

Original Article

New methods for determining proton pumping ability and electron transfer activity of the cytochrome *bc*₁ complex

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Abstract

It is important to determine the electron transfer activity and proton pumping ability of the cytochrome *bc*₁ complex for better understanding its structure and function. In this study, several methods for determining the electron transfer and proton pumping of the *bc*₁ complex, including the traditional and the new methods, are presented and evaluated. For determining the proton pumping ability of the *bc*₁ complex, the new stopped-flow method has a higher accuracy than the traditional pH meter method, and the new spectrophotometer method is more convenient than the traditional pH meter method. In measuring the electron transfer activity of the *bc*₁ complex, the new stopped-flow method is more accurate and has a higher separating capacity than the traditional spectrophotometer method.

Key words: cytochrome *bc*₁ complex, electron transfer, proton pumping, method

Introduction

The cytochrome *bc*₁ complex, also known as complex III or ubiquinol-cytochrome *c* oxidoreductase, is an essential segment of the electron transfer chain in mitochondria and photosynthetic bacteria [1]. The complex catalyzes electron transfer from quinol to cytochrome *c* (*c*₂ in some bacteria) with concomitant translocation of protons across the inner membrane of mitochondria or the cytoplasmic membrane of bacteria. Although the *bc*₁ complexes from different sources vary in their polypeptide compositions, they all contain four redox prosthetic groups: two *b*-type cytochromes (*b*_L and *b*_H), one *c*-type cytochrome (*c*₁), and one high potential Rieske iron-sulfur cluster (2Fe-2S).

Recently, the three-dimensional crystal structures of *bc*₁ complexes from bovine heart [2,3], chicken heart [4], yeast [5], and bacteria [6,7], which contain 1–9 supernumerary subunits in addition to the three core subunits, have been obtained. The structural information for the *bc*₁ complex establishes the location of the redox centers, the number of trans-membrane helices, quinone binding at the *Q*_N site, and inhibitor binding at both the *Q*_P and *Q*_N sites [2–4,8,9]. Moreover, these crystal structures suggest mobility of the extrinsic head domain of iron-sulfur protein (ISP) during *bc*₁ catalysis. Strong evidence in support of this movement has been reported [10–13,14–18].

The ‘proton-motive Q-cycle’ is the most favored mechanism for electron and proton transfer in this complex [19,20]. The central feature of the Q-cycle mechanism is the bifurcation of electrons from ubiquinol at the *Q*_P site. The first electron of ubiquinol is transferred to the ‘high potential chain,’ consisting of the Rieske (2Fe-2S) cluster, housed in the ISP, and heme *c*₁, housed in cytochrome *c*₁. Then the second electron of ubiquinol is passed through the ‘low potential chain’ consisting of heme *b*_L and heme *b*_H, both housed in the cytochrome *b* subunit.

It is important to determine the electron transfer activity and proton pumping ability of the *bc*₁ complex. The history for studying this question goes back to 1967 when Peter Mitchell tried to account for something that we now know is untrue. Mitchell did not believe that any protons were pumped by cytochrome oxidase in site IV. There are two energy coupling sites for electrons passing through the *bc*₁ complex en route to O₂: one at complex III and one at site IV. At each site, two protons are needed to form each ATP. To solve the problem of no ATP formation at site IV, he came up with the Q cycle that could pump four protons at site III, making up for the missing two protons from site IV. Subsequently, in the face of evidence that two protons were pumped by cytochrome oxidase, Mitchell agreed that

he was wrong. Since that time, a controversy arose as to whether two or four protons were pumped at site III. With the four protons from site III and four from site II, a total of six protons should be pumped with succinate as substrate. But, Lehninger's laboratory reported that eight pumped protons were observed. For at least 10 years, the best laboratories throughout the world tried various new improved approaches to determine whether the number was really 6 or 8. In 1987, Hendler and Shrager [21] performed a detailed analysis on all of the procedures that had been used, pointed out various deficiencies leading to the different results, and described an approach to avoid all of the traps.

At present, it has been established that for every electron transferred through the *bc*₁ complex, two protons are translocated across the membrane. This 2H⁺/e⁻ stoichiometry has been verified using pH measurements in a wide variety of species *in vitro* by studying *bc*₁ complexes in phospholipid (PL) vesicles [22,23]. However, this method has some disadvantages: (i) too many instruments are required; (ii) the determining procedure is complex and long; and (iii) the reliability and reproducibility is low. Usually, the electron transfer activity of the *bc*₁ complex is determined by monitoring the reduction of cytochrome *c* spectrophotometrically. However, it is difficult to gain the highest value with this method because the initial enzyme reaction is very fast.

In order to make an accurate and quick determination of the proton pumping and electron transfer activity of the *bc*₁ complex, we developed several new methods in this study. The advantages and disadvantages of every method were evaluated.

It should be noted that as soon as protons are pumped across the vesicle, a local membrane potential is built, which retards further proton release and causes some pumped protons to be pulled back. This backward flux is called leakage. It becomes progressively worse as more protons are pumped and membrane potential is built. This phenomenon happens in the presence of valinomycin, because the rates of pumping are faster than the compensatory movement of K⁺ across the membrane. As a result, the H⁺/e⁻ value can be underestimated to some extent. The methods presented here are to get an average proton pumping value, which includes the very high H⁺/e ratio at zero time and all of the slower ratios that follow.

Materials and Methods

Materials

Cytochrome *c* (horse heart, type III), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and valinomycin were purchased from Sigma (St Louis, USA). 8-Hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (pyranine) was from Acros Organics (Geel, Belgium). Asolectin was obtained from Associated Concentrates (New York, USA) and purified according to the method of Kagawa and Racker [24]. *N*-Dodecyl-β-D-maltopyranoside (DM) was from Anatrace (Maumee, USA). Nickel-nitrilotriacetic acid gel was from Qiagen (Hilden, Germany). 2,3-Dimethoxy-5-methyl-6-(10-bromodecyl)-1,4-benzoquinol (Q₀C₁₀BrH₂) was prepared as described previously [25]. All other chemicals were of the highest purity commercially available.

Enzyme preparations

The bovine *bc*₁ complexes were prepared from bovine heart as reported previously [26]. The *Rhodobacter sphaeroides* *bc*₁ complexes, including the wild-type and mutant R94N, were purified as described previously [27]. The *bc*₁ complex samples were stored at -80°C in the presence of 10% glycerol. Protein concentrations were determined by measuring the absorbance at 280 nm, using a converting factor of 1 OD₂₈₀ = 0.56 mg/ml. The concentrations of cytochromes *b*, *c*, and *c*₁

were determined spectrophotometrically using published molar extinction coefficients [28–30].

Preparation of protein–PL vesicles

Protein–PL vesicles were prepared by the cholate dialysis method of Kagawa and Racker [24]. Bovine heart *bc*₁ complex was mixed with 1 ml of asolectin micellar solution to give an asolectin/protein (w/w) ratio of 40. The asolectin micellar solution was prepared by sonicating 200 mg of acetone-washed asolectin in 4 ml of 50 mM sodium phosphate buffer, pH 7.4, containing 2% sodium cholate (SC) and 100 mM KCl, in an ice-water bath. Sonication was performed in an anaerobic environment by continually passing argon into the vessel. The *bc*₁ complex–PL mixtures were incubated at 0°C for 30 min before overnight dialysis at 4°C against 100 volumes of 50 mM sodium phosphate buffer, pH 7.4, containing 100 mM KCl with three changes of buffer. The mixture was then dialyzed against 100 volumes of 150 mM KCl for 3–4 h.

Determination of proton pumping ability of *bc*₁ complex–PL vesicles by the traditional pH meter method

Proton translocation coupled to electron flow through the bovine *bc*₁ complex–PL vesicles was measured at room temperature using an Accumet model 10 pH meter and a model 13-620-96 combination pH electrode. Twenty-five nanomoles of Q₀C₁₀BrH₂ was added to the 1.6-ml reaction mixture containing 150 mM KCl, 4 μM ferricytochrome *c*, 1 μM valinomycin, and an appropriate amount of *bc*₁–PL vesicles (30–50 μl). Electron flow was initiated by the addition of 5 nmol of ferricyanide, which oxidized the cytochrome *c*, and thus provided an electron acceptor for the complex. Electron flow under conditions where no transmembrane ΔpH had formed was measured in an identical manner except that the protonophore, CCCP, was present at a concentration of 2 μM to make the vesicles permeable to protons. Proton pumping (H⁺/e⁻) was calculated as the ratio of the decrease in pH upon ferricyanide addition to *bc*₁–PL vesicles before and after treatment with CCCP.

Determination of proton pumping ability of *bc*₁ complex–PL vesicles by the new spectrophotometer method

The determination was carried out at room temperature in a Shimadzu UV-2401 PC spectrophotometer by monitoring the absorbance changes of pyranine at 457 nm. After 5.7 μl of 14 mM pyranine was added to 1.6 ml of 150 mM KCl solution, the absorbance was set to zero. Then 2 μl of 2 mM ferricytochrome *c*, 2 μl of 0.2 mg/ml valinomycin, 5 μl of 5 mM Q₀C₁₀BrH₂ and an appropriate amount of bovine *bc*₁–PL vesicles (30–50 μl) were added to the reaction solution, respectively. The mixture was then equilibrated for a short time until the baseline was almost flat. Electron flow was initiated by the addition of 5 nmol of ferricyanide. Electron flow under conditions where no transmembrane ΔpH formed was measured in an identical manner except that the protonophore, CCCP, was present at a concentration of 2 μM. Proton pumping (H⁺/e⁻) was calculated as the ratio of the decrease in absorbance at 457 nm upon ferricyanide addition to *bc*₁–PL vesicles before and after treatment with CCCP.

Determination of proton pumping ability of *bc*₁ complex–PL vesicles by the new stopped-flow method

The measurement was performed in a stopped-flow Applied Photophysics SX.18MV spectrometer (Leatherhead, England) with a

photodiode array scan between 600 and 500 nm. The reaction was started by mixing equal volumes of solution A and solution B at room temperature. Solution A contained 150 mM KCl, 0.5 μ M valinomycin, 0.1 mM pyranine (a kind of dye), 35.7 μ g/ml of bovine *bc*₁ complex (based on protein, embedded in PL vesicles), and 0.036 mM Q₀C₁₀BrH₂. Solution B contained 150 mM KCl and 2.9 μ M ferricytochrome *c*. Solutions A and B were both adjusted carefully to pH 7.0 with 2 mM KOH. Proton pumping (H^+/e^-) was determined as the ratio of protons pumped (calculated from the absorbance changes of pyranine at 457 nm) to electrons transferred (calculated from the absorbance changes of cytochrome *c* at 550 nm).

Determination of electron transfer activity of *bc*₁ complex by the traditional spectrophotometer method

To determine the electron transfer activity, purified *bc*₁ complex was diluted with 50 mM Tris-Cl, pH 8.0, containing 200 mM NaCl and 0.01% DM to a final cytochrome *b* concentration of 1 μ M. Appropriate amounts of the diluted samples were added to 1 ml of assay mixture containing 100 mM of sodium phosphate buffer, pH 7.4, 0.3 mM EDTA, 50 μ M ferricytochrome *c*, and 25 μ M Q₀C₁₀BrH₂. Activities were determined by measuring the reduction of cytochrome *c* according to the increase in the absorbance at 550 nm, in a Shimadzu UV-2101 PC spectrophotometer (Kyoto, Japan) at 23°C, using a millimolar extinction coefficient of 18.5 $\text{mM}^{-1}\text{cm}^{-1}$ for calculations. The non-enzymatic oxidation of Q₀C₁₀BrH₂, determined under similar conditions in the absence of the enzyme, was subtracted during calculations for the specific activity. Specific activity was expressed as μ moles cytochrome *c* reduced/nmol cytochrome *b*/min.

Determination of electron transfer activity of *bc*₁ complex by the new stopped-flow method

To assay *bc*₁ complex activity, the *bc*₁ complex was diluted with 50 mM Tris-Cl, pH 8.0, containing 200 mM NaCl and 0.01% DM to a final cytochrome *b* concentration of 1 μ M. The measurement was performed in a stopped-flow apparatus, and the reactions were carried out at 23°C by mixing 1 : 1 solutions A and B. Solution A contained 100 mM Na⁺/K⁺ phosphate buffer, pH 7.4, 0.3 mM EDTA, 100 μ M cytochrome *c*, and 50 μ M Q₀C₁₀BrH₂. Solution B contained 100 mM Na⁺/K⁺ phosphate buffer, pH 7.4, 0.3 mM EDTA. Before assay, 3–10 μ l diluted enzyme solution was added to 1 ml of solution B. Activities were determined by monitoring the reduction of cytochrome *c* according to the increase in the absorbance at 550 nm. The slope of the initial part of the cytochrome *c* reduction curve or the K_1 value indicated the electron transfer activity of the *bc*₁ complex.

Results

Proton pumping ability of *bc*₁ complex-PL vesicles determined by traditional pH meter method

Figure 1 shows a typical proton translocation activity assay for PL vesicles embedded with the bovine mitochondrial *bc*₁ complex. When the pH of the mixture containing Q₀C₁₀BrH₂ reached equilibrium, valinomycin, ferricytochrome *c*, and bovine *bc*₁ complex-PL vesicles, an aliquot of ferricyanide solution (addition 1), were added to initiate electron flow from Q₀C₁₀BrH₂ to cytochrome *c*. After pH equilibrium was attained, protonophore CCCP was added (addition 2) to make the liposome membrane freely permeable to protons, and then a second aliquot of ferricyanide was added (addition 3). The ratio (x/y)

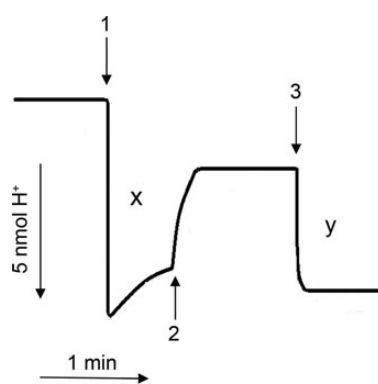


Figure 1. The proton pumping of bovine *bc*₁ complexes embedded in PL vesicles determined by traditional pH meter method Arrows indicate the points of addition of 5 nmol of ferricyanide (arrow 1), 2 μ M CCCP (arrow 2), and 5 nmol of ferricyanide (arrow 3). The proton pumping ratio (H^+/e^-) is x/y .

of the pH changes produced by the addition of equal amounts of ferricyanide, before and after the addition of CCCP, was taken as a measure of the H^+/e^- ratio for the proton translocation activity of the *bc*₁ complex. The protons released after CCCP addition were the 'scalar' protons only (namely the net proton production caused by quinol oxidation), as no contribution from the accumulation of 'vectorially' translocated protons was possible. Ratios between 1.6 and 2.0 were routinely observed with liposomes containing the bovine *bc*₁ complex. The average value of 1.8 is equal, within the experimental variations of the measurements, to the value of 2.0 predicted by the Q-cycle model for electron flow [21].

Proton pumping ability of *bc*₁ complex-PL vesicles determined by the new spectrophotometer method

Pyranine is a kind of pH-sensitive dye, which has long been used to measure delta pH across vesicular compartments. Figure 2 shows a typical proton translocation activity assay for PL vesicles embedding the bovine *bc*₁ complex using the new spectrophotometer method. In this example, the proton pumping ratio (H^+/e^-), namely x/y , is ~ 1.84 .

Proton pumping ability of *bc*₁ complex-PL vesicles determined by the new stopped-flow method

Figure 3 shows the proton pumping of bovine *bc*₁ complexes embedded in PL vesicles and the cytochrome *c* reduction in the reaction solution. The *bc*₁ complex catalyzes electron transfer from quinol to cytochrome *c* with concomitant translocation of protons across the membrane of the vesicle. The data from our preliminary experiments showed that the OD change at 457 nm was ~ 0.0251 if 1 nmole H^+ was released in the reaction mixture (0.4 ml, volume of the reaction cell). So the amount of H^+ released is 1.017 nmole (Fig. 3). The millimolar extinction coefficient of the reduced cytochrome *c* at 550 nm is 18.5 $\text{mM}^{-1}\text{cm}^{-1}$, so concentration of the reduced cytochrome *c* is 1.474 μ M (Fig. 3), and amount of the reduced cytochrome *c* is 0.5896 nmole. Thus, the proton pumping activity (H^+/e^-) is 1.725. The equations can be summarized as follows:

$$P = (\Delta A_{457} \times 18.5) / (0.0251 \times 1000 \times 0.4 \times \Delta A_{550}) \\ = 1.8426 \times \Delta A_{457} / \Delta A_{550}$$

where P is value of the proton pumping ability, ΔA_{457} and ΔA_{550} are the absorbance changes of pyranine and cytochrome *c*, respectively.

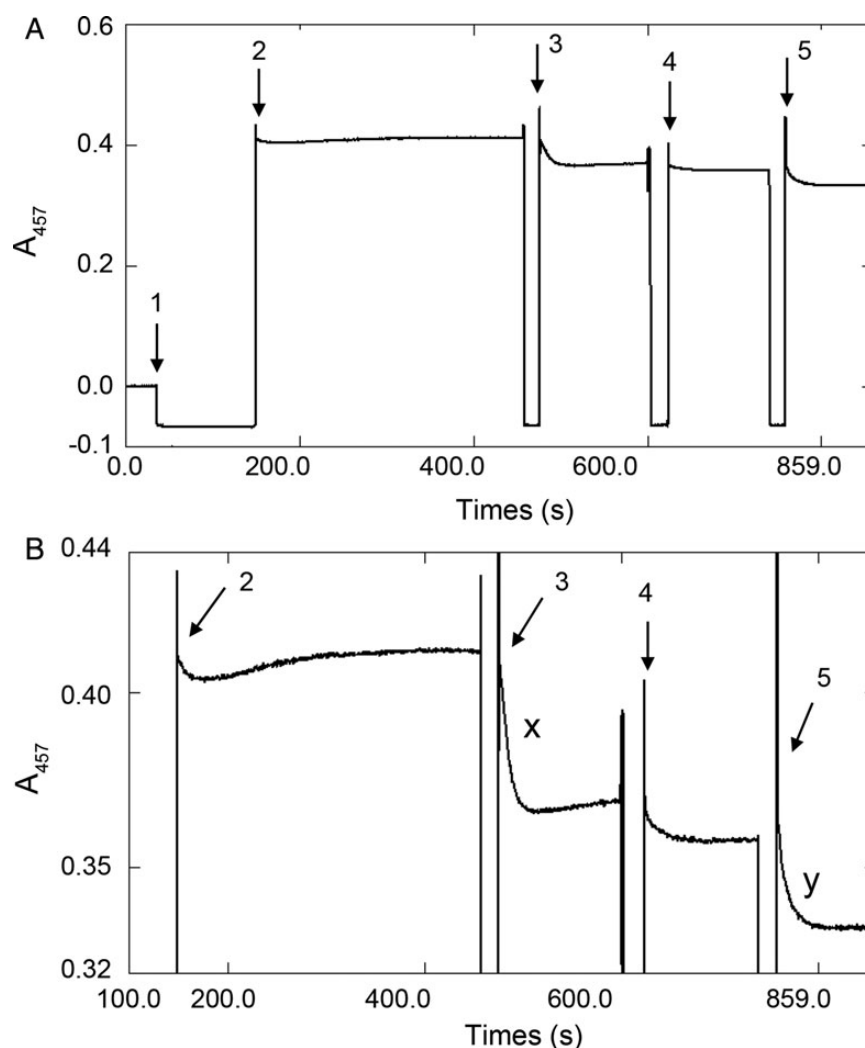


Figure 2. The proton pumping of bovine *bc*₁ complexes embedded in PL vesicles determined by the new spectrophotometer method (B) Amplification of the upper of (A). Arrow1 indicates the addition of 5.7 μ l of 14 mM pyranine to the 150 mM KCl solution; arrow2 indicates the addition of ferricytochrome *c*, valinomycin, $Q_0C_{10}BrH_2$, and vesicle; arrow3 indicates the addition of ferricyanide; arrow 4 indicates the addition of CCCP; arrow 5 indicates the addition of ferricyanide. The proton pumping ratio (H^+/e^-) = x/y ; the vertical line is the noise signal when the sample cell door of the spectrophotometer is opened or closed.

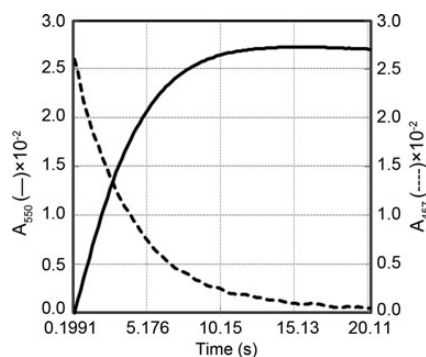


Figure 3. The proton pumping of bovine *bc*₁ complexes embedded in PL vesicles and cytochrome *c* reduction in the reaction solution determined by the new stopped-flow method The dotted trace indicates the absorbance changes of pyranine at 457 nm; the solid trace indicates the absorbance changes of cytochrome *c* at 550 nm.

Electron transfer activity of the *bc*₁ complex determined by the new stopped-flow method

The new stopped-flow method was performed on a stopped-flow instrument, allowing the fast reaction course to be recorded. For example, the dead time of the stopped-flow instrument we used (Applied Photophysics SX.18MV spectrometer) was only 2 ms. With this method, the cytochrome *c* reduction rate can reflect the *bc*₁ activity more accurately than that with the spectrophotometer method. The slope of the initial part of the cytochrome *c* reduction curve can reflect the activity clearly and qualitatively.

Because the concentration of ubiquinol used was much higher than that of the *bc*₁ complex, the reactions between *bc*₁ and ubiquinol were treated as pseudo first-order reactions. Time traces of the cytochrome *c* reduction can be fitted with a first order rate equation to obtain the pseudo first order rate constants K_1 by Kaleidagraph. Thus the K_1 value can reflect the activity quantitatively.

We have prepared a mutant of R94N on the cytochrome b of bc_1 complex from *R. sphaeroides*, whose electron transfer activity was not so different from that of the wild type. So it is difficult to compare the activities of the mutant and the wild type by using the spectrophotometer method. Figure 4 shows the result by using the new stopped-flow method. The activities of the two samples are remarkably different, as seen in the slopes (or the K_1 values) of the two curves.

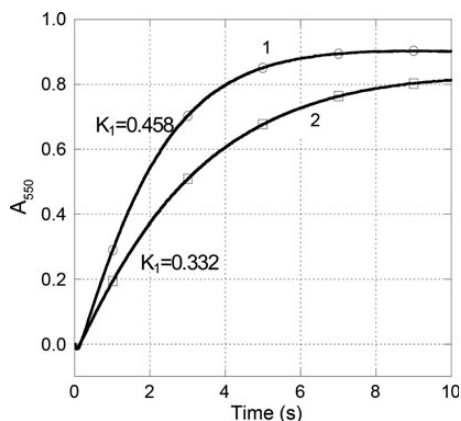


Figure 4. The electron transfer activity of bc_1 complex from *R. sphaeroides* determined by the new stopped-flow method Curves (1) and (2) indicate the cytochrome c reduction by wild-type and mutant R94N, respectively. The electron transfer activity is reflected by the slope of the curve or the K_1 value.

Discussion

The reason why vesicle is used in this study is that it allows the use of high concentrations of bc_1 complex. In addition, compared with the mitochondria membrane, there is no ion channel on the vesicle membrane, so the H^+ leakage can be ignored.

In the vesicle membrane, the orientation of the quinol oxidation side of the bovine bc_1 complex may be outward in its native state (Fig. 5A) or inward (Fig. 5B). If it is inward, the electron cannot be transferred from bc_1 complex to cytochrome c because cytochrome c exists in the outside solution but not inside the vesicle. So the proton pumping cannot be initiated. Namely, proton pumping is only related to the bc_1 complexes that are outward in the vesicle.

As mentioned above, the specific activity of the bc_1 complex is expressed as $\mu\text{moles cytochrome } c \text{ reduced/nmol cytochrome } b/\text{min}$. So the electron transfer activity of the bovine bc_1 complex embedded in the vesicle is decreased. Our experiment result showed that the activity decreased to 1.50 ± 0.05 compared with the free ones (7.60 ± 0.15). These data implied that about only 20% of the bc_1 complexes were outward in the vesicle. What is the reason for this proportion? It is known that the surface of the extramembrane region of the bc_1 complex is hydrophilic. The extramembrane region of the bc_1 complex in the quinol oxidation side (the smaller circle in Fig. 5) is smaller than that in the quinone reduction side (the larger circle in Fig. 5). In addition, the hydrophilicity of the microenvironment inside the vesicle is smaller than that of the solution outside the vesicle, and the space inside the vesicle is much smaller than that outside the vesicle. So, most of the bc_1 complexes are inward.

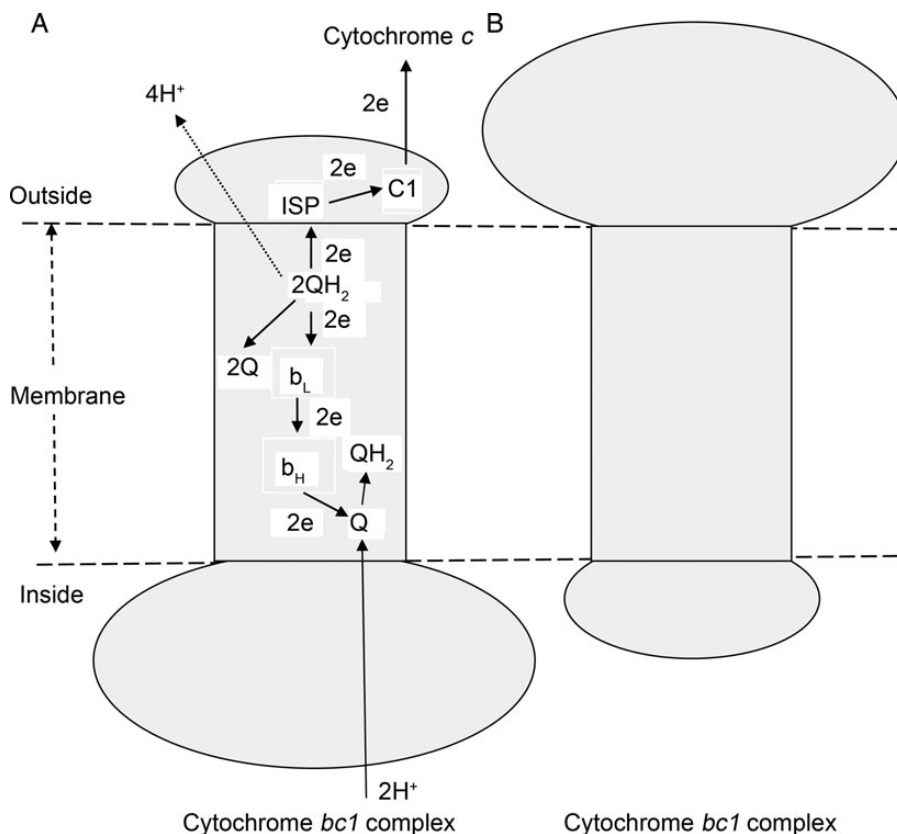


Figure 5. The schematic presentation of the orientation of bc_1 complexes in the vesicle membrane and the concerted Q-cycle mechanism (A) indicates the native state of the bc_1 complex; (B) indicates the inverse state of the bc_1 complex. The smaller or larger circle indicates the extramembrane region of the bc_1 complex in the quinol oxidation or quinone reduction side, respectively. ISP, iron-sulfur protein; QH_2 , 2,3-dimethoxy-5-methyl-6-(10-bromodecyl)-1,4-benzoquinol.

We have treated the vesicle with SC, which can destroy the vesicle. It was found that the electron transfer activity of the *bc*₁ complex on the vesicle restored to its normal level after the treatment. This implied that the *bc*₁ complex was embedded in the vesicle in its native structure and kept its normal function.

As discussed above, the *bc*₁ complex may be inserted into the vesicle outward or inward (Fig. 5), and proton pumping is only related to the outward *bc*₁ complexes. However, this does not affect the evaluation of the proton pumping ability of the *bc*₁ complex.

Our preliminary experiment results showed that the decreases in pH were the same when the same amount of HCl was added to the reaction mixture successively. It implied the reliability of the traditional pH meter method in theory. In this method, errors caused by the delayed response times can be eliminated, because (i) the proton pumping value is a ratio of 'X/Y', and the two successive pH change measurements are made with the same glass electrode; (ii) 'X' and 'Y' are both average values.

The pH meter method requires the use of too many instruments, including a recorder, a pH meter, and a magnetic stirrer, etc. The environmental physical changes around the instruments, for example, air blowing, electromagnetic waves, and physical vibration, can affect the instruments' stability. In addition, several kinds of reagents with micro amounts must be added manually during the process, and the procedure is too complex and time-consuming. Thus, the errors resulting from these conditions may accumulate and become significant.

The new spectrophotometer method is very simple and convenient compared with the pH meter method because only one instrument, the spectrophotometer, is needed. Spectrophotometers are stable, and less affected by physical changes in the environment.

In the spectrophotometer method, ~5 nmol of H⁺ is released when the first aliquot of ferricyanide solution (addition 3 in Fig. 2) is added. To examine the reliability of this method, 5 nmol of HCl was continually fed into 1.6 ml of 150 mM KCl solution with 50 mM pyranine for two separate times. It was found that the absorbance changes were the same, which ensured the accuracy and reliability of this method. For this method, the reaction mixture solution cannot be mixed continually with a magnetic stirrer as in the pH meter method. Thus, equilibrium is not easy to attain, as can be seen from Fig. 2.

The new stopped-flow method is performed in a stopped-flow apparatus with a photodiode array scan, so the absorbance changes of pyranine and cytochrome *c* can be obtained at the same time, which increases the accuracy of this method. The volume of the reaction cell was 0.4 ml for the stopped-flow instrument we used, and the volume is only 20 µl for the most advanced equipment at present. The small volume of the sample ensures a rapid mixing and the subsequent high accuracy.

The electron transfer activity of the *bc*₁ complex can be determined by the traditional spectrophotometer method or the new stopped-flow method. For the spectrophotometer method, the electron transfer activity of the *bc*₁ complex is determined by measuring the reduction of cytochrome *c* spectrophotometrically. It is difficult to get the exact activity value using this method because the initial reaction rate is very fast. The obtained activity data depend on the speed of operating the equipment. Therefore, the reproducibility is low, and it is difficult to differentiate between samples with similar activity values. In spite of these disadvantages, this method is convenient and easy to perform because it does not require complex instruments or operations.

The stopped-flow method has advantages in precisely determining the activity of the *bc*₁ complex, and in differentiating samples with similar activity values. In spite of these advantages, this method is not convenient or easy to use, compared with the spectrophotometer method because it requires a stopped-flow instrument and relatively complex operations.

In summary, every method has its own limitations. To better understand the mechanism of electron transfer and proton pumping in *bc*₁ complex, appropriate methods should be selected based on lab conditions and the purpose of the study.

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References

1. Trumpower BL, Gennis RB. Energy transduction by cytochrome complexes in mitochondrial and bacterial respiration: the enzymology of coupling electron transfer reactions to transmembrane proton translocation. *Annu Rev Biochem* 1994; 63: 675–716.
2. Xia D, Yu CA, Kim H, Xia JZ, Kachurin AM, Zhang L, Yu L, *et al.* Crystal structure of the cytochrome *bc*₁ complex from bovine heart mitochondria. *Science* 1997; 277: 60–66.
3. Iwata S, Lee JW, Okada K, Lee JK, Iwata M, Rasmussen B, Link TA, *et al.* Complete structure of the 11-subunit bovine mitochondrial cytochrome *bc*₁ complex. *Science* 1998; 281: 64–71.
4. Zhang Z, Huang L, Shulmeister VM, Chi YI, Kim KK, Hung LW, Crofts AR, *et al.* Electron transfer by domain movement in cytochrome *bc*₁. *Nature* 1998; 392: 677–684.
5. Hunte C, Koepke J, Lange C, Rossmanith T, Michel H. Structure at 2.3 Å resolution of the cytochrome *bc*(1) complex from the yeast *Saccharomyces cerevisiae* co-crystallized with an antibody Fv fragment. *Structure (Lond.)* 2000; 8: 669–684.
6. Esser L, Elberry M, Zhou F, Yu CA, Yu L, Xia D. Inhibitor-complexed structure of the cytochrome *bc*₁ from the photosynthetic bacterium *Rhodobacter sphaeroides*. *J Biol Chem* 2008; 283: 2846–2857.
7. Xia D, Esser L, Elberry M, Zhou F, Yu L, Yu CA. The road to the crystal structure of the cytochrome *bc*₁ complex from the anoxygenic, photosynthetic bacterium *Rhodobacter sphaeroides*. *J Bioenerg Biomembr* 2008; 40: 485–492.
8. Gao X, Wen X, Esser L, Quinn B, Yu L, Yu CA, Xia D. Structural basis for the quinone reduction in the *bc*₁ complex: a comparative analysis of crystal structures of mitochondrial cytochrome *bc*₁ with bound substrate and inhibitors at the Q_i site. *Biochemistry* 2003; 42: 9067–9080.
9. Esser L, Quinn B, Li YF, Zhang M, Elberry M, Yu L, Yu CA, *et al.* Crystallographic studies of quinol oxidation site inhibitors: a modified classification of inhibitors for the cytochrome *bc*₁ complex. *J Mol Biol* 2004; 341: 281–302.
10. Tian H, Yu L, Mather MW, Yu CA. Flexibility of the neck region of the Rieske iron-sulfur protein is functionally important in the cytochrome *bc*₁ complex. *J Biol Chem* 1998; 273: 27953–27959.
11. Tian H, White S, Yu L, Yu CA. Evidence for the head domain movement of the Rieske iron-sulfur protein in electron transfer reaction of the cytochrome *bc*₁ complex. *J Biol Chem* 1999; 274: 7146–7152.
12. Xiao K, Yu L, Yu CA. Confirmation of the involvement of protein domain movement during the catalytic cycle of the cytochrome *bc*₁ complex by the formation of an intersubunit disulfide bond between cytochrome *b* and the iron-sulfur protein. *J Biol Chem* 2000; 275: 38597–38604.
13. Darrouzet E, Valkova-Valchanova M, Christopher C, Moser P, Dutton L, Daldal F. Uncovering the [2Fe2S] domain movement in cytochrome *bc*₁ and its implications for energy conversion. *Proc Natl Acad Sci USA* 2000; 97: 4567–4572.
14. Kim H, Xia D, Yu CA, Xia JZ, Kachurin AM, Zhang L, Yu L, *et al.* Inhibitor binding changes domain mobility in the iron-sulfur protein of the mitochondrial *bc*₁ complex from bovine heart. *Proc Natl Acad Sci USA* 1998; 95: 8026–8033.
15. Berry EA, Guergova-Kuras M, Huang LS, Crofts AR. Structure and function of cytochrome *bc* complexes. *Annu Rev Biochem* 2000; 69: 1005–1075.

16. Nett JH, Hunte C, Trumpower BL. Changes to the length of the flexible linker region of the Rieske protein impair the interaction of ubiquinol with the cytochrome *bc*₁ complex. *Eur J Biochem* 2000, 267: 5777–5782.
17. Obungu VH, Wang Y, Amyot SM, Gocke CB, Beattie DS. Mutations in the tether region of the iron-sulfur protein affect the activity and assembly of the cytochrome *bc*₁ complex of yeast mitochondria. *Biochim Biophys Acta* 2000, 1457: 36–44.
18. Ghosh M, Wang Y, Ebert CE, Vadlamuri S, Beattie DS. Substituting leucine for alanine-86 in the tether region of the iron-sulfur protein of the cytochrome *bc*₁ complex affects the mobility of the [2Fe2S] domain. *Biochemistry* 2001, 40: 327–335.
19. Mitchell P. Possible molecular mechanisms of the protonmotive function of cytochrome systems. *J Theor Biol* 1976, 62: 327–367.
20. Brandt U, Trumpower B. The protonmotive Q Cycle in mitochondria and bacteria. *Crit Rev Biochem Mol Biol* 1994, 29: 165–197.
21. Hendler RW, Shrager RI. Problems in the experimental determination of substrate-specific H⁺/O ratios during respiration. *J Bioenerg Biomembr* 1987, 19: 551–569.
22. Leung KH, Hinkle PC. Reconstitution of ion transport and respiratory control in vesicles formed from reduced coenzyme Q-cytochrome *c* reductase and phospholipids. *J Biol Chem* 1975, 250: 8467–8471.
23. Güner S, Robertson DE, Yu L, Qui ZH, Yu CA, Knaff DB. The *Rhodospirillum rubrum* cytochrome *bc*₁ complex: redox properties, inhibitor sensitivity and proton pumping. *Biochim Biophys Acta* 1991, 1058: 269–279.
24. Kagawa Y, Racker E. Partial resolution of the enzymes catalyzing oxidative phosphorylation. *J Biol Chem* 1971, 246: 5477–5487.
25. Yu CA, Yu L. Syntheses of biologically active ubiquinone derivatives. *Biochemistry* 1982, 21: 4096–4101.
26. Yu CA, Yu L. Resolution and reconstitution of succinate-cytochrome *c* reductase: preparations and properties of high purity succinate dehydrogenase and ubiquinol-cytochrome *c* reductase. *Biochim Biophys Acta* 1980, 591: 409–420.
27. Tian H, Yu L, Mather MW, Yu CA. The involvement of serine 175 and alanine 185 of cytochrome *b* of *Rhodobacter sphaeroides* cytochrome *bc*₁ complex in interaction with iron-sulfur protein. *J Biol Chem* 1997, 272: 23722–23728.
28. Berden JA, Slater EC. The reaction of antimycin with a cytochrome *b* preparation active in reconstitution of the respiratory chain. *Biochim Biophys Acta* 1970, 216: 237–249.
29. Yu CA, Yu L, King TE. Preparation and properties of cardiac cytochrome *c*₁. *J Biol Chem* 1972, 247: 1012–1019.
30. Yu L, Dong JH, Yu CA. Characterization of purified cytochrome *c*₁ from *Rhodobacter sphaeroides* R-26. *Biochim Biophys Acta* 1986, 852: 203–211.