HHS Public Access

Author manuscript

Methods Enzymol. Author manuscript; available in PMC 2020 August 12.

Published in final edited form as:

Methods Enzymol. 2009 ; 456: 459–473. doi:10.1016/S0076-6879(08)04425-X.

ANALYSIS OF ELECTRON TRANSFER AND SUPEROXIDE GENERATION IN THE CYTOCHROME bc_1 Complex

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Abstract

During the electron transfer through the cytochrome bc_1 complex (ubiquinol-cytochrome *c* oxidoreductase or complex III), protons are translocated across the membrane, and production of superoxide anion radicals ($O_2^{\bullet -}$) is observed. The bc_1 complex is purified from broken mitochondrial preparation prepared from frozen heart muscles by repeated detergent solubilization and salt fractionation. The electron transfer of the purified complex is determined spectrophotometrically. The activity depends on the choice of detergent, protein concentration, and ubiquinol derivatives used. The proton translocation activity of $2H^+/e^-$ is determined in the reconstituted bc_1-PL vesicles. The $O_2^{\bullet -}$ production by bc_1 is determined by measuring the chemiluminescence of the 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazol[1,2-1]pyrazin-3-one hydro-chloride (MCLA)-$O_2^{\bullet -}$ adduct during a single turnover of bc_1 complex, with the Applied Photophysics stopped-flow reaction analyzer SX.18MV, by leaving the excitation light source off and registering the light emission. Production of $O_2^{\bullet -}$ by bc_1 is in an inverse relationship to its electron transfer activity. Inactivation of the bc_1 complex by incubating at elevated temperature (37°C) or by treatment with proteinase K results in an increase in $O_2^{\bullet -}$-generating activity to the same level as that of the antimycin A–inhibited complex. These results suggest that the structural integrity of protein subunits is not required for $O_2^{\bullet -}$-generating activity in the bc_1 complex.

1. INTRODUCTION

The mitochondrial electron transport chain is a major intracellular source of superoxide anion radical ($O_2^{\bullet -}$) production (Chance *et al.*, 1979). The cytochrome bc_1 complex (ubiquinol: cytochrome *c* oxidoreductase, or complex III) has been identified as one of the major $O_2^{\bullet -}$ production sites in the mitochondrial respiratory chain (Mclennan and Esposti, 2000; Turrens and Boeris, 1980; Turrens *et al.*, 1985). The cytochrome bc_1 complex (bc_1) is a multisubunit integral membrane protein complex that catalyzes electron transfer from ubiquinol to cyt *c* with concomitant translocation of protons across the membrane to generate a membrane potential and proton gradient for ATP synthesis (Trumpower and

Gennis, 1994). This complex has been purified and its 3-D structure determined (Iwata *et al.*, 1998; Xia *et al.*, 1997, 2007).

The purified bovine bc_1 complex is in a dimeric and oxidized form. Each monomer contains a full complement of all 11 protein subunits (3 redox subunits and 8 supernumerary subunits) with a slight excess of cyt c_1. Three redox subunits are essential and found in the bc_1 complex from different species: the cyt *b* subunit housing hemes b_L and b_H (low and high potential hemes), the cyt c_1 subunit containing a heme c_1, and the iron sulfur protein (ISP) housing a high potential 2Fe-2S cluster. All additional subunits, referred to as supernumerary subunits, are believed to contribute to the increased stability of these complexes (Ljungdahl *et al.*, 1987; Yu *et al.*, 1999). The absorption ratio of Soret over UV is approximately 0.95 in the purified preparation compared with that in crystalline form of 0.88 (Yue *et al.*, 1991). The purified complex catalyzes electron transfer from ubiquinol to cyt *c* with a specific activity of 24 μmol cyt *c* reduced per nmol cyt *b* at room temperature, pH 7.4, with 2,3-dimethoxy-5-methyl-6-(10-bromodecyl)-1,4-benzoquinol ($Q_0C_{10}BrH_2$) as the substrate. When the complex is embedded in phospholipid vesicles, it translocates 2 H^+ per electron transferred. This preparation is very stable; it lasts for days at 0° or months at −80 °C without activity loss. Thus, it is suitable for crystallization in the presence or absence of inhibitors. The crystals grown from this preparation diffracted X-rays up to 2.1 Å resolution at best (Esser *et al.*, 2006).

Production of $O_2^{\bullet -}$ during electron transfer through the bc_1 complex is thought to result from leakage of electrons from their normal pathways to react with molecular oxygen. Under normal catalytic conditions, only a very small number of electrons leak from the complex to form $O_2^{\bullet -}$ (Sun and Trumpower 2003; Zhang *et al.*, 1998). This $O_2^{\bullet -}$-generating activity increases when the electron transfer is blocked by antimycin or when the electron transport chain becomes overreduced (Zhang *et al.*, 1998). The electron leakage (or superoxide production) site has been speculated at ubisemiquinone of the Qp site (Dröse and Brandt, 2008; Muller *et al.*, 2003) or reduced cytochrome b_L (Nohl and Jordan, 1986; Yang *et al.*, 2008), depending on the mechanism by which bifurcation of ubiquinol proceeds in the Q-cycle model (Brandt and Trumpower, 1994; Crofts, 2004; Mitchell, 1976; Yu *et al.*, 2008). If bifurcation of quinol at the Qp site proceeds by the sequential mechanism, semiquinone formed at the Qp site (Cape *et al.*, 2007; De Vries *et al.*, 1981) and reduced heme b_L would both be the electron leakage sites during bc_1 catalysis. If bifurcation of ubiquinol at the Qp site proceeds by the concerted mechanism (Snyder *et al.*, 2000; Zhu *et al.*, 2007), reduced heme b_L would be the only electron leakage site.

This chapter describes methods for measuring the electron transfer, proton translocation, and $O_2^{\bullet -}$ production activities in the cytochrome bc_1 complex. In addition, a large-scale preparation of the cytochrome bc_1 complex from frozen heart muscles is described. A reversed relationship between electron transfer activity and $O_2^{\bullet -}$ production activity in the bc_1 complex is established.

2. MATERIALS

Fresh beef hearts were obtained from Wellington Quality Meat Company at Wellington, Kansas; sodium cholate, deoxycholic acid, cytochrome *c* (horse heart, type III), superoxide dismutase, antimycin A, valinomycin, and asolectin were from Sigma. Proteinase K was from Invitrogen. MCLA was from Molecular Probes Inc. n-Dodecyl-*β*-D-maltopyranoside (DM) was from Antrace. 2,3-Dimethoxy-5-methyl-6-geranyl-1,4-benzoquinol (Q_2H_2), 2,3-dimethoxy-5-methyl-6-isoprenyl-1,4-benzoquinol (Q_1H_2), 2,3-dimethoxy-5-methyl-6-heptyl-1,4-benzoquinol ($Q_0C_7H_2$), 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinol ($Q_0C_{10}H_2$), and 2,3-dimethoxy-5-methyl-6-(10-bromodecyl)-1,4-benzoquinol ($Q_0C_{10}BrH_2$) were prepared as previously reported (Yu and Yu, 1982). The His_6-tagged four-subunit wild-type complex (Tian *et al.*, 1998), the three-subunit core complex (the complex lacking subunit IV) (Tso *et al.*, 2000), and the [ISP (H131C, H152C)] mutant complex (the complex lacking the iron-sulfur cluster) (Gurung *et al.*, 2005) from *Rhodobacter sphaeroides* were prepared according to methods previously reported. All other chemicals were of the highest purity commercially available.

3. PURIFICATION OF THE CYTOCHROME bc_1 COMPLEX FROM BOVINE HEART SUBMITOCHONDRIAL PARTICLES

3.1. Preparation of submitochondrial particles (SMP) from frozen bovine heart muscles

Bovine hearts removed from carcasses of the slaughtered animals were immediately immersed in an ice water bath and transported to the laboratory. The fat tissues and vesicular tubes were trimmed off; chunks of neat muscles approximately 1 to 2 inches in size were put into plastic bags, 10 pounds each, and placed in a −20° freezer. A bag of frozen meat was taken out from the freezer and thawed by placing it at room temperature for 2 h and then in the cold room overnight. The thawed meat was passed through a meat grinder in a cold room. The ground meat was divided into portions of 600 g each. Each portion was mixed with 1.8 L of 10 m*M* K_2HPO_4 in a Warren blender and homogenized at high speed for two 30-sec periods. A 6 to 10 ml amount of 6 *N* NaOH was added during blending to maintain the pH at 6.8 to 7.0. The blended mixtures were combined and centrifuged at 1600*g* for 15 min and the supernatant was poured through an eight-layer cheesecloth. The pellets were resuspended in half of the volume of 20 m*M* K/Na-phosphate buffer, pH 7.4, and centrifuged at 1600*g* for 15 min. The supernatants were again collected in the same manner as the first centrifugation step, and all supernatants were combined and divided into two portions. The supernatants were acidified to pH 5.5 with 2 *N* acetic acid and centrifuged immediately at 3300*g* for 20 min. The precipitates were immediately washed with a half volume of 20 m*M* K/Na-phosphate buffer and centrifuged at 3300*g* for 25 min. The precipitates, referred to as the submitochondrial particles (SMP) or broken mitochondria, were homogenized in 0.1 *M* phosphate-borate buffer, pH 7.8, to a cyt *b* concentration of approximately 11 *μM* (4 to 5 L) and stored in a cold room 3 to 4 days before proceeding to the preparation of succinate: cytochrome *c* oxidoreductase.

3.2. Preparation of succinate: cytochrome *c* oxidoreductase from SMP

SMP, aged for 3 to 4 days, were stirred in a cold room for 20 h before 45 ml of a 20% sodium cholate solution was slowly added for every liter of SMP. Pulverized ammonium sulfate (AS), 21.2 g/100 ml of solubilized SMP (37.5% saturation), was slowly added under constant stirring and, after the addition, the stirring continued for another 20 min. The mixture was then centrifuged at 14,000*g* for 90 min. While the precipitates were saved and stored in a deep freezer (−80°C) for the preparation of cyt *c* oxidase (Yu *et al.*, 1975), the supernatant solutions were combined, and ammonium sulfate (8.9 g/100 ml) was slowly added under constant stirring. To maintain the pH of the mixture, 5 μl of concentrated ammonium hydroxide was added concurrently for every gram of ammonium sulfate used. The stirring was continued for 20 min before the mixture was centrifuged at 14,000*g* for 20 min. The resulting precipitates containing crude succinate-cytochrome *c* oxidoreductase (complexes II and III) were dissolved in PES (50 m*M* phosphate buffer, pH 7.4, containing 1 m*M* EDTA and 0.25 *M* sucrose) buffer (~1/6 vol of the SMP used) to a protein concentration of 25 to 30 mg/ml and stored at 0° for 8 h followed by another centrifugation at 96,000*g* for 40 min. The precipitates, containing mostly cyt *c* oxidase, were discarded. The supernatants were combined and dialyzed overnight with one change of buffer against 50 m*M* Na/K phosphate buffer, pH 7.4, containing 1 m*M* EDTA. The crude succinate: cyt *c* oxidoreductase appearing as precipitates in the dialysates were recovered by centrifuging at 28,000*g* for 30 min and homogenized in 50 m*M* Tris-HCl buffer, pH 7.2, to a protein concentration of 20 mg/ml. Protein concentration was estimated by optical absorption at 278 nm in 1% SDS using a converting factor of $1OD_{280}$ = 0.75 mg/ml. This crude succinate cyt *c* oxidoreductase can be stored at −80°C for future use or subjected to further purification.

The crude preparation, under constant stirring, was slowly mixed with deoxycholate solution (10%) to 0.35 mg/mg protein. The pH of the mixture was maintained at 7.3 to 7.4 with 1 *N* NaOH or HCl. The mixture was then subject to ammonium acetate fractionation with a 50% saturated solution. The mixture was brought up to 8.3, 12, 15, and 33% saturation, in steps, by slowly adding saturated ammonium acetate solution. Each fractionation step was followed by stirring for 10 min before the solution was centrifuged for 15 min at 28,000*g* to remove unwanted precipitates. Purified succinate: cyt *c* oxidoreductase was recovered in the precipitates of the final fractionation step by centrifuging at 96,000*g* for 30 min with 40% yield. The purified succinate: cyt *c* oxidoreductase was dissolved in 50 m*M* Na/K phosphate buffer, pH 7.4, containing 0.25 *M* sucrose and 1 m*M* EDTA to a protein concentration of approximately 20 mg/ml. The preparation was then dialyzed against the same buffer extensively over 2 days with two changes of buffer and stored at −80°C.

3.3. Preparation of cytochrome bc_1 particles from succinate: cytochrome *c* oxidoreductase

The extensively dialyzed, frozen succinate: cyt *c* oxidoreductase was thawed and diluted with an equal volume of 50 m*M* Na/K phosphate buffer, pH 7.4, and centrifuged at 158,000*g* for 60 min. The precipitates were collected and homogenized under an argon atmosphere in 50 m*M* borate-phosphate buffer, pH 7.8, containing 20 m*M* succinate. The pH of the suspension was adjusted to 10 with 1 *N* NaOH and stirred for further 10 min under argon before it was centrifuged at 96,000*g* for 45 min. The supernatant solution was saved for the

preparation of succinate dehydrogenase (Yu and Yu, 1980), and the precipitates were homogenized in borate-phosphate buffer, and the preceding pH 10 treatment was repeated. The precipitates (cyt bc_1 particles, yield >90%) were collected and homogenized in 50 m*M* Tris-HCl buffer, pH 8.0, to a protein concentration of approximately 20 mg/ml.

3.4. Preparation of cyt bc_1 complex from cyt bc_1 particles

The bc_1 particles as prepared previously were partially reduced. To purify a fully oxidized bc_1 complex, the homogenate was treated with active cyt *c* oxidase (1% w/w, Yu *et al.*, 1975) and cyt *c* (2 μM) at 4°C overnight (or until all cyt c/c_1 was oxidized). Potassium deoxycholate (10% solution) was slowly added to the mixture under constant stirring to a ratio of 0.35 mg/mg protein. The solution was centrifuged at 28,000*g* for 20 min to remove any formed precipitate. The supernatant was then subject to a 10-step ammonium acetate fractionation with 50% saturated solution. The volumes of ammonium acetate solution used for each step were 10, 10, 5, 4, 3.5, 3, 2, 1, 1, and 12% (v/v) of the protein solution, respectively. After each addition, the mixture was stirred continuously at 4°C for 30 min before it was centrifuged to remove any precipitates. The solutions of the first two fractionation steps were centrifuged at 96,000*g* for 30 min to remove both floating, unwanted fat and precipitates. For steps 3 through 9, the mixtures were centrifuged at 28,000*g* for 20 min, and for the final step it was centrifuged at 96,000*g* for 30 min. The purified bc_1 complex was recovered in the precipitates with a yield of approximately 40%. The precipitates were dissolved in 50 m*M* Tris-HCl buffer, pH 8.0, containing 0.66 *M* sucrose and stored at −80°C until use. This preparation is suitable for protein crystallization. The crystals obtained diffract X-rays to 2.9 Å resolution in the absence of inhibitor (Xia *et al.*, 1997) and improved to 2.1 Å in the presence of inhibitor (Esser *et al.*, 2006).

4. ELECTRON TRANSFER ACTIVITY IN THE PURIFIED bc_1 COMPLEX

The ubiquinol oxidation activity in purified bc_1 is determined by following the reduction of cyt *c* spectrophotometrically. The assay mixture contains 50 m*M* Na/K-phosphate buffer, pH 7.4, 100 μM cyt *c,* 1 m*M* EDTA, and 25 μM ubiquinol. Diluted cyt bc_1 complex (3 to 5 μl) containing 0.1 μM cyt *b* in the presence of 0.01% DM is added to start the reaction after an initial scan of the mixture for 10 sec at 550 nm for the nonenzymatic reduction of cyt *c* by the substrate. A difference millimolar extinction coefficient of 18.5 is used for the calculation of cyt *c* reduction. The substrate, ubiquinol, is prepared by hydrogenation of ubiquinone with Pt-C as catalyst. The ubiquinol stock solution (5 m*M*) is made in 95% ethanol containing 1 m*M* HCl. The diluted HCl is used to slow down the autooxidation of substrate. The determination of cyt bc_1 activity depends heavily on the physical state of the complex. Proper dilution of the complex in the presence of the right amount of detergent is the key to obtaining the best activity. Fig. 25.1 shows the activity determined under different protein concentrations. As indicated in Table 25.1, ubiquinol and its derivatives give rise to different activities; of all the derivatives tested; $Q_0C_{10}BrH_2$ shows the best activity.

5. PROTON TRANSLOCATION IN THE PURIFIED bc_1 COMPLEX

Phospholipid (PL) vesicles embedded with cyt bc_1 complex were prepared essentially according to the cholate dialysis method (Gurung *et al.*, 2005; Kagawa and Racker, 1971).

Asolectin micellar solution was prepared by sonicating 200 mg of acetone-washed asolectin in 4 ml of 50 m*M* sodium phosphate buffer, pH 7.4, containing 2% sodium cholate and 100 m*M* KCl in an ice-water batch under an anaerobic environment maintained by continuously passing argon into the vessel. The cyt bc_1 complex (1.25 mg) was mixed with 1 ml of asolectin micellar solution to give an asolectin/protein ratio of 40. The bc_1 complex-PL mixtures were incubated at 0° for 30 min before overnight dialysis at 4°C against 100 × volumes of 50 m*M* sodium phosphate buffer, pH 7.4, containing 100 m*M* KCl with three changes of buffer. The mixture was then dialyzed against 100 × volume of 150 m*M* KCl (without buffer) for 3 to 4 h.

Proton translocation coupled to electron flow through the bc_1 complex-PL vesicles is measured at room temperature with an Accumet Model 10 pH meter and a Model 13-620-96 combination pH electrode. Twenty-five nmol of $Q_0C_{10}BrH_2$ were added to 1.6 ml reaction mixture containing 150 m*M* KCl, 4 *μM* ferricytochrome *c*, 1 *μM* valinomycin, and an appropriate amount of bc_1-PL vesicles (30 to 50 *μl*). Electron flow is initiated by the addition of 5 nmol of ferricyanide, which oxidized cyt *c*, and thus provided an electron acceptor for the complex. Electron flow is also measured in an identical manner except for the presence of the protonophore, m-chloro carbonyl cyanide phenylhydrazone (CCCP), at a concentration of 2 *μM*, under which the vesicles are permeable to protons and no cross-membrane ΔpH exists. Proton-pumping stoichiometry (H^+/e^-) of 2 is calculated as the ratio of the decrease in pH on ferricyanide addition to bc_1-PL vesicles before and after treatment with CCCP (Fig. 25.2 A, also Gurung *et al.*, 2005). When proton-pumping activity is determined with PL-vesicles coembedded with bovine bc_1 and a *R. sphaeroides* mutant complex lacking the iron-sulfur cluster [ISP (H131C-H152C)] (Fig. 25.2B), a H^+/e^- of 1 is obtained, indicating that a proton leakage channel is provided by this mutant complex (Gurung *et al.*, 2005).

6. SUPEROXIDE GENERATION BY THE PURIFIED bc_1 COMPLEX

Although the rate of $O_2^{\bullet -}$ production by the cyt bc_1 complex can be determined by measuring the decrease in rate of cyt *c* reduction in the presence of superoxide dismutase under conditions of continuous turnover of the bc_1 complex (Muller *et al.*, 2002), the small rate of $O_2^{\bullet -}$ formation, compared with the normal rate of cyt *c* reduction, compromises the accuracy of this method. MCLA has a high sensitivity for $O_2^{\bullet -}$ in the neutral pH range (Nakano, 1990). The MCLA-$O_2^{\bullet -}$ chemiluminescence method has been widely used to detect $O_2^{\bullet -}$ (Midorikawa *et al.*, 2001; Uehara *et al.*, 1993; Zhang *et al.*, 1998). However, when the MCLA-$O_2^{\bullet -}$ chemiluminescence method was adopted to determine the $O_2^{\bullet -}$ production during continuing turnover of the bc_1 complex (in the presence of ubiquinol and cytochrome *c*) with Lumac/3M Biocounter (model 2110A), a high background of $O_2^{\bullet -}$ production, resulting from the nonenzymatic oxidation of ubiquinol by cytochrome *c*, was encountered. This makes it difficult to accurately measure $O_2^{\bullet -}$ production by the cyt bc_1 complex.

This difficulty has finally been overcome by measuring the chemiluminescence of the MCLA-$O_2^{\bullet -}$ adduct during a single turnover of bc_1 complex, with Applied Photophysics stopped-flow reaction analyzer SX.18MV (Leatherhead, England). By leaving the excitation light source off, the chemiluminescence of MCLA-$O_2^{\bullet -}$ generated when cyt bc_1 complex is mixed with ubiquinol and MCLA is registered in light emission (Denicola *et al.*, 1995). Because the system contains no cytochrome *c*, chemiluminescence of MCLA-$O_2^{\bullet -}$, resulting from nonenzymatic oxidation of ubiquinol by cytochrome *c*, is eliminated. This method enables us to unambiguously compare $O_2^{\bullet -}$ production by various bc_1 complexes.

Experimentally, the reaction is carried out at 25° by mixing solutions A and B in a 1:1 ratio. Solution A contains 100 m*M* Na^+/K^+ phosphate buffer, pH 7.4, 1 m*M* EDTA, 1 m*M* KCN, 1 m*M* NaN_3, 0.1% bovine serum albumin, 0.01% DM, and an appropriate concentration of bovine cyt bc_1 or other systems. Solution B contains 125 μM $Q_0C_{10}BrH_2$ and 4 μM MCLA in the same buffer. Once the reaction starts, the produced chemiluminescence, in voltage, is consecutively monitored for 2 sec. $O_2^{\bullet -}$ generation is expressed in xanthine oxidase (XO) units. One XO unit is defined as chemiluminescence (maximum peak height of light intensity) generated by 1 unit of XO, which equals 2.0 V from an Applied photophysics stopped-flow reaction anlyzer SX.18MV, when solution A containing 100 m*M* Na^+/K^+ phosphate buffer, pH 7.4, 100 μM hypoxanthine, 4 μM MCLA, and 1 m*M* NaN_3 is mixed with solution B containing 100 m*M* Na^+/K^+ phosphate buffer, pH 7.4, 1 m*M* NaN_3 and 1 unit of XO.

7. COMPARISON OF $O_2^{\bullet -}$ PRODUCTION BY THE bc_1 COMPLEXES WITH VARYING ELECTRON TRANSFER ACTIVITIES

Under normal catalytic conditions only a very small number of electrons leak from the bovine complex to form $O_2^{\bullet -}$ (Fig. 25.3A). The $O_2^{\bullet -}$ production significantly increases (fivefold) when the electron transfer reaction is blocked by antimycin A (Fig. 25.3A). This inhibitor effect is also observed in the bacterial complex (Fig. 25.3B). Because no ubisemiquinone can be detected in antimycin A–inhibited complexes in the presence of ubiquinol and such systems feature an increase in the reduction level of cyt b_L, the reduced cyt b_L is most probably the electron source for the superoxide generation.

When $O_2^{\bullet -}$ productions in cyt bc_1 complexes with varying electron transfer activities, a reversed relationship between electron transfer activity and $O_2^{\bullet -}$ production is revealed (Fig. 25.4). The specific activities, μmol cyt *c* reduced/min/nmol *b*, for the bovine complex, the four subunit, wild-type, reconstituted, and the three-subunit core complexes of *R. sphaeroides* are 24 , 2.2, 2.0, and 0.6., respectively, whereas the $O_2^{\bullet -}$ production, XO unit/nmol *b*, by these four complexes are 0.03, 0.063, 0.067, and 0.25, respectively (calculated from Fig. 25.4). Inactivation of the bc_1 complex by incubating at elevated temperature (37°C) or treatment with proteinase k results in an increase in superoxide production to the same level as that of the antimycin A–inhibited complex (Fig. 25.3). These results suggest

that the structural integrity of protein subunits is not required for superoxide generating activity.

ACKNOWLEDGMENTS

This research was supported in part by an NIH grant (GM 30721) to C. A. Y. by the Oklahoma Agricultural Experiment Station (Projects #1819 and #2372), Oklahoma State University, and by the Intramural Research Program of National Cancer Institute, Center for Cancer Research, NIH.

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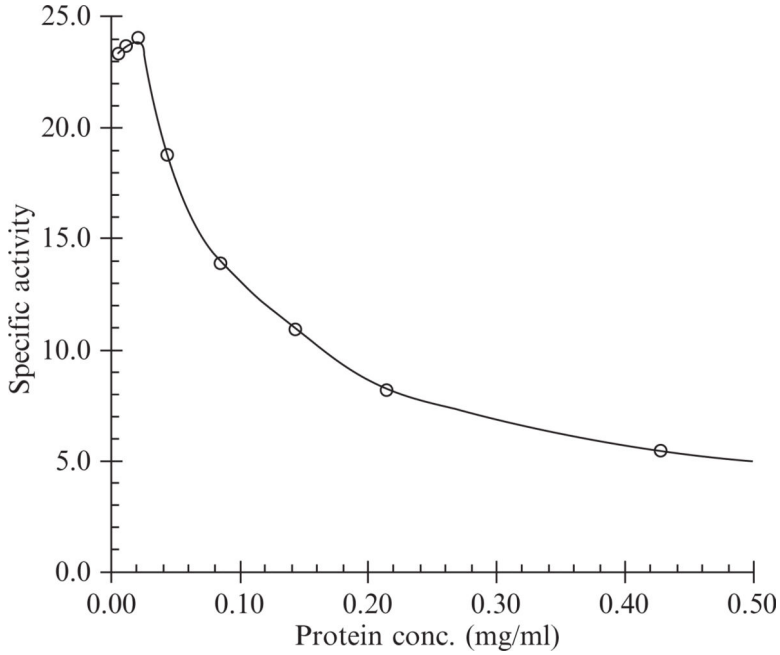

Figure 25.1.

Effect of protein concentration on the electron transfer activity measurement of cyt bc_1 complex. Cytochrome bc_1 complex preparation was dissolved in 50 m*M* Tris-HCl buffer, pH 8.0, containing 0.66 *M* sucrose and 0.01% DM at indicated protein concentrations. Three- to five-microliter aliquots were used for activity determination at room temperature. Specific activity is expressed as μmoles cytochrome *c* reduced/nmol cytochrome *b*/min.

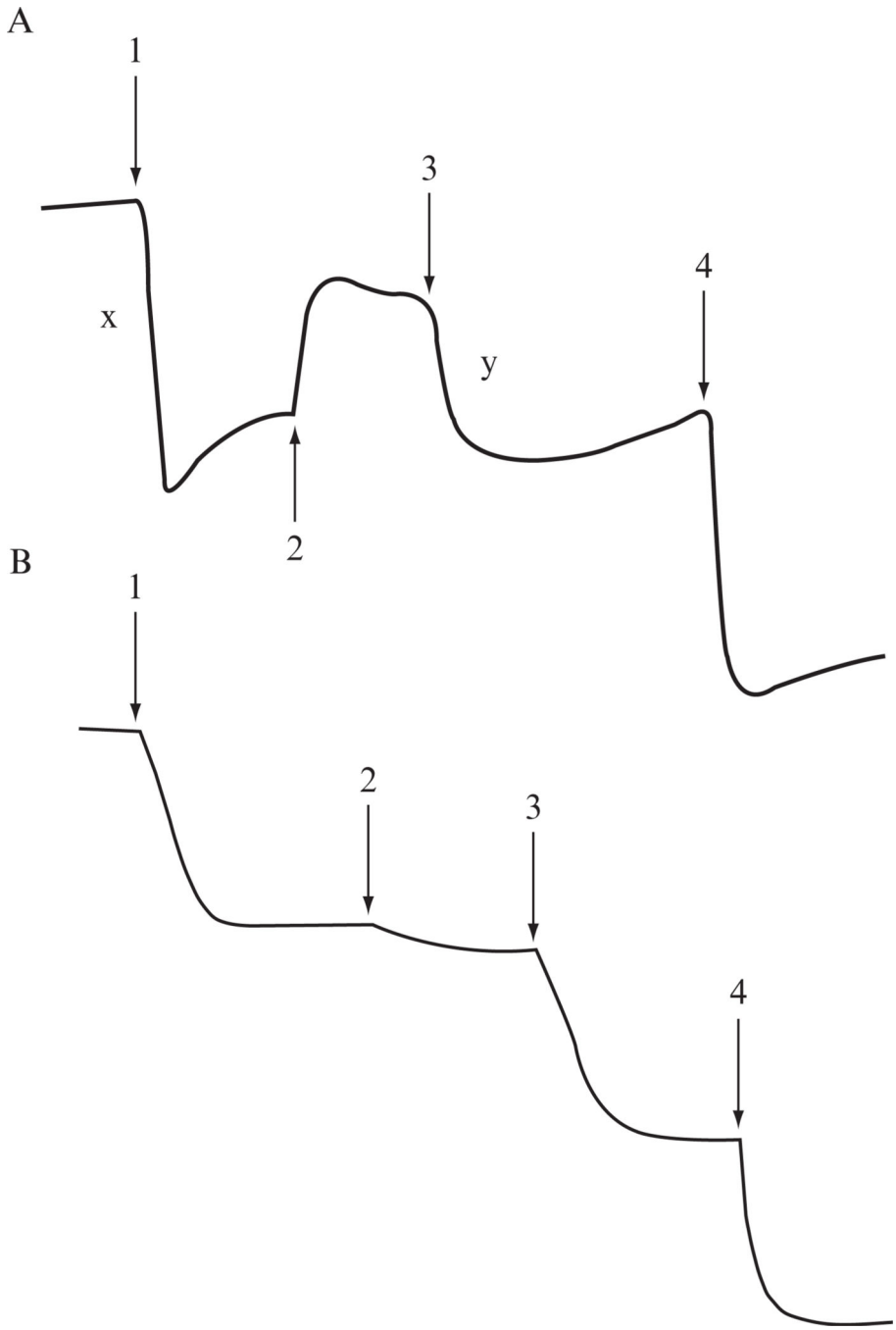

Figure 25.2.

Proton-pumping of cyt bc_1 complexes embedded in PL vesicles. Measurement of pH change as an indicator for the proton-pumping activity of PL vesicles embedded with (A) mitochondria bc_1 complex only, (B) with mitochondrial bc_1 complex and the 2Fe-2S cluster lacking mutant complex, H131C-H152C, with ubiquinol as the substrate. Arrows indicate the points of addition of 5 nmol of ferricyanide (1), 3 μM CCCP (2), 5 nmol of ferricyanide (3), and 5 nmol HCl (4). *Note*: proton-pumping ratio (H^+/e^-) = x/y.

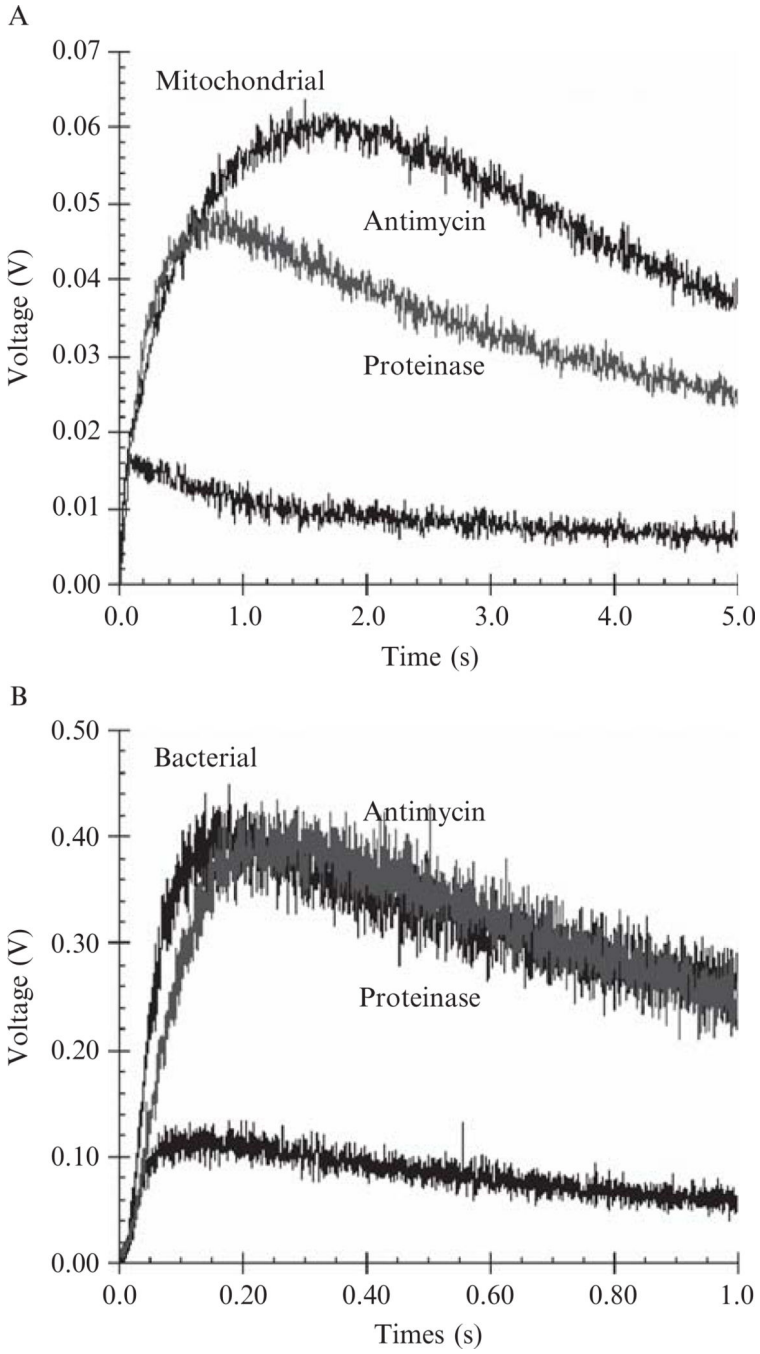

Figure 25.3.

Generation of $O_2^{\bullet -}$ in intact, antimycin A–inhibited and proteinase K-treated cytochrome bc_1 complexes. (A) Data for the mitochondrial bc_1 complex and (B) for R. *sphaeroides* four-subunit, wild-type bc_1 complex. The concentrations of bovine and *R. sphaeroides* bc_1 complexes in solution A were 5 μM. Detailed experimental conditions are given in Methods for determining superoxide generation. To digest subunits of the cyt bc_1 complex, the bc_1 solution was diluted with 50 m*M* Tris-HCl buffer, pH 7.4, containing 0.01% DM, to a protein concentration of 20 mg/ml and incubated with 0.4 mg/ml of proteinase K at room

temperature. The electron transfer activity and superoxide generation activity were followed during the course of incubation. When electron transfer activity was completely lost, the incubated mixture was subjected to SDS-PAGE to confirm the protein digestion and to determine $O_2^{\bullet -}$ production.

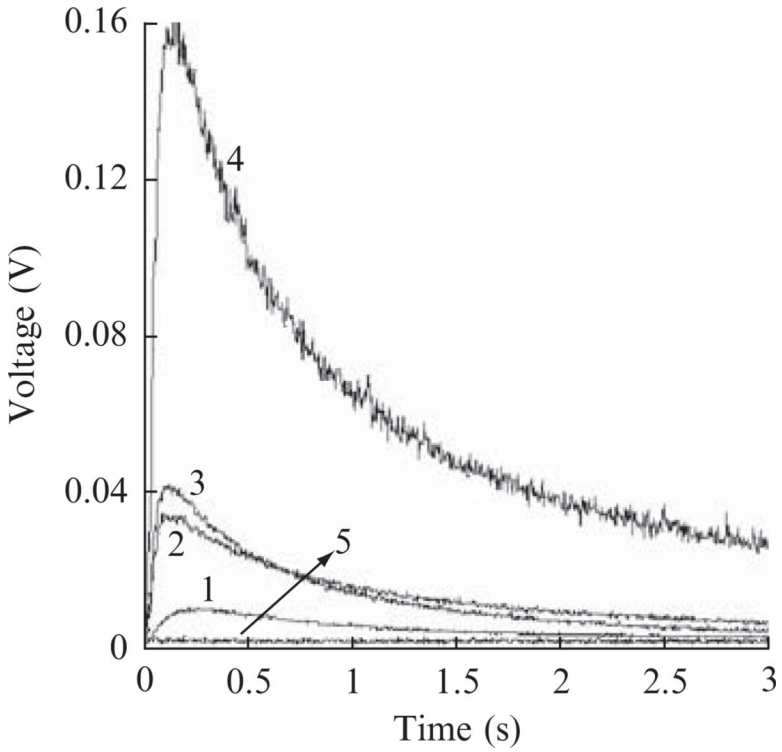

Figure 25.4.

Time traces of $O_2^{\bullet -}$ *production by various* bc_1 *complexes*. Curves 1 to 4 represent the bovine and *R. sphaeroides* complexes of wild-type, reconstituted, and three-subunit core, respectively. Reconstituted complex refers to the complex reconstituted from the core complex and recombinant wild-type subunit IV. The concentrations of bc_1 complexes used in solution Awere 3 μM. Curve 5 is for control experiment when no bc_1 complexes nor $Q_0C_{10}BrH_2$ is present in the system. A similar curve was obtained when 300 unit/ml superoxide dismutase was added to the complete system.

Table 25.1

Relative effectiveness of ubiquinol derivatives in the electron transfer reaction of cyt bc_1 complex

Substrate	Relative activity, %
Q_2H_2	100
Q_1H_2	35
$Q_0C_7H_2$	65
$Q_0C_{10}H_2$	105
$Q_0C_{10}BrH_2$	120