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Histidine Cycle Mechanism for the Concerted Proton/Electron Transfer from Ascorbate to the Cytosolic Haem *b* Centre of Cytochrome b_{561}: A Unique Machinery for the Biological Transmembrane Electron Transfer

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Cytochromes b_{561} are a family of transmembrane proteins found in most eukaryotic cells and contain two haem *b* prosthetic groups per molecule being coordinated with four His residues from four different transmembrane α-helices. Although cytochromes b_{561} residing in the chromaffin vesicles has long been known to have a role for a neuroendocrine-specific transmembrane electron transfer from extravesicular ascorbate to intravesicular monodehydroascorbate radical to regenerate ascorbate, newly found members were apparently lacking in the sequence for putative ascorbate-binding site but exhibiting a transmembrane ferrireductase activity. We propose that cytochrome b_{561} has a specific mechanism to facilitate the concerted proton/electron transfer from ascorbate by exploiting a cycle of deprotonated and protonated states of the $N_{\delta 1}$ atom of the axial His residue at the extravesicular haem center, as an initial step of the transmembrane electron transfer. This mechanism utilizes the well-known electrochemistry of ascorbate for a biological transmembrane electron transfer and might be operative for other type of electron transfer reactions from organic reductants.

Key words: ascorbate, cytochrome b_{561}, electron transfer, haem axial His residue, membrane protein.

Abbreviations: AsA, ascorbate; MDA, monodehydroascorbate; DEPC, diethylpyrocarbonate; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight.

Cytochrome b_{561} residing in the chromaffin vesicle membranes participates in the transmembrane electron transfer from cytosolic ascorbate (AsA) to intravesicular monodehydroascorbate (MDA) radical to supply electron equivalents to intravesicular copper-containing monooxygenases. These are essential reactions for the production of various neurotransmitters within the vesicles (*1–4*). For this purpose, the cytochrome contains two haem centres on each side of the membranes (*5–12*). Site-directed mutagenesis studies on mouse (*13*) and bovine (*14*) cytochromes b_{561} confirmed our previous assignment of two pairs of His residues as the haem axial ligands locating on four different α-helices: His88(helix 3)-His161(helix 5) on the cytosolic side and His54(helix 2)-His122(end of helix-4) on the intravesicular side (Fig. 1) (*8*, *15*). There were two fully conserved regions in the neuroendocrine cytochrome b_{561} sequences; the first one (^{69}ALLVYRVFR^{77}) was considered to be present on the extravesicular side of α-helical segment, and the second one (^{120}SLHSW^{124}) to reside in an intravesicular loop connecting two α-helical segments, respectively (*8*). These conserved sequences may form a part of the putative binding sites for cytosolic AsA and intravesicular MDA radical, respectively. Thus, these two haems *b* reside on each side of the membranes in close contact with these binding sites, respectively (*8*) (Fig. 1).

As the whole genome projects for various organisms proceed, we gradually realized that there are so many akin and distant members of the cytochrome b_{561} family in animals (*16–18*), plants (*19*, *20*) and even in other primitive eukaryotes (*21*). Many pieces of evidence suggested that they constitute a novel class of transmembrane electron transport system. Since most of them are likely to have a role(s) for transmembrane electron transfer reactions, clarification of their physiological electron donor/acceptor is of a prime importance. For plant cytchrome b_{561}, AsA is postulated as a physiological electron donor (*22*, *23*) as found for neuroendocrine (bovine) cytochrome b_{561}. For other members of the cytochrome b_{561} protein family, AsA is also considered as a primary candidate (*17*, *18*, *24*). However, the molecular mechanism of such electron transfer reactions from AsA has been scarcely understood due to the absence of X-ray crystal structure of this very hydrophobic protein. Therefore, elucidation of the precise mechanism of the electron transfer to/from neuroendocrine cytochrome b_{561} is increasingly important and will be very valuable

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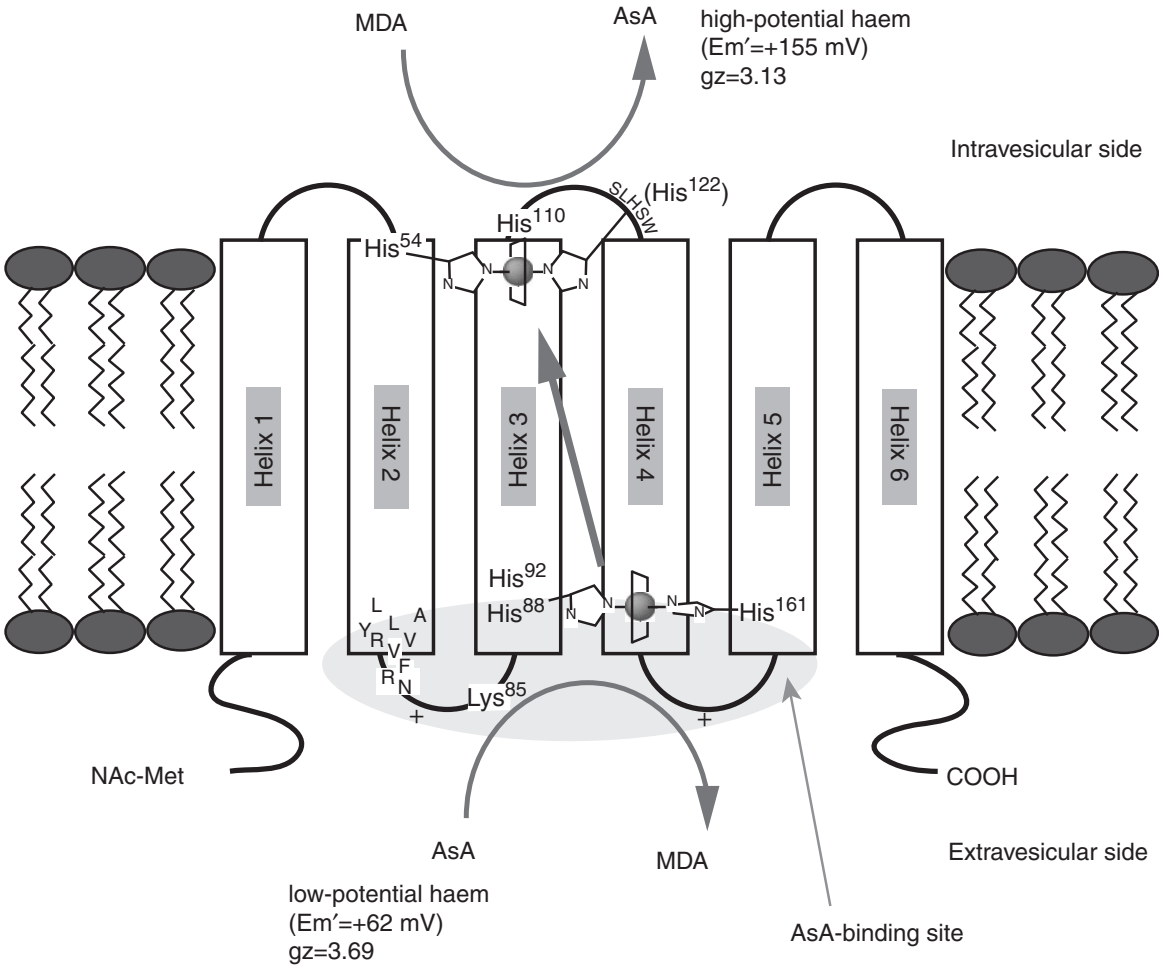

Fig. 1. **Transmembrane structural model of bovine cytochrome b_{561}.** Two well-conserved sequences (^{69}ALLVYRVFR^{77} and ^{120}SLHSW^{124}) and five conserved His residues (His54, His88, His110, His122 and His161) are indicated based on the model of (*8*). His54 and His122 are the haem axial ligands on the intravesicular side, whereas His88 and His161 are the haem axial ligands on the cytolic side. The haem *b* on the cytosolic side has a function for the electron acceptance from AsA; on the other hand, the haem *b* on the intravesicular side may donate electron equivalent to MDA radical to reproduce AsA. The assignment that the high-potential haem to intravesicular side and the low-potential haem to extravesicular (cytoplasmic) side was based on our following experimental results; (i) EPR analysis on the partially reduced state (*12*), (ii) EPR analysis on the alkaline-treated form (*8*, *12*), in which the electron donating ability to MDA radical was lost (*7*), (iii) EPR analysis on the DEPC-treated form, in which the haem centre ($g_z = 3.69$) on the extravesicular side could not be reduced with AsA (*10*), and (iv) EPR analysis on the 4-PDS-modified form, in which two Cys resides locating close to the intravesicular haem centre were selectively modified, showing perturbation on the $g_z = 3.14$ species only (*11*). Although our assignment is not consistent with a recent proposal by Kamensky *et al*. (*14*), in which the low-potential haem was assigned to the intravesicular side on the basis of EPR spectra of the haem axial ligand mutants, our model is in fully accordance with the notion of the direction of the physiological electron transfer (*i.e*. cytosolic AsA, low-potential haem, high-potential haem and intravesicular MDA radical) and is much more feasible.

to clarify the exact physiological roles of these new members of the di-haem containing transmembrane protein.

Tsubaki *et al*. (*25*) found that the electron accepting ability of bovine cytochrome b_{561} from AsA was selectively inhibited by the treatment with diethylpyrocarbonate (DEPC), a well-known His-specific reagent (*26*). However, the electron donating activity from reduced haem *b* centre to MDA radical was retained after the treatment (*25*). MALDI-TOF mass analyses revealed that two fully-conserved His residues (His88 and His161, the haem axial ligands on the cytosolic side) (*8*) and one well-conserved Lys residue (Lys85) were the major modification sites (*25*). Further, the electron-accepting ability from AsA could be protected by the presence of AsA during the DEPC-treatment, suggesting presence of an AsA-binding site on the cytosolic side (*27*) (Fig. 1). Interestingly, upon the modification with DEPC, there was neither a release of the haem *b* prosthetic group from the protein moiety nor any significant spectral perturbation of the two haem *b* centres as judged from the sodium dithionite-reduced visible absorption spectra (*25*). Since DEPC attacks the deprotonated nitrogen atom of an imidazole group (*26*), we postulated that the

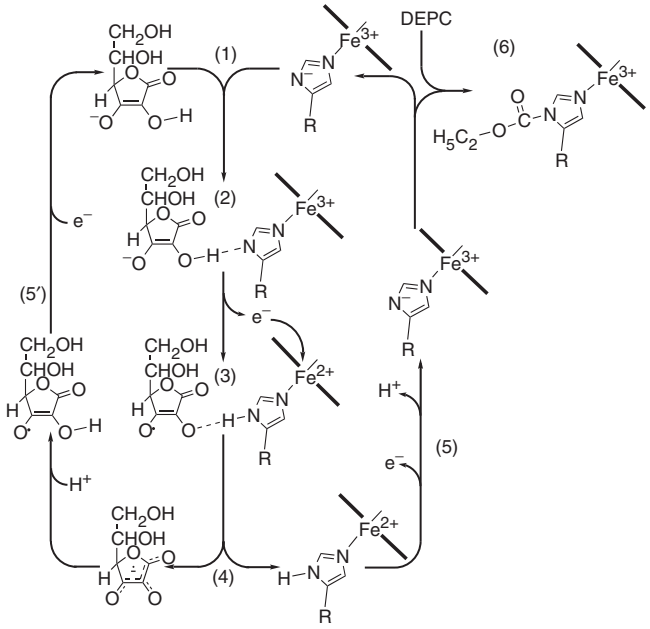

Fig. 2. **Postulated histidine cycle mechanism for the concerted proton/electron transfer reaction from AsA to the cytosolic haeme *b* centre of cytochrome b_{561}.** Details are described in the text. This scheme is based on the original model of Njus *et al*. (*41*, *46*, *47*) with modifications.

non-coordinated nitrogen atom of the coordinating imidazole group of the cytosolic haem centre might be specifically *N*-carbethoxylated (*25*). This specific *N*-carbethoxylation might be directly coupled to the inhibition of electron acceptance of the cytosolic haem centre from AsA.

In the present article, we propose a novel hypothetical mechanism for the AsA-specific electron transfer reaction catalysed by the cytochrome b_{561} protein family. Since the electron donation from AsA to the cytosolic haem center is the first step of the succeeding multiple electron transfer events in cytochrome b_{561}, its understanding might be a great importance for the elucidation of other type of electron transfer events catalysed by the members of this protein family.

SUPPORTING EVIDENCE

Mechanism of the DEPC Reactivity for Axial His Residues—The neutral imidazole ring of a His residue can exist in two tautomeric forms; either the $N_{\varepsilon 2}$-H tautomer or the $N_{\delta 1}$-H tautomer (*28*). DEPC is well known for its reactivity to a deprotonated nitrogen atom of an imidazole group (*26*). The reactivity of an imidazole group with DEPC will be influenced by (i) the steric accessibility of the imidazole to the solvent, (ii) the hydrogen-bonding interactions with other amino acid residues and (iii) the pK_a of the imidazole group. However, it must be noted that these factors will be mutually influenced. Majority of surface-exposed His residues were easily *N*-carbethoxylated upon treatment with DEPC as in cytochrome b_5 (*29*, *30*) or other hydrophilic proteins including α_1-acid glycoprotein (*31*), angiotensin II, insulin (*32*) and a myoglobin mutant (Nakanishi *et al*., unpublished data).

Axial His residues in haemoproteins, on the other hand, did not show such high reactivity to DEPC at all (*29*, *30*, *33*). The major cause of the low DEPC reactivity is the coordination bond between haem iron and axial imidazole group, which occurs through its $N_{\varepsilon 2}$ atom. Only a few exceptions are known to have a Fe–$N_{\delta 1}$ bond, such as the *c*-type low-spin haem-I in the tetra-haem cytochorme c_{554} from the bacterium *Nitrosomonas europaea* (*34*) and the Met65His mutant of the haem domain of cellobiose dehydrogenase (*35*). Accordingly, $N_{\delta 1}$ atom of the axial His residue is in a protonated state and this state is usually further stabilized by a hydrogen bonding by nearby amino acid residues (*36*). In addition, the haem prosthetic group is usually buried deeply in the protein moiety, leading to a significant shielding of the imidazole group from DEPC reagents (*30*).

Anomalous DEPC Reactivity of the Haem Axial Ligands of Cytochrome b_{561} Purified from Chromaffin Vesicles—As described in the introduction section, we observed distinct anomalies for the reaction of DEPC with cytochrome b_{561} purified from bovine chromaffin vesicles. These were as follows. (i) Treatment of oxidized cytochrome b_{561} with a lower concentration of DEPC (0.5 mM) caused a specific *N*-carbethoxylation of the haem-coordinating histidyl imidazole ligands (His88 and His161) on the cytosolic side and significant losses in the fast electron acceptance ability from AsA and in the final reduction level (*25*). *N*-carbethoxylations of only the two axial His residues were sufficient to cause a significant decrease (~1/20) in the electron transfer rate from AsA (*27*, *37*). (ii) Although small but distinct spectral changes occurred upon the DEPC treatment both in the oxidized (*25*) and in the reduced states (Nakanishi *et al*., unpublished data), there was no release of haem *b* prosthetic group from the protein moiety at all (*25*). (iii) Since DEPC reagent specifically attacks a deprotonated nitrogen atom of an imidazole ring as described above, it is indicative that the non-coordinating nitrogen atom (most likely $N_{\delta 1}$) of the coordinating imidazole group at the cytosolic haem centre is in a deprotonated form (anion form, or imidazolate form) at physiological pH and, therefore, might be specifically *N*-carbethoxylated. (iv) The efficient and specific reactivity of the axial His residues at the cytosolic haem centre to DEPC showed a remarkable contrast to the non-reactivity of the axial His residues (His54 and His122) at the intravesicular haem centre of cytochrome b_{561} under the same experimental condition (*25*).

Anomalous DEPC Reactivity of the Haem Axial Ligands of Plant Cytochrome b_{561}—One plant cytochrome b_{561} from *Zea mays* (=b561Z.mays/AB182641) showed a moderate similarity in amino acid sequences with bovine cytochrome b_{561} (37.7% identity) as found for other plant cytochromes b_{561}, suggesting a common 6-transmembrane α-helices with two haem centres (*21*). However, the conservation of the sequence corresponding to the putative AsA-binding site (^{67}AIMVYRVLP^{75}) was only marginal, although the putative MDA-binding sequence was well conserved (*21*) as found for other plant cytochromes b_{561} (*20*). Very recently, we succeeded

in the heterologous expression of *Zea mays* cytochrome b_{561} in *Pichia pastris* and its purification. We found that the purified protein contained two haem centres with distinct EPR signals ($g_z = 3.69$ and $g_z = 3.21$) and had an electron-donating ability to MDA radial (Nakanishi *et al*., unpublished data) as found for bovine cytochrome b_{561} (*12*). Very importantly, the anomalous DEPC reactivity was also conserved for *Zea mays* cytochrome b_{561}; *i.e*. DEPC-treatment of the purified protein in the oxidized form caused specific modifications of axial His residues (His86 and His159) at the cytosolic haem centre and one Lys residue (Lys83, corresponding to Lys85 of bovine cytochrome b_{561}) resulting in the inhibition of electron acceptance from AsA both in the rate of electron transfer and in the final haem reduction level (Nakanishi *et al*., unpublished data), as observed for bovine cytochrome b_{561} (*25*). Inhibition of the electron transfer from AsA upon the DEPC treatment was also reported for the partially purified form of an *Arabidopsis thaliana* cytochrome b_{561} (=At4g25570/Q8L856/b561A.tha5/Artb561-1; 67.0% identity with *Zea mays* cytochrome b_{561} and 33.9% identity with bovine cytochrome b_{561}) (*38*). These results suggest that anomalous environments at the cytosolic haem centre might be a common property of the cytochrome b_{561} protein family, at least, among the neuroendocrine and plant subgroups (*21*).

Presence of Substrate (AsA)-Binding Site—As one possible cause for such an anomalous DEPC reactivity to the $N_{\delta1}$ atom, presence of positively charged residue(s) at the putative substrate-binding site might be considered. Indeed, we have postulated presence of substrate (AsA)-binding site comprised with well-conserved Lys and Arg residues near the cytosolic haem centre (*21*, *27*). Such positively charged residues might have prime importance for the approach of AsA to the putative substrate-binding site and its accommodation. Further, presence of positive charges in the immediate vicinity of the $N_{\delta1}$ atom of the axial His residues might cause a significant decrease in its microscopic pK_a, leading to the stabilization of a deprotonated form of the $N_{\delta1}$ atom. The importance of these positively charged residues around the putative substrate-binding site for the electron transfer from AsA was supported by site-directed mutagenesis studies on mouse cytochrome b_{561} (*24*, *39*). Our stopped-flow analyses for the site-directed mutants of *Zea mays* cytochrome b_{561} confirmed the importance of Lys83; in which both K83A and K83E mutants showed a significant decrease in electron accepting rate from AsA, although their final haem reduction levels were almost the same as that of the wild-type protein (Nakanishi *et al*., unpublished data).

One may argue that presence of AsA will protect the reactive amino acid residues at the substrate-binding site from the DEPC-modification with steric hindrance. As expected, presence of AsA during the DEPC-treatment inhibited the modification of the axial His residues and protected the electron-acceptance ability from AsA significantly both in bovine (*25*, *27*) and *Zea mays* cytochromes b_{561} (Nakanishi *et al*., unpublished data). The protective effect of AsA against the DEPC-modification of Lys85 (of bovine cytochrome b_{561}) residue was, however, not so significant, leaving Lys85 as the sole *N*-carbethoxylated residue (*27*). These results support the notion that (i) the haem axial His residue(s) is likely to have a direct interaction with a bound AsA molecule at the substrate-binding site and, therefore, is a part of the substrate-binding site itself and (ii) Lys85 does not have a strong interaction with a bound AsA molecule, but may have a role for initial recognition of AsA or for assisting the approach of AsA to the substrate-binding site. It might be noted that the properties of the K83A and K83E mutants of *Zea mays* cytochrome b_{561} in the electron transfer reaction from AsA as described in previous section were very similar to that of the bovine cytochrome b_{561} pre-treated with DEPC in the presence of AsA, in which only Lys85 residue was *N*-carbethoxylated (*27*).

Role of the Haem Axial His Residues in Cytochrome b_{561} for the Electron Acceptance from AsA—Existence of the deprotonated $N_{\delta1}$ atom only at the axial imidazole rings of the cytosolic haem centre could explain the preferable modification of this site with a relatively low concentration of DEPC. Further, such unusual environments around the axial His residues of the cytosolic haem centre suggest a specific physiological role of the axial His residues during the AsA-related electron transfer reaction. Since the concomitant proton transfer from AsA is a prerequisite step for the fast biological electron transfer from AsA to an electron donor (*40*, *41*), we speculated that a cycle of deprotonated and protonated forms of axial imidazole $N_{\delta1}$ atom at the cytosolic haem centre might be essential for the fast electron acceptance from AsA in cytochrome b_{561}, as an initial step of the transmembrane electron transfer (*27*, *37*). Indeed, such a specific role of the deprotonated $N_{\delta1}$ atom in the axial imidazole group for the electron acceptance from AsA was inferred with a specific inhibition of the electron acceptance from AsA upon the $N_{\delta1}$-carbethoxylation (*25*, *27*). It is our intention to correlate the well-known electro-chemistry of AsA with a unique mechanism for the biological transmembrane electron transfer in the present article.

Hypothesis–Histidine Cycle Mechanism for the Concerted Proton/Electron Transfer Reaction (Fig. 2)—(i) In the oxidized state cytochrome b_{561}, the axial His ligand of the cytosolic haem *b* centre is in 'deprotonated' form.(ii) Binding of AsA to the substrate-binding site near the cytosolic haem *b* centre causes a hydrogen-bond formation from AsA to the deprotonated $N_{\delta2}$ atom of the axial His residue. (iii) Concerted proton/electron transfer occurs leading to a proton transfer to the imidazole $N_{\delta2}$ atom and the reduction of the cytosolic haem *b* centre. (iv) The resulting MDA radical at the substrate-binding site is eventually released into the medium. (v) The electron at the cytosolic haem *b* centre is further transferred to the other haem *b* centre after the transmembrane (intramolecular) electron transfer reaction and the proton at the axial imidazole group is transferred to outer surface of cytochrome b_{561} molecule *via* an unknown relay system. (v) The MDA radical in the cytosolic medium is reduced back to AsA by a specific reductase and is ready to bind the substrate-binding site

of cytochrome b_{561}. (vi) Treatment of oxidized cytochrome b_{561} with DEPC causes a specific *N*-carbethoxylation of the axial imidazole, which leads to an inhibition of electron acceptance from bound AsA due to the inability to accept a proton from AsA.

DISCUSSION

Spectral Change in the Haem Electronic Absorption Spectra after N_δ-Carbethoxylation of the Haem Axial His Residues—One may argue that the observed change in the haem electronic absorption spectra might be too small if it was actually caused by N_δ-carbethoxylation of the axial His residues (*25*). However, we thought that the observed minor perturbation in the electronic absorption spectra might be reasonable and was comparable to those found for H93G proximal cavity mutant of myoglobin, where the proximal His has been replaced with Gly, creating a cavity which can be occupied by a variety of exogenous ligands (*42*, *43*). Exogenously added imidazole could coordinate haem iron of H93G myoglobin as the proximal ligand, showing almost identical spectroscopic characteristics with those of wild-type myoglobin, despite of a significant rotation (~45°) of imidazole plane and a decreased distance between the haem and the proximal imidazole (*42*). Further, when imidazole was replaced with other small organic ligands, such as pyridine and methyl-substituted imidazoles, relatively minor absorption spectral changes occurred (*43*). Based on these previous studies, we concluded that the observed changes in the haem electronic absorption spectra were caused by the $N_{\delta1}$-carbethoxylation of the axial His residue at the cytosolic haem centre with maintaining its $N_{\varepsilon2}$–Fe bond (*25*).

A Marked Contrast in the DEPC Reactivity between the Two Haem Centers in Cytochrome b_{561}—We noticed a clear difference in the reactivity of DEPC reagent to the axial His residues between the cytosolic and the intravesicular haem centres (*25*, *27*). This fact might have a great importance to consider the physiological function and mechanism of cytochrome b_{561}. Most importantly, this observation excluded the possibility that the unusually high reactivity of DEPC towards the His residues of the cytosolic haem centre was caused by the intrinsic nature of cytochrome b_{561}. Locations of the two haem *b* groups are just on the opposite side of a cytochrome b_{561} molecule (Fig. 1) and, therefore, availability of the His residues at each haem centre to DEPC reagents would not be so much different each other, as far as the cytochrome b_{561} molecules being in a detergent-solubilized state. Accordingly, cause of the difference in the DEPC reactivity should be ascribed to the differences in local structures of the two haem centres. Such differences in the local structure might lead to the differences in EPR signals (*10*, *12*), in redox potentials (*27*), in stabilities (*8*), and in functional roles for the transmembrane electron transfer (*7*, *11*). Although both MDA radical reductase activity to regenerate AsA (*7*, *11*) and ferrireductase activity (*17*, *22*, *24*, *44*) might be retained at the intravesicular haem centre, detailed discussions about their physiological roles and mechanism might be outside the scope of our current article.

Substrate-Binding Site on the Cytosolic Side—It is very reasonable to assume that the haem prosthetic group on the cytosolic side might be lie in parallel to the membrane normal (*45*). Therefore, only one of the two haem axial His residues (His88 and His161) might actually have a role for accepting a proton from the bound AsA, according to our present hypothesis. Although both His residues were modified with DEPC in a significant extent (*25*) and were protected from the modification in the presence of AsA (*27*), we favour His88 to have such a role. This is based on (a) a relative spatial closeness of Lys85, which might be a part of the substrate (AsA)-binding site (*25*), to His88 and (b) a relatively stronger protective effect of AsA against the DEPC-modification to His88 than to His161 (Takeuchi *et al*., unpublished observation).

Mechanism of Proton Transfer from $N_{\delta1}$ of the Axial His Residue—After an event of the concerted proton/electron transfer from a bound AsA, the electron equivalent residing on the cytosolic haem centre will be transferred to the other haem centre on the intravesicular side (*via* intramolecular pathways) and the resultant MDA radical molecule will remain bound at the substrate-binding site. The $N_{\delta1}$ proton of the axial His residue will also remain bound until a new AsA molecule approaches. The retention of the $N_{\delta1}$ proton might be appropriate for preventing the backflow of electron equivalents to MDA radical. Upon binding of a new AsA molecule at the substrate-binding site, however, the proton on the $N_{\delta1}$ atom must be released to the cytosolic side. Although details of the mechanism are unknown, existence of such machinery was inferred from our detailed stopped-flow studies for both bovine (*37*) and *Zea mays* (Nakanishi *et al*., unpublished data) cytochromes b_{561}. The studies showed a significant time lag before the actual electron transfer occurs after the mixing of oxidized cytochrome b_{561} with AsA in a lower pH region (<6.0). These observations could be explained as the diminishing of proton-accepting ability of the machinery, which might constitute of a series of proton acceptor(s) with a low pK_a, such as propionic acid groups of the haem periphery, Asp and Glu residues, and even a water molecule(s).

Distinction from a Previous Proposal—Njus and coworkers proposed previously a 'concerted proton/electron transfer model' based on the observation of the inhibition of electron transfer from extravesicular AsA in chromaffin granule ghosts by the treatment with DEPC (*41*, *40*, *46*, *47*). Although they did neither prove that cytochrome b_{561} was the target of the DEPC-modification nor specify the site(s) in cytochrome b_{561} for the modification with DEPC, their proposed mechanism was a starting point of our current hypothesis. According to their original scheme (*41*, *46*, *47*), it was assumed that only one haem *b* prosthetic group exists in a molecule and the His residue responsible for the interaction with and receiving proton from AsA is distinct from the haem axial His residues. Thus, obviously, the Njus's model is not

appropriate for our current view of cytochrome b_{561}. Further, the separation between the proton-accepting site and the redox haem centre is not in accordance with the 'concerted proton/electron transfer model'. Our model has a clear advantage than the system comprising two different His residues with separated roles (*40*, *47*). In our current model, changes in the redox states of the haem iron can be directly (and pronouncedly) transmitted to the imidazole ring to control its protonation state and further to the protonation state of a substrate (and, reversely, the protonation state of the substrate will influence on the axial His residue, which can control the redox state of the haem iron).

Examples of Axial His Residues having a Role for Substrate-Binding and Proton-Transfer—It might be very instructive to look at several examples in X-ray structures showing bindings of substrates (organic reductants) to the axial His ligands in other type of di-haem containing membrane proteins with four helix bundles (*48*). The first group, 'two-helix motif', in which four His ligands from two different transmembrane helices are employed for the haem-binding, consists of cytochrome bc_1 complex (*49*, *50*), cytochrome b_6f complex (*51*) and quinol-nitrate reductase A (NarGHI) (*52*). The second group 'three-helix motif', in which four His ligands from three transmembrane helices are employed for the haem-binding, consists of hydrogenase and formate dehydrogenase (*53*). The third group 'four-helix motif', where four His ligands from four different transmembrane helices are employed for the haem-binding, consists of di-haem succinate:quinone oxidoreductase and quinol: fumarate reductase (*54*, *55*). It must be noted that, although the cytochrome b_{561} family has a 'four-helix motif' too, the architecture of the di-haem binding is very distinct and should be considered as an independent motif (*21*).

Among these examples, particular interest is a quinol-binding and oxidation site (Q-site) in NarGHI (*52*), where menaquinol or ubiquinol is postulated to have a direct hydrogen bond with one of the haem-coordinating histidyl imidazole. In the structure of the menaquinone-binding and reduction site of formate dehydrogenase-*N*, menaquinone is thought to have a hydrogen bond to the $N_{\delta 1}$ atom of the haem-coordinating His residue (H169) (*53*). In the menaquinol-binding and oxidation site of *Wolinella succinogenes* quinol: fumarate reductase, the bound menaquinol was proposed in a close proximity to the axial His residue of $haem_D$ centre (*48*, *56*). On the other hand, at the ubiquinol-binding and oxidation site (Q_o-site) in the cytochrome bc_1 complex, ubiquinol was found to have a hydrogen bond to His161 (*49*, *57*). His161 is also a ligand of the 2Fe-2S cluster (*49*). In these structural examples, the $N_{\varepsilon 2}$ atom is used for the coordination to metal centres and the $N_{\delta 1}$ atom has a role to form a hydrogen bond with the substrate molecule. Thus, the redox change at the metal centre is directly transmitted to the substrate molecule *via* the coordinating imidazole group and *vice versa*. Although the machinery of this type of system is not mechanistically proved or clarified due to the low resolution of the bound substrate in the X-ray structures, it is clear that our postulation has a rigid basis in the structures of transmembrane proteins.

CONCLUSIONS AND FUTURE STUDIES

- The proposed mechanism is based on the previous proposal of Njus and co-workers (*41*, *46*, *47*). However, we have accommodated the haem binding by proposing that the axial His residue is in the anion (imidazolate) form. The dual role of the His residue might give the kind of concerted proton/electron transfer rates as observed. Free imidazole group alone did accelerate AsA oxidation (*40*) but, apparently, it is not enough to account for the reaction rates observed in cytochrome b_{561}. The imidazolate form, presumably induced by the haem and surrounding amino acid residues, seems more likely to achieve the concerted proton/electron transfer.
- The proposed mechanism accounts for many of experimental results obtained for bovine and *Zea mays* cytochromes b_{561} and might be applicable for other type of cytochrome b_{561}; *i.e*. Dcytb and other plant cytochromes b_{561}, in which AsA is expected to work as a physiological electron donor (*17*).
- Other type of organic reductants in biomembranes, such as ubiquinols, menaquinols, plastoquinols and vitamin E, also lose a proton upon the electron donation (*40*, *48*). This fact suggests that a similar mechanism utilizing the metal-bound imidazolate form might be in operation in other type of transmembrane electron transfers including those of distant subgroups of the cytochrome b_{561} family.
- The proposed mechanism requires the direct proton transfer from AsA to the axial His residues during the electron transfer events. In this case, there should be a significant deuterium isotope effect when deuterated AsA is used as a substrate.
- X-ray structural study is apparently required to prove our hypothesis. We are currently trying to obtain such protein crystals suitable for high-resolution analyses.

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