pH-induced Intramolecular Electron Transfer between the Iron-Sulfur Protein and Cytochrome c1 in Bovine Cytochrome bc1 Complex*

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†The abbreviations used are: $E_m$, redox midpoint potential; ISP, iron-sulfur protein; MOA-stilbene, (E)-β-methoxyacrylate-stilbene; UHDBT, 5-n-undecyl-6-hydroxy-4,7-dioxobenzothiazole; $t_{1/2}$, reaction half-life.

Structural analysis of the bc1 complex suggests that the extramembrane domain of iron-sulfur protein (ISP) undergoes substantial movement during the catalytic cycle. Binding of Qo site inhibitors to this complex affects the mobility of ISP. Taking advantage of the difference in the pH dependence of the redox midpoint potentials of cytochrome c1 and ISP, we have measured electron transfer between the [2Fe-2S] cluster and heme c1 in native and inhibitor-treated partially reduced cytochrome bc1 complexes. The rate of the pH-induced electron transfer between these redox centers can be estimated by conventional stopped-flow techniques ($t_{1/2}$, 1–2 ms), whereas the rate of cytochrome c1 oxidation is too high for stopped-flow measurement. These results suggest that oxidized ISP has a higher mobility than reduced ISP and that the movement of reduced ISP may require an energy input from another component. In the 5-n-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT)-inhibited complex, the rate of cytochrome c1 reduction is greatly decreased to a $t_{1/2}$ of approximately 2.8 s. An even lower rate is observed with the stigmatellin-treated complex. These results support the idea that UHDBT and stigmatellin arrest the [2Fe-2S] cluster at a fixed position, 31 Å from heme c1, making electron transfer very slow.

The cytochrome bc1 complex is a segment of the mitochondrial respiratory chain which catalyzes antimycin-sensitive electron transfer from ubiquinol to cytochrome c (1, 2). The reaction is coupled to the translocation of protons across the mitochondrial inner membrane to generate a proton gradient and membrane potential for ATP synthesis (2–4). The purified cytochrome bc1 complex contains 11 protein subunits, 2165 amino acid residues, and 4 prosthetic groups with a total molecular mass of 248 kDa, without counting the bound phospholipids. The amino acid sequences of all subunits are available from either peptide or nucleotide sequencing (5, 6). The essential redox components of the complex are: two b-type cytochromes (heme b1, $E_m = -30$ mV, heme b1H, $E_m = 90$ mV), one c-type cytochrome (heme c1, $E_m = 227$ mV), one high potential [2Fe-2S] cluster (FeS, $E_m = 280$ mV), and a ubiquinone.

The three-dimensional structure of cytochrome bc1 complex at 2.9 Å resolution was first reported in 1997 (5, 7). More complete structures, from a different crystalline form, have been reported recently (8–10). The crystal structure of the complex exists as a closely interacting functional dimer. There are 13 transmembrane helices in each monomer, 8 belong to cytochrome b1, and one each to cytochrome c1, ISP, and subunits 7, 10, and 11. The 21 Å between heme b1H and heme b1H and 27 Å between heme b1 and the [2Fe-2S] cluster accommodate well the observed fast electron transfer between these redox centers (11, 12). However, the 31 Å between the [2Fe-2S] cluster and heme c1 makes it difficult to explain the high electron transfer rates between them.

During analysis of x-ray diffraction data of the crystalline complex (7), it was noticed that the extramembrane region of ISP and cytochrome c1, on the cytoplasmic side of the inner membrane, has a lower electron density than other regions of the complex. This suggests that the ISP is flexible and perhaps is under constant motion. Binding of various Qo site inhibitors (10, 13) produces different effects on the anomalous signals of the [2Fe-2S] cluster, suggesting that these inhibitors are affecting the mobility of the [2Fe-2S] cluster and, perhaps, the whole extramembrane domain of ISP (10). Binding of the Qo-II or Qo-III inhibitors, such as UHDBT and stigmatellin, increases the $E_m$ of ISP and decreases the motion of ISP, whereas binding of the Qo-I inhibitors, such as myxothiazol and MOA-stilbene, causes a red shift in the optical spectrum of cytochrome b1 and an increase in the motion of ISP. The mobility of ISP is also supported by the observation of a decrease in electron transfer activity and an increase in activation energy of the cytochrome bc1 complex in mutants with increased rigidity in the neck region of ISP (14, 15).

Because the $E_m$ of ISP is pH-dependent, with a slope of about −60 mV per pH unit (16), and that of cytochrome c1 is not, electron transfer between ISP and cytochrome c1 occurs, without input of electrons, when the pH of the solution changes. At pH 8.0, the $E_m$ values of ISP and cytochrome c1 are about the same. Decreasing the pH of the partially reduced cytochrome bc1 complex causes cytochrome c1 oxidation, and increasing pH causes cytochrome c1 reduction at the expense of ISP reduction or oxidation. The rate of these acid- or base-induced cytochrome c1 oxidations or reductions can be determined if a fast mixing device is available. Although the currently available fast mixing apparatus, with a millisecond dead time, is marginally suitable for measuring electron transfer rate from the [2Fe-2S] cluster to heme c1, it is too slow for measuring electron transfer from heme c1 to [2Fe-2S] cluster.

Electron transfer rate is exponentially dependent on distance (17). A variation of 20 Å in the distance between donors...
and acceptors changes the electron transfer rate by 10^{12}-fold (18). The anomalous scattering data indicate that in the oxidized cytochrome bc₁ complex less than 50% of ISP is in the fixed state, and the rest is in the loose state. Binding of Qo-II or Qo-III inhibitors to the cytochrome bc₁ complex increases the amount of ISP in the fixed state, a distance of 31 Å from heme c₁. Hence great reduction in the electron transfer rate is expected in the Qo-II or Qo-III inhibitor-treated complex. However, because the binding of Qo-I inhibitors favors ISP in the loose state, no significant change in electron transfer rate is expected.

In this paper we report the pH-induced electron transfer rates between the [2Fe-2S] cluster and heme c₁ in the presence and absence of various Qo site and Qi site inhibitors. Also, we describe a simple way to assess the binding affinity of Qo site inhibitors by measuring their competition against stigmatellin or UHDBT.

**EXPERIMENTAL PROCEDURES**

Cytochrome c₁, type III, and sodium cholate were purchased from Sigma. 2,3-Dimethoxyl-5-methyl-6-geranyl-4-benzoquinone (Q₂), its reduced form (Q₂H₂) (19), and UHDBT (20, 21) were synthesized as previously reported. Other chemicals were of the highest purity commercially available.

Purified cytochrome bc₁ complex was prepared as previously reported (22). It was dissolved in 50 mM Tris-Cl buffer, pH 7.8, containing 0.66 M sucrose to protein concentration of 20 mg/ml and frozen at −80 °C until use. The purified enzyme contains 10 nmol of cytochrome b and 5.7 nmol of cytochrome c₁ per mg of protein. The concentrations of cytochromes b and c₁ were determined spectrophotometrically, using a difference millimolar coefficient of 28.5 and 17.5 for cytochromes b and c₁, respectively.

For activity assay, the cytochrome bc₁ complex was diluted with 50 mM phosphate buffer, pH 7.4, containing 1 mM EDTA and 0.01% dodecyl maltoside to a protein concentration of 0.25 mg/ml. Five μl of the diluted enzyme solution was added to 1 ml of an assay mixture containing 100 mM phosphate buffer, pH 7.4, 1 mM EDTA, 100 μM cytochrome c, and 25 μM Q₂H₂. Activity was determined by measuring the reduction of cytochrome c₁ (by the increase of absorbance at 550 nm) in a Shimadzu UV 2101 PC spectrophotometer at room temperature. A millimolar extinction coefficient of 18.5 was used to calculate specific activity. Nonenzymatic oxidation of Q₂H₂, determined under the same conditions in the absence of the cytochrome bc₁ complex, was subtracted.

For the determination of electron transfer rates between the [2Fe-2S] cluster and heme c₁, the complex was diluted in 20 mM phosphate buffer, pH 6.8 or 9.2, containing 0.05% dodecyl maltoside and 100 mM KCl to a cytochrome bc₁ complex concentration of around 10 μM and mixed with buffers of various pHs at room temperature in an Olis stopped-flow rapid scanning spectrophotometer. Oxidation or reduction of cytochrome c₁ was monitored by the decrease or increase of absorbance at 553 nm minus 545 nm. When an inhibitor was used, the cytochrome bc₁ complex was treated with 5- to 10-fold molar excess of the inhibitor for 10 min prior to the pH-induced electron transfer experiment. For determination of the displacement rate of the inhibitors, the UHDBT-treated cytochrome bc₁ complex was diluted in 20 mM Tris-Cl, pH 8.3, containing 0.05% dodecyl maltoside and 100 mM KCl to a cytochrome c₁ concentration of 10 μM and mixed with an equal volume of the buffer containing MOA-stilbene in the same apparatus.

The redox state of the ISP was determined by EPR (23), using a Bruker 200D, and by circular dichroism (24, 25), using a JASCO J-715 spectropolarimeter. Experimental conditions used were: step resolution, 1 μM; scan speed, 100 mm/min; accumulation, 10 traces for averaging; response, 1 s.; bandwidth, 1.0 nm; sensitivity, 10 mdeg; slit width of 500 μm.

**RESULTS AND DISCUSSION**

**pH-dependent Oxidation of ISP and Reduction of Cytochrome c₁ in the Purified Cytochrome bc₁ Complex**—Without special treatment during purification, the purified complex ends up with partially reduced cytochrome c₁ and ISP. At pH 8.0, both ISP and cytochrome c₁ are 58% of reduced, indicating both components having the same redox potential, 227 mV. Cytochrome c₁ reduction increases as pH increases and decreases as pH decreases. Electrons for cytochrome c₁ reduction come from ISP. A linear relationship between ISP oxidation and cytochrome c₁ reduction is observed (see Fig. 1A). When the pH of the complex is decreased from pH 8.0 to 7.0, cytochrome c₁ reduction decreases from 58.0 to 33.5%, whereas ISP reduction increases from 58.0 to 84.0%. When the pH is increased from 8.0 to 9.0, cytochrome c₁ becomes more reduced, from 58.0 to 76.0%, and ISP becomes more oxidized, the reduction changes from 58.0% to 35.0%. The pH-induced oxidation and reduction of ISP is basically coupled to the reduction and oxidation of cytochrome c₁ when the pH is between the two pKₐ values of ISP (16). As shown in Fig. 1B, the reduction of ISP increases with pH.
Intramolecular Electron Transfer in Cytochrome bc1 Complex

The electron transfer from cytochrome $c_1$ to ISP is faster than a conventional stopped-flow apparatus can measure. Because the $E_m^c$ of ISP is pH-dependent, increasing pH of cytochrome $b_6$ complex will cause electron transfer from ISP to cytochrome $c_1$ if the latter is in the oxidized or partially reduced state. By the same reasoning, acidification will cause oxidation of cytochrome $c_1$ and reduction of ISP if they are in the partially reduced states. This behavior allows one to measure the rate of electron transfer between ISP and cytochrome $c_1$ by rapid mixing of cytochrome $b_6$ with different pH buffer solutions in a stopped-flow apparatus. Unfortunately, the pH-induced electron transfer from cytochrome $c_1$ to ISP is too fast to be followed in the conventional stopped-flow apparatus (Fig. 2A). Electron transfer is completed within the mixing time, indicating $t_{1/2}$ of less than 1 ms. The degree of cytochrome $c_1$ oxidation, however, can be determined spectrophotometrically. Because cytochrome $c_1$ is not autooxidizable, the extent of redox change of ISP can be estimated, assuming electrons only moving between cytochrome $c_1$ and ISP.

However, when the pH is increased, the electron transfer rate from ISP to cytochrome $c_1$ can be followed by stopped-flow apparatus, though marginally, as about 50% of pH-induced cytochrome $c_1$ reduction is completed during the mixing time (Fig. 2B). By extrapolating the tracing time to 0 or to the absorbance of cytochrome $c_1$ complex mixed with an equal volume of the buffer with the same pH, one estimates a $t_{1/2}$ of the reaction to be 1 to 2 ms, corresponding to a first order rate constant of $K_t = 346 \text{ s}^{-1}$. This rate constant is lower than the turnover number of 800 $\text{s}^{-1}$ for the cytochrome $b_6$ complex, calculated from activity determinations at room temperature (25 °C). A turnover number as high as 3000 $\text{s}^{-1}$ has been reported for the complex at higher temperatures (26, 27). A partial reaction rate lower than the turnover rate of an enzyme reaction indicates that this step is somehow inhibited or is not in the native state. Because the back reaction, electron transfer from cytochrome $c_1$ to ISP, is faster than the turnover, assuming a $t_{1/2}$ of about 0.5 ms, the extramembrane domain of oxidized ISP is apparently capable of rapid motion. The mobility of this domain in reduced ISP may be governed by an interacting partner, such as cytochrome $b$. In other words, an energy input is needed for effective electron transfer from ISP to cytochrome $c_1$. The most likely energy source would be a conformational change in cytochrome $b$ during electron transfer from $b_1$ to $b_H$. Under the experimental conditions described no redox state changes in cytochromes $b_1$ and $b_H$ are involved and thus no energy input is involved. In the normal electron transfer, ubiquinol is the substrate; oxidation of the ubisemiquinone by cytochrome $b_L$ and subsequent electron transfer from $b_L$ to $b_H$ involves significant conformational change which may provide the energy needed to facilitate the fast movement of the extramembrane domain of ISP.

To be sure that the fast electron transfer from ISP to cytochrome $c_1$ observed is because of the intramolecular electron movement and is not a result of the intermolecular electron exchange, the effect of the concentration of the cytochrome $b_6$ complex on the electron transfer rates were examined. When the pH-induced cytochrome $c_1$ reduction was carried out with various concentrations (final concentration of 2.8, 4, and 8 $\mu$M) of partially reduced cytochrome $b_6$ complex, no apparent difference in rate constant was observed. A similar concentration-independent rate constant was observed in the inhibitor (azoxystrobin)-treated complex. These results confirm that the observed fast electron transfer is indeed an intramolecular event because an intermolecular event would be concentration-dependent.

Determination of Electron Transfer Rate between ISP and Cytochrome $c_1$ in the Stigmatellin-Inhibited Cytochrome $b_6$ Complex—Binding of stigmatellin greatly elevates the $E_m^c$ of ISP in the cytochrome $b_6$ complex but has no effect on that of cytochrome $c_1$. When a partially reduced cytochrome $b_6$ complex at neutral pH is treated with stigmatellin, cytochrome $c_1$ becomes almost completely oxidized. Adjust the pH of the stigmatellin-treated complex from 6.5 to 8.6 and a small part of cytochrome $c_1$ becomes reduced (Fig. 3), but at a very slow rate. It takes more than 10 min to complete the reaction, with an estimated $t_{1/2}$ of 6 min. Re-oxidation of ISP could be due to pH-induced electron transfer or to release of the bound inhibitor. When the complex is treated with less than stoichiometric amounts of inhibitor, a fast phase is observed. The extent of the fast phase is inversely dependent in the amount of inhibitor.

**Fig. 2.** Time trace of the redox change of cytochrome $c_1$ in the cytochrome $b_6$ complex upon decreasing or increasing the pH from 8.84 to 6.96 or from 6.91 to 8.96. For the acidification experiment, cytochrome $b_6$ complex was diluted in 20 mM Puffer, pH 8.84, containing 0.05% deoxycholate and 100 mM KCl to a concentration of 10.0 $\mu$M (based on cytochrome $c_1$). For alkalization, the complex was diluted in the same buffer with pH 6.91. The diluted complex was rapidly mixed with an equal volume of the buffer containing enough HCl or NaOH to cause the indicated pH change, at room temperature, in an Olis stopped-flow rapid scanning spectrophotometer. The oxidation (A) or reduction (B) of cytochrome $c_1$ was monitored (553 nm minus 545 nm). The diluted complex against an equal volume of the same buffer was used as a base line in both systems.
used, indicating that it results from the free (uninhibited) enzyme complex. When the amount of stigmatellin used is more than the stoichiometric amount, the rate is not very sensitive to the concentration of the inhibitor. The electron transfer observed probably results from the pH-induced change of the $E_m$ of the ISP/stigmatellin complex. From structural studies of crystals of inhibitor/bc$_1$ complex, it is known that stigmatellin arrests the movement of ISP and forces the [2Fe-2S] cluster to remain in a fixed position, closer to $b_1$, and far away (31 Å) from heme c$_1$. This distance accounts for the slow electron transfer.

**Determination of Electron Transfer Rate between ISP and Cytochrome c$_1$ in the UHDBT-Inhibited Cytochrome bc$_1$ Complex—**Binding of UHDBT to the cytochrome bc$_1$ complex also causes an increase of the $E_m$ of ISP but to a much lesser extent than with stigmatellin. Because UHDBT also arrests the movement of ISP, the pH-induced electron transfer rates between the [2Fe-2S] cluster and heme c$_1$ should be comparable with those observed with stigmatellin. However, there is a complication in using UHDBT because inhibitor binding is pH-dependent. At alkaline pH, UHDBT is released. The observed electron transfer from ISP to cytochrome c$_1$ following a pH change from 6.77 to 8.91 may be due to inhibitor-free cytochrome bc$_1$ or to inhibitor-loaded cytochrome bc$_1$ complex with a $t_{1/2}$ of 2.8 s (Fig. 4A). The contribution of inhibitor-free electron transfer can be decreased by increasing the concentration of inhibitor used. The back electron transfer from cytochrome c$_1$ to ISP, oxidation of cytochrome c$_1$ or to inhibitor-loaded cytochrome bc$_1$ complex with a $t_{1/2}$ of 2.8 s (Fig. 4A). The contribution of inhibitor-free electron transfer can be decreased by increasing the concentration of inhibitor used. The back electron transfer from cytochrome c$_1$ to ISP, oxidation of cytochrome c$_1$ or to inhibitor-loaded cytochrome bc$_1$ complex with a $t_{1/2}$ of 2.8 s (Fig. 4A).

**Antimycin, Myxothiazol, or MOA-stibene Has Little Effect on the Rate of Intramolecular Electron Transfer between ISP and Cytochrome c$_1$.**—As expected, because antimycin binds at the Qi site, remote from Qo pocket, it has no effect on the redox potentials of ISP or cytochrome c$_1$. MOA-stibene and myxothiazol which bind closer to cytochrome $b_1$, heme, although without direct contact, cause some spectral changes in cytochrome $b_1$ but have no effect on the $E_m$ of ISP or heme c$_1$. Therefore they do not cause electron transfer between these two redox centers. In the presence of these inhibitors, pH-induced electron transfer between ISP and cytochrome c$_1$ is as fast as in the uninhibited complex (data not shown). This agrees with structural data indicating that these inhibitors increase the mobility of ISP in the crystal.

**Binding Competition of Qo Inhibitors—**Because binding of stigmatellin or UHDBT causes cytochrome c$_1$ oxidation by raising the $E_m$ of ISP, the binding competition between the Qo inhibitors which do not cause an $E_m$ elevation, and stigmatellin or UHDBT, can be investigated by following cytochrome c$_1$ oxidation or reduction. The relative binding strength of these inhibitors can be assessed by measuring their ability to reverse the effect of stigmatellin or UHDBT. Although binding competition of the Qo inhibitors was observed by the EPR signals of the [2Fe-2S] cluster (28), the approach discussed here is simple and effective. If an inhibitor is bound to the cytochrome bc$_1$ complex more tightly than UHDBT, addition of such an inhibitor to the UHDBT-treated partially reduced complex will cause cytochrome c$_1$ to become more reduced. From the structural data it is clear that the binding sites of most of the Qo
Induced oxidation of cytochrome c. One ml of partially reduced cytochrome bc₁ complex in a solution containing 20 mM Tris Cl, pH 7.6, 100 mM KCl, 0.05% dodecyl maltoside was treated sequentially with different inhibitors (5-fold molar excess). The redox state of cytochrome c₁ was monitored (553 – 545 nm). Cytochrome c₁ was detected before addition of inhibitor. The final concentration of cytochrome c₁ was 5 μM.

Inhibitors overlap to some extent. The effect of UHDBT on the ISP can be reversed by addition of other Qo inhibitors (stigmatellin, myxothiazol, and MOA-stilbene), suggesting the binding of UHDBT to the complex is weakest (Fig. 5). However stigmatellin and myxothiazol have about equal binding affinities for the complex because stigmatellin has little effect on redox state of cytochrome c₁ in a complex already treated with myxothiazol. Conversely, myxothiazol does not replace the bound stigmatellin as it does not cause re-reduction of oxidized cytochrome c₁ in the presence of stigmatellin.

The kinetics of the displacement of UHDBT by MOA-stilbene was examined in the stopped-flow rapid mixing apparatus (see Fig. 6). Addition of MOA-stilbene to the UHDBT-treated complex induces a slow reduction of cytochrome c₁, with a t₁/₂ of approximately 10 s. This result indicates that the binding of MOA-stilbene is stronger than that of UHDBT and the second order rate constant for MOA-stilbene binding is $2 \times 10^3$ m⁻¹ s⁻¹. Binding of UHDBT results in ISP being held in a conformation where the [2Fe-2S] cluster is 31 Å from heme c₁. This position is called the fixed state of tetragonal crystals (7, 10) or the $b$-state of hexagonal crystals (8, 9). When MOA-stilbene replaces UHDBT, ISP changes conformation such that the [2Fe-2S] cluster is somewhere between the heme c₁ and fixed positions and, presumably, is fast moving. This conformation is referred to as the loose state of the tetragonal crystals (7, 10). Unlike in the fixed position, in the loose position ISP can move closer to the heme c₁, making electron transfer between the [2Fe-2S] cluster and heme c₁ possible.

From the x-ray crystallographic data, it is clear that in the fully oxidized state (7) some of the complex molecules are in the fixed state. The rest are in the loose states. In the partially reduced cytochrome bc₁ complex, the location of the [2Fe-2S] cluster is not established. Probably it is somewhere between the “c₁-state” (9) and the fixed position (7, 10) and is constantly moving because at the loose positions no electron density of the [2Fe-2S] cluster of ISP is observed. Movement makes electron transfer between the [2Fe-2S] cluster and heme c₁ possible when the $E_m$ of ISP is raised by acidification, inducing cytochrome c₁ oxidation, or lowered by alkalization, causing cytochrome c₁ reduction (29). The pH-induced electron transfer between the [2Fe-2S] cluster and heme c₁ is very fast in the uninhibited complex. It becomes very slow when the complex is pretreated with UHDBT or stigmatellin, indicating that these inhibitors arrest the head domain movement of ISP regardless of the redox state of the [2Fe-2S] cluster or heme c₁.

The rates determined for MOA-stilbene binding, by competition with stigmatellin or UHDBT, may be slower than the actual rate because binding of stigmatellin or UHDBT arrests the [2Fe-2S] cluster in the fixed position. Unlike in the free complex, bound UHDBT is released by MOA-stilbene at a position where ISP is 31 Å from the heme c₁. This ISP will have longer distance to move before electron transfer can take place, hence the slower rate.

Whether or not the rate of electron transfer between heme c₁ and the [2Fe-2S] cluster depends solely on the distance between them is unknown at present. Electron transfer could be significantly faster than expected, from point to point, if an aromatic amino acid residue is in the electron transfer path. Several such amino residues (Phe-97, Tyr-156, Tyr-157, and His-164 of ISP and Phe-153 of cytochrome c₁) are located between the [2Fe-2S] cluster and heme c₁.

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