (mean ± SD).

transfer reaction to occur (Figs. 2B and 3). The soluble form of DBH might also form a transient bimolecular complex with cytochrome b_{561} on the membrane surface, although the nature of the interaction might be very different from that of cytochrome *c* and, therefore, not be suitable for efficient monooxygenase activity. Physiologically, however, the direct electron transfer from cytochrome b_{561} to the soluble form of DBH will play only a minor role since the concentration of AsA in bovine chromaffin vesicles is around 20 mM (*35*). Ferricyanide (physiologically, the MDA radical) might shunt the electron from cytochrome b_{561} to the soluble form of DBH, leading to a significant increase in monooxygenase activity (Fig. 7 and Table 1).

The topology of cytochrome b_{561} in artificial bilayer membranes is consistent with the present results of the reconstituted enzymatic activities. We found that cytochrome b_{561} is inserted into vesicle membranes in an inside-out orientation (Fig. 6). The inverted orientation enables electron transfer from intravesicular AsA to the extravesicular surface *via* an authentic route (*i.e.*, the same route in the protein moiety with that in intact chromaffin vesicles). It must be stressed that previous studies using reconstituted artificial membranes (*2*, *33*) did not consider the topology of cytochrome b_{561} at all. In cases using AsA-loaded chromaffin vesicle "ghosts" (*4*, *10*, *36*, *37*), the direction of electron transfer was likely reversed or there might have been a mixture of both directions.

In our previous studies, we postulated that both the carboxy- and the amino-terminus of cytochrome b_{561} are exposed to the extravesicular side (*16*), and that the side containing the conserved sequence (^{69}ALLVYRVFR^{77}) is the electron-accepting site from AsA, whereas the other side is responsible for the electron donation to the MDA radical (*17*, *18*). We found further that the electron accepting ability from AsA was selectively destroyed by the DEPC modification at His88, His161 and Lys85 (*18*) without affecting the electron donating activity from the reduced heme *b* center to the MDA radical. These previous observations are fully consistent with our present results for the AsA-loaded vesicles with cytochrome b_{561}. We found later that the presence of AsA during the reaction with DEPC suppresses the carbethoxylation of the heme-coordinating histidyl (His88 and His161) residues, whereas the level of modification of Lys85 was not affected (*20*). Concomitantly, when AsA was used as a reductant, the final reduction level of heme *b* was protected, although the fast reduction process was not fully-restored (*19*, *20*). This result suggests the importance of the conserved Lys85 residue for the initial recognition and interaction with AsA on the extravesicular side (*19*, *20*). In the present study, however, we found that the transmembrane electron transfer rate for vesicles reconstituted with DEPC-treated cytochrome b_{561} in the presence of AsA showed no difference from (or an even higher rate) the vesicles reconstituted with native cytochrome b_{561}. This unexpected observation might be explained if the rate of the transmembrane electron transfer by cytochrome b_{561} is limited by the intramolecular electron transfer between the two heme *b* centers; in this case, the slow reduction of the heme *b* on the extravesicular side (in the reconstituted vesicles, this heme *b* resides on the intravesicular side) caused by the carbethoxylation of Lys85 must be compensated for by a very high intravesicular AsA concentration (100 mM).

In conclusion, we show that cytochrome b_{561} in reconstituted phospholipid-cholesterol vesicle membranes catalyzes transmembrane electron transfer. Pre-treatment of cytochrome b_{561} with DEPC suppresses the reduction of external cytochrome *c* significantly. Trypsin treatment followed by SDS-PAGE and MALDI-TOF-MS analyses showed that cytochrome b_{561} was reconstituted into the vesicles in an inside-out orientation and, therefore, the cytochrome could transport electron equivalents through the authentic route. Further, we show that cytochrome b_{561} in the AsA-loaded vesicle membrane can supply electron equivalents to support DBH activity in the extravesicular medium without the addition of any mediator, but that this activity is enhanced significantly upon the addition of ferricyanide.

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