# MOLECULAR CHARACTERIZATION AND REACTION MECHANISM OF BOVINE HEART CYTOCHROME BC1 COMPLEX

A Doctoral Thesis

by

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February, 1993

## ACKNOWLEDGMENTS

The present work has been carried out under the direction of Professor Hiroshi Matsubara of Faculty of Science, Osaka University. I would like to thank him for his incessant guidance and encouragement throughout this work. I am deeply indebted to Professor Shinya Yoshikawa, Himeji Institute of Technology, for his invaluable advice and discussion. I am also grateful to Dr. Kyoko Itoh, Himeji Institute of Technology, for her technical advice and helpful suggestion.

I am deeply indebted to Associate Professor Keiichi Fukuyama, Osaka University, for his incessant encouragement and helpful advice to X-ray crystallography. I am also grateful to Professor Tomitake Tsukihara, Tokushima University, for his valuable suggestion.

I am deeply grateful to Professor Hajime Hirata, Himeji Institute of Technology, for extensive analysis on proton pump activity. I am also deeply grateful to Dr. Hiroshi Hori, Osaka University, for EPR analysis.

I like to thank Mr. Tsurusawa, Municipal Abattoir, Kobe, for kind donation of fresh bovine heart.

I express my thanks to all members in Matsubara's and Yoshikawa's laboratories for kind assistances and encouragement.

Finally, I thank my parents for their incessant understanding and encouragement.

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# Chapter-1.
# General Introduction

The cytochrome $bc_1$ complex, an oligomeric membrane protein, is the most common type of pump responsible for transfer of protons across energy-transducing membranes, coupled with electron transfer from quinol to *c*-type cytochrome (Trumpower, 1990). The proton gradient produced by this pump is utilized for ATP synthesis. This enzyme is widely distributed among living organisms from bacteria to mammals. Analogous enzyme, *b*6*f* type, occurs in photosynthetic apparatus, chloroplast and so on (Hauska *et al.*, 1983). They all contain four redox centers, two *b*-type and one *c*-type cytochromes and a Rieske-type iron-sulfur center. However, the number of subunits is diverse; ranging from three in the *Paracoccus denitrificans* enzyme (Yang & Trumpower, 1986) to 11 in the mammalian one (Weiss, 1987) (schematically shown in Fig. 1.1).

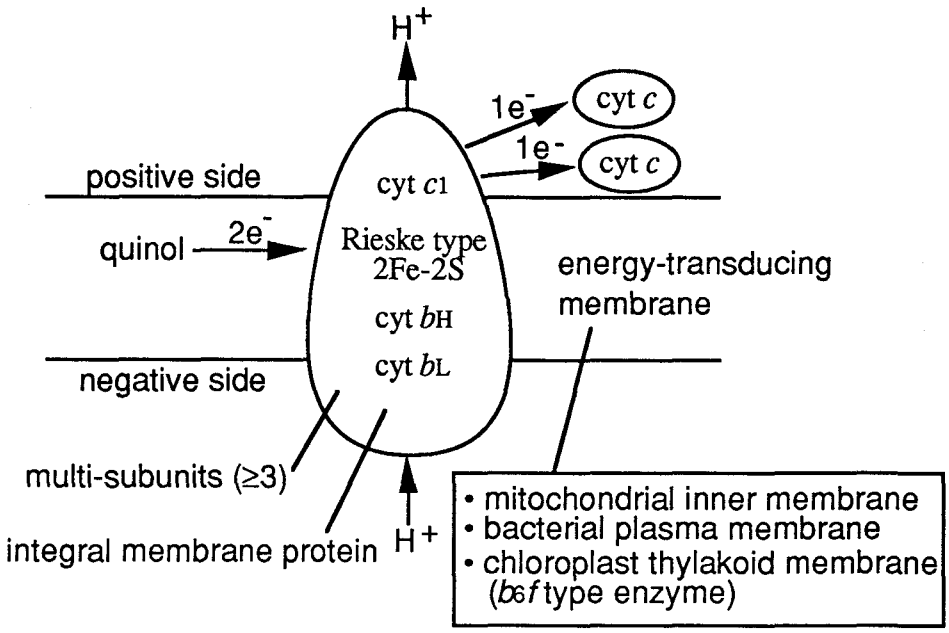



**Figure 1.1. Schematic representation of profile of cytochrome $bc_1$ complex.**

Mitochondrial cytochrome $bc_1$ complex (E.C. 1.10.2.2) is an integral membrane protein located in the inner membrane, which catalyzes the electron transfer from ubiquinol to cytochrome *c* coupling with proton translocation across the membrane (Hatefi, 1985). This enzyme constructs the energy coupling site II in the respiratory chain, so that it has attracted interest of many investigators. In 1960's, the enzyme was isolated from bovine heart by Hatefi *et al.* (1962) for the first time. The improvement of purification of the enzyme has enabled to identify the enzyme compositions; molecular weight of a functional unit was estimated to be ca. 250k by fluid physical techniques (Tzagoloff *et al.*, 1965; Weiss & Kolb;, 1979). The unit consists of 11 different polypeptides (Weiss, 1987), in which 2 protohemes, 1 heme C and 1 [2Fe-2S] iron-sulfur cluster were contained as prosthetic groups (Rieske, 1976). By determination of the amino acid sequences of subunits I and II by cDNA technique (Gencic *et al.*, 1991), the primary structures of all subunits have been completed (Anderson *et al.*, 1982; Wakabayashi *et al.*, 1982a; 1982b; 1985; Schägger & von Jagow, 1983; Borchart *et al.*, 1985; 1986; Schägger *et al.*, 1985; 1987). The molecular weight of the functional unit estimated from the amino acid sequences is 230k, assuming that it contains one mole of each subunit. Dimeric nature, so-called a diprotomer which is constituted by two of a functional unit composed of 11 subunits, was reported from electron microscopic analysis using *Neurospora* enzyme (Karlsson *et al.*, 1983). The subunit arrangement in the enzyme was proposed from protein chemical analysis using techniques of photoaffinity or chemical labeling, proteolysis in solubilized and membrane bound form and cross-linking (Gonzalez-Halphen *et al.*, 1988). The environment of each redox center is predicted from the primary structures of the subunits associating redox centers (Hauska, 1988) and various physicochemincal analyses (Simpkin *et al.*, 1989; Gurbiel *et al.*, 1991).

The redox behavior of the purified enzyme, as well as membrane bound form, has been studied most extensively among others using various inhibitors (Hatefi, 1985). The results obtained, especially the oxidant-induced reduction of cytochrome *b* in the presence of antimycin A (Rieske, 1971), strongly suggest a cyclic electron transfer

reaction within the enzyme molecule. Several mechanisms have been proposed for the electron transfer within the molecule, coupled with proton translocation (Croft *et al.*, 1983; de Vries, 1986; Wikström & Krab, 1986). Here, the Q-cycle mechanism (Mitchell, 1976) is presented as one of these working hypotheses (Fig. 1.2).

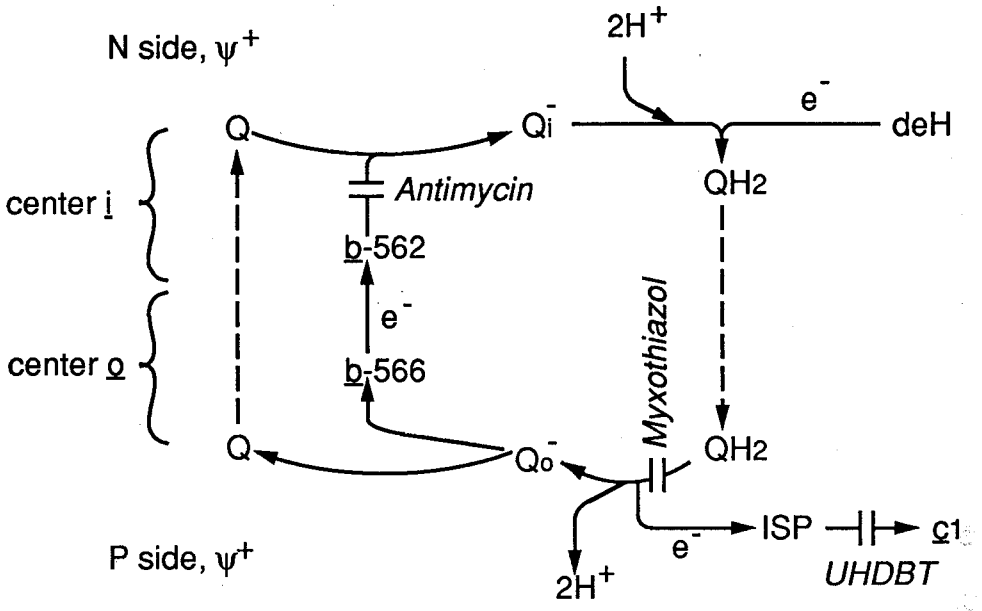



**Figure 1.2. The proposed protonmotive Q-cycle of cytochrome *bc*1 complex.** The dashed arrows designate the limited mobilities of ubiquinone and ubiquinol. The subscripts i and o designate specific species of ubisemiquinone anion, which react at the electronegative N (matrix) side (center i) or electropositive P (cytosolic) side (center o) of the inner mitochondrial membrane. ISP, iron-sulfur protein; deH, dehydrogenase. From (Hatefi, 1985).

In spite of extensive structural and functional approaches, detailed reaction mechanism of the enzyme has not been understood due to lack of three-dimensional structure at the atomic resolution. X-ray crystallography is the most powerful technique for determination of three-dimensional structure of whole protein. Thus, crystallization of cytochrome $bc_1$ complex was undertaken. The crystals were successfully grown applicable of X-ray crystallography, as described in Chapter-2. In Chapter-3, characteristics of the crystalline preparation was investigated to identify the intrinsic

nature of cytochrome $bc_1$ complex. The results indicate that crystallization is effective procedure to purify this enzyme, a multisubunit membrane protein, without perturbing the intactness of the protein. Chapter-4 deals with kinetical approach for the enzyme mechanism under initial steady-state conditions. The unique results was obtained, indicating a Hexa-Uni ping-pong kinetics involving strong repulsive interaction between the two substrate binding sites.

These studies would be initial and fundamental studies on elucidation of the reaction mechanism of cytochrome $bc_1$ complex on the basis of three-dimensional structure.

# Chapter-2.
# Crystallization of Cytochrome $bc_1$ Complex

## 2.1. INTRODUCTION

In the biological system, membrane system possesses most important functions. In order to understand biological phenomena, function of membrane system must be understood. For this purpose, it is necessary to clarify structure and function of many membrane proteins giving a characteristics of membrane system. However, in spite of important and fundamental functions of membrane proteins, there are only a few instances of determination of three-dimensional structure of membrane protein at atomic resolution (Kühlbrandt, 1988; Weiss *et al.*, 1990; Deisenhofer & Michel, 1989). It is very difficult to crystallize membrane protein purified in detergent-protein mixed micelle, that is entirely different from soluble globular proteins with respect to the chemical behavior. However, crystallization is unavoidable process for application of X-ray crystallography, which is the most powerful technique for determination of three-dimensional structure of whole protein at atomic resolution. Therefore, the crystallization of membrane proteins has been one of the most important and challenging subjects for elucidation of the functional mechanism. Recently, Kühlbrandt (1988) reviewed the general approach and some of the specific problems of crystal growth of membrane proteins. Also, it is pointed out by Michel (1991) and Garavito (1991) that membrane proteins are able to be crystallized by approach similar for globular protein, although micellar behavior should be in mind. They showed helpful and encouraging guideline for crystallization of membrane proteins.

Many different approaches have been adopted in various laboratories for elucidation of the reaction mechanism of cytochrome $bc_1$ complex. However, the molecular mechanism of the enzyme reaction cannot be understood without any stereochemical information about four redox centers, substrate-binding sites, proton

channels, and so on. A gross three-dimensional structure of a dimeric nature at far lower than atomic resolution has been reported for the *Neurospora crassa* enzyme on the basis of an electron microscopic study (Leonard *et al.*, 1981). In order to obtain more detailed structural information, we attempted to grow single crystals suitable for X-ray crystallographic analysis. Here we describe the crystallization of bovine heart cytochrome $bc_1$ complex and characterization of the crystals by X-ray diffraction.

### 2.1.1. Strategy for Protein Crystallization

A solubility diagram is helpful for a rational consideration of strategy for protein crystallization as given in Figure 2.1 (McPherson, 1991). A crystal growth proceeds three phases. i) forming nuclei from extremely high supersaturated protein solution, in 'labile region' in Figure 2.1. ii) growth of nuclei to form crystals in supersaturated solution. For crystal growth, a protein solution should be in supersaturating phase, 'metastable region', where crystal growth occur but formation of new nuclei does not. iii) termination of growth in unsaturated region, reaching to equilibrium.

Most definitive step is forming nuclei. This step is influenced by a degree of supersaturation, a manner of preparation of a supersaturated solution, and various other crystallization conditions. In order to obtain a few large well-ordered crystals, nucleation should be limited as few as possible. The optimum of saturation level is often narrow, so that it is difficult to produce a few large crystals in a vessel; no nucleus will be formed at lower level than the optimum, and many microcrystals will be deposited at higher than the optimum.

Such discussion is for soluble proteins. However, it is pointed out that membrane proteins are able to crystallize in the similar mechanism to soluble proteins, although micellar properties strongly affect crystal growth of membrane proteins (Kühlbrandt, 1988).

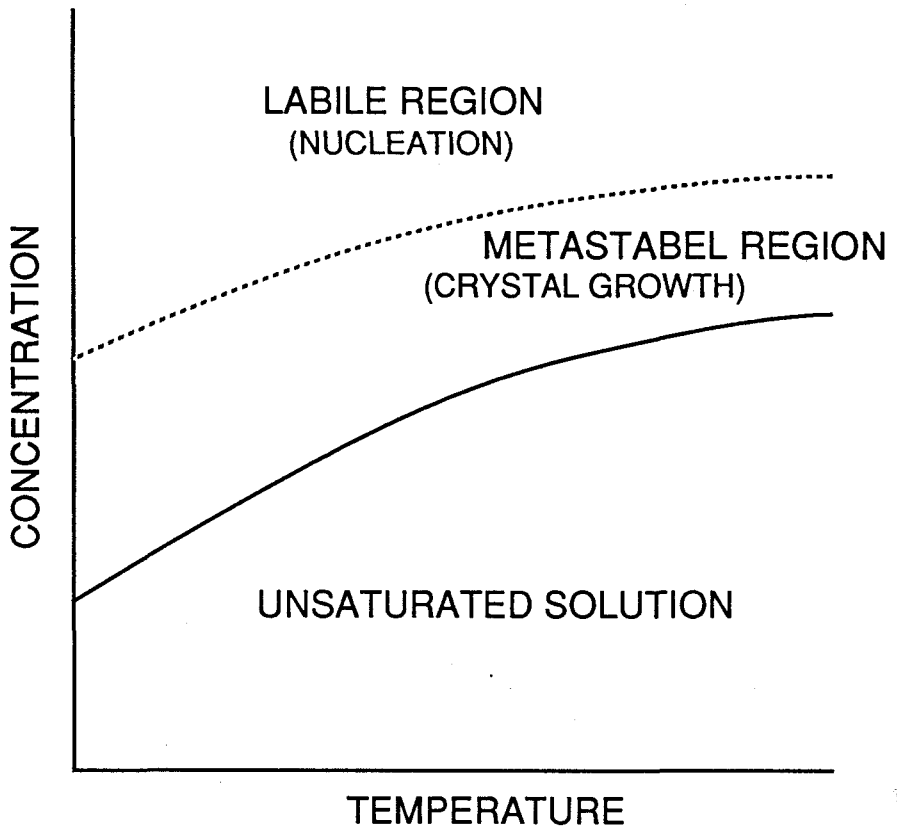



**Figure 2.1. A typical solubility diagram showing the labile, metastable, and unstaturated region.** The solid line represents the saturation boundary. This figure is cited from the reference (McPherson, 1991).

## 2.2. EXPERIMENTAL AND RESULTS

For crystallization of cytochrome $bc_1$ complex, many factors were examined. These are listed in Table 2.I as well as the results.

### 2.2.1. Chemical

Sucrosemonolaurate (>99% monoester pure) was a product of MITSUBISHI-KASEI FOOD Co. Ltd. Other detergents, β-octylglucoside, β-decylglucoside and β-

dodecylglucoside, were purchased from Sigma. Polyethyleneglycol (PEG)-4000[1] was a reagent of MERK Co. Ltd. for gas chromatography. PEG-4000 used in this work was that from the same batch. PEGs of other size were Extra Pure Reagents of WAKO Co.Ltd. Horse heart cytochrome *c* (Type VI) was a product of Sigma. Other reagents were of the highest grade commercially available.

**Table 2.I. Summary of survey of crystallizing conditions of cytochrome $bc_1$ complex.**

| factor | conditions surveyed | optimum conditions |
|---|---|---|
| purity of protein | modification of preparation, recrystallization | recrystallization |
| method of crystallization | batchwise, vapor diffusion, microdialysis | batchwise |
| surface of vessel | glass, polymer, siliconized | glass |
| surfactant or detergent | alkyl-suger type | sucrosemonolaurate |
| concn. of detergent | 0.04 ~ 2 % (w/v) | no effect (0.5 %) |
| temperature | 4°C, room temp. | room temp. |
| concentration of protein | 100 μM ~ 450 μM | 350 μM |
| species of precipitant | AS, PEG(2000~20000), MPD | PEG-4000 (MERK) |
| pH and species of buffer | Table 2.IV, 2.V and 2.V | KPi pH 8.0 |
| buffer concn. | 10 mM ~ 100 mM Tris<br>30 mM ~ 50 mM KPi | different from batch to batch |
| additive | Table 2.III | 660 mM sucrose |
| substrate or inhibitor | cytochrome c<br>antimycin, myxothiazol | none<br>no effect |

[1] Abbreviations: CMC, critical micellar concentration; DMSO, dimethylsulfoxide; PEG, polyethyleneglycol; Pi, phosphate.

### 2.2.2. Preparation of Cytochrome $bc_1$ Complex

*Purification* — The enzyme was prepared from fresh bovine heart muscle by the method of Rieske (1967) with slight modifications. This method consists of 4 steps; i) preparation of mitochondria, ii) extraction with deoxycholate and KCl, iii) fractionation with ammonium acetate in the presence of deoxycholate to obtain a succinate-cytochrome *c* reductase rich fraction, and iv) fractionation with ammonium sulfate in the presence of cholate to remove succinate dehydrogenase. A Keilin-Hartree preparation (Yoshikawa *et al.*, 1977) instead of a mitochondrial preparation was used as the starting material. The Keilin-Hartree preparation was made up from fresh bovine heart within 4 h after slaughter. Freshness of bovine heart seems critical for the consistency of the preparation. The Keilin-Hartree preparation was suspended into 50 mM Tris-HCl (pH 8.0), 0.66 M sucrose and 1 mM L-histidine (buffered sucrose), when protein concentration was increased to 35 mg/ml from 25 mg/ml for the original method. Extraction of membrane proteins was carried out with the same conditions as the original method, except for the protein concentration. In this conditions, the resulting extract was red and almost free from cytochrome *c* oxidase. If the extract contains cytochrome *c* oxidase, any later procedure can hardly remove the oxidase. Even though the oxidase was removed, no crystal was obtained from such enzyme preparation. This indicates that extraction step from the membrane is a key procedure for the purification of cytochrome $bc_1$ complex. Reproducibility of the extraction was improved by rapid preparation of the Keilin-Hartree preparation from bovine heart, careful control of extraction time and protein concentration.

Modification of starting material and its protein concentration resulted in change in the following fractionations. The final fraction with ammonium acetate in the presence of deoxycholate exhibited NADH dehydrogenase activity which was not shown in the fraction by original method (Hatefi *et al.*, 1961; Rieske, 1967). In the following fractionation with ammonium sulfate in the presence of cholate, fraction of 0-35% sat. had mainly a NADH-cytochrome *c* reductase activity, and 35%-42% fraction showed mainly succinate-cytochrome *c* reductase activity. Remaining fraction between

42%-49% had only ubiquinol : cytochrome *c* reductase activity, free from NADH- or succinate-cytochrome *c* reductase activity.

The yield of final product was 300~600 mg from 800 g of minced bovine heart. The preparation showed high molecular activity as ubiquinol : cytochrome *c* oxidoreductase, >600 ($s^{-1}$) using ubiquinol-2 as a substrate (Chapter-3). This had the same absorption spectrum (not shown; see Chapter-3) as that reported by other groups (Yu *et al.*, 1974), indicating similar heme content to that prepared with the original method judged from ratio of Soret-band height to absorbance at 280 nm. Moreover, the subunit composition was the same as reported by Schägger *et al.* (1986) judged from SDS-polyacrylamido-gel-electrophoresis, that is, the preparation composed of 11 subunits (Chapter-3).

Reduction of the time for the enzyme preparation from bovine heart from 4 days in the original method to 2 days improved the crystal growth as well as yield or reproducibility of purification. Also, quality of bovine heart, i.e. the content of lipid, protein and other substances, seems critical for the preparation. The yield of the final product was diverse among preparations (300mg~600mg).

*Exchange of the detergents with non-ionic detergent* — Ionic detergents may interfere crystallization of membrane protein due to repulsive force between charges. In fact, any crystal of membrane protein has not been obtained from the protein solution containing ionic steroid detergents, cholate or deoxycholate. The ionic detergents, deoxycholate and cholate, were used in the present purification procedure. The fact that the enzyme could be dispersed in detergent free buffer system suggests that the enzyme preparation contains a small amount of the ionic detergent tightly bound to the enzyme. Therefore, exchange of the detergents with non-ionic detergents was performed in order to remove the detergents completely.

The precipitation with PEG in the presence of the new detergent, whose concentration was ca. 10 times higher than critical micellar concentration (CMC), was employed as a method of exchange of the detergents. Exchange with dialysis required a long time (> 48 h) for complete replacement of detergents, often resulting in

denaturation of the protein. Gel filtration or adsorption chromatographic technique does not seem suitable for preparation of membrane proteins with multi-subunits, like cytochrome $bc_1$ complex, as described in discussion. PEG-4000 (Extra Pure Grade) was used as a precipitant instead of ammonium sulfate, because of instability of the enzyme in a buffer system with high ionic strength in the presence of a detergent.

Sucrose was present in the preparative buffer solution during all procedure for purpose of stabilization. Detergents of the alkyl-suger type, oligo-saccharide derivative, were tested. This type of detergents was used in the many cases for crystallization of membrane proteins. Alkyl-suger detergents with longer alkyl chain and bulkier hydrophilic head are milder (Kühlbrandt, 1988). Treatment with β-octylglucoside, β-decylglucoside and β-dodecyl-glucoside, harsher detergents, caused deterioration of absorption spectrum of cytochrome *b*. As expected, no crystal was formed from protein solutions replaced by these detergents. Sucrosemonolaurate, constructed by a longer alkyl chain and a bulkier hydrophilic head, was the mildest detergent to solubilize of cytochrome $bc_1$ complex as far as tested.

The purified enzyme diluted with buffered sucrose to 2 ~ 4 mg/ml was precipitated with 30% (w/v) PEG-4000 (Extra Pure Reagent of WAKO Co.Ltd.) in the presence of 0.1% (w/v) sucrosemonolaurate. The precipitate collected by centrifugation was dissolved in the buffered sucrose containing 0.5% sucrosemonolaurate and washed with the same medium in an ultrafiltration apparatus employing an ADVANTEC UK-200 membrane (Toyo Roshi Co. Ltd.). The final product was dialyzed against appropriate buffer solution for crystallization.

### 2.2.3. Standard Methods of Crystallization

To prepare a supersaturated protein solution, solid PEG-4000 (MERK), ca. 4~7% (w/v), was added into the protein solution containing more than 350 μM protein (ca. 80 mg protein/ml) to give slight turbidity. The enzyme concentration was expressed in terms of cytochrome $c_1$ concentration determined from the absorbance difference for the fully reduced form (dithionite-reduced form) between 554 nm and 544 nm minus

that for the fully oxidized form, using the difference extinction coefficient of 19 $mM^{-1}cm^{-1}$. The resulting solution was stored at room temperature. These conditions are shown in Table 2.II. Numerous needle like microcrystals were immediately deposited without any amorphous material. After 1-3 weeks, large red parallelepiped crystals were obtained reproducibly without any amorphous material as shown in Figure 2.2, and concomitantly microcrystals were dissolved. These crystals belong to monoclinic. A large crystal diffracted X-rays to 7.5 Å resolution, but the crystals were fragile and sensitive to mechanical touch, and most of them were twin. Therefore, this crystal form was not suitable for X-ray crystallography. The another conditions should be sought to get a more suitable crystal form.

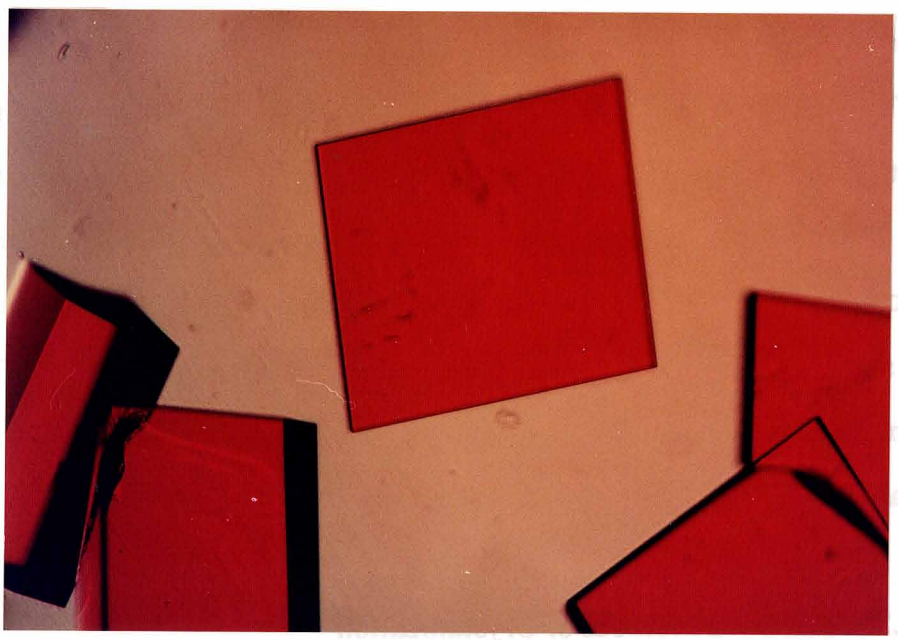


**Figure 2.2. Momoclinic crystals of cytochrome *bc*1 complex.** Cytochrome *bc*1 complex was crystallized from protein solution (88 mg protein/ml) containing 0.5% sucrosemonolaurate and 6.42% PEG-4000 in buffered sucrose at room temperature. The other conditions are listed in Table 2.II. The overall dimension of the area in the photograph is 1.4 × 2.1 mm.

**Table 2.II. Standard conditions of crystallization of cytochrome $bc_1$ complex.**

| | |
|---|---|
| temperature | room temp. |
| pH | 8.0 |
| method | batchwise |
| precipitant | PEG-4000 |
| protein concn. | 350 μM |
| detergent | 0.5% sucrosemonolaurate |
| buffer | 50 mM Tris-HCl |
| additive | 660 mM sucrose |

### 2.2.4. Survey of Crystallizing Conditions

*Crystallization method* — Protein crystals grow in a supersaturated protein solution. A supersaturated protein solution is prepared usually by increase of a precipitant concentration by a vapor diffusion method, micro-dialysis method, or careful addition of a precipitant, so-called batchwise method. The batchwise method is used for testing conditions for crystallization of cytochrome $bc_1$ complex. Because no change in concentration of all solute occurs during incubation, this method is suitable for judging the conditions tested. The slide glass with large hole, 1.5 cm × 2 mm (ca. 150 μl), was used together with cover glass to monitor the crystal growth. The large crystals were grown (Fig. 2.2) in this type of the hole slide glass in the condition as described above.

Various methods and vessels for crystallization were examined. A vapor diffusion method with sitting drop caused denaturation by liquid surface effects, probably by surface tension. As a result, no well-ordered crystals and/or cluster of needle like crystals were grown. Unfortunately and unexpectedly, no crystal was obtained by the micro-dialysis method using small dialysis cells made of acrylic

plastics. Moreover, even by batchwise method, no single crystal was obtained in micro-tubes (0.3 × 1.5 cm) or glass capillary for X-ray diffraction experiments. These results suggest that material and shape of crystallization vessel seem to be critical factors. The surface of the hole slide glass may serve nucleation points. This consideration was supported by the results of the experiments using siliconized hole slide glass and cover glass (with SurfaSil siliconizing agent, PIERCE), where no large crystal was formed. Also, the narrow inter-glass space between slide and cover glasses might provide suitable conditions for crystallization. Consequently, hole slide glass was used in all experiments as described below.

*Preparation of a supersaturated protein solution* — In crystallization, preparation of a supersaturated solution is a key procedure. Too high saturation level in labile phase in Figure 2.1 results in 'crystals showers' or many microcrystals. While, no crystal is deposited from a solution in lower saturation level in metastable region in Figure 2.1. Thus, it is very difficult to prepare a supersaturated solution to form a few nuclei. In batchwise method applied for crystallization of cytochrome $bc_1$ complex, in order to prepare a supersaturated protein solution, a small amount of precipitant was added by degrees into the enzyme solution by monitoring the change in color of the solution by bare eyes. At a concentration just to start precipitating, the color of the solution changed from red brown to ashed red. This method was not very reproducible because of the low sensitivity of bare eye determination. Thus, the several batches were prepared at several points of the concentration of the precipitant selected in a narrow range of concentration (± 0.5 % (w/v)) including the point determined by bare eyes. Such improvement on the precipitant concentration increased significantly the reproducibility in obtaining the large crystals in one series of crystallization experiment.

*Concentration of the detergents* — The detergent concentration must be higher than CMC to solubilize a membrane protein. The CMC of sucrosemonolaurate is ca. 0.02% (w/v). The crystallization conditions at various concentration of the detergent higher than 0.04% were tested. As a results, crystal growth was not affected on the concentration of the detergent so long as it was higher than CMC by up to 2%. These

indicate that the enzyme which was once well dispersed did not aggregate even at low detergent level, that might reflect that this enzyme was relatively hydrophilic as compared to other smaller integral membrane proteins, which were crystallized from the protein solution containing the detergent at extremely higher concentration than CMC (1~10 mM above CMC) (Kühlbrandt, 1988).

*Protein concentration* — The protein concentration was one of the critical factors for crystallization of cytochrome $bc_1$ complex. Nucleation is dependent on concentration of the enzyme. In addition, speed of crystal growth, final size, stability of the enzyme, etc. should be affected on the protein concentration. It is considered that the protein concentration should be reduced as low as possible above the saturating point because lesser nucleation will occur and the rate of crystal growth will be lower at lower protein concentration. According to expectation, at the lower protein concentration, the higher precipitant concentration was requisite to give rise to supersaturation. At less than 300 μM protein, few microcrystals were obtained, not followed by forming large crystals. At more than 350 μM, large parallelepiped crystals formed. At higher concentration crystals were grown with less flaws judged by investigation by a light microscope under polarized light. However, at high protein concentration, a large amount of the enzyme preparation was consumed. Hence, all crystallization experiments were carried out at 350 μM protein unless otherwise stated.

*Temperature* — The solubility of cytochrome $bc_1$ complex to buffer system used is higher at lower temperature. It is well known that the behavior of the detergent-protein mixed micelle is extremely dependent on temperature. Thus, temperature could be very important factor for any membrane protein crystallization (Kühlbrandt, 1988; Prof. Shirakihara, personal communication). However, all crystallization experiments in the present study were performed at room temperature unless otherwise stated. The crystal growth was not affected significantly by the fluctuation of room temperature (± 2°C). So far, membrane proteins were often crystallized at room temperature or at temperature not specified (Kühlbrandt, 1988). Cytochrome $bc_1$ complex was not an exceptional case.

*Effects of additive* — Effects of some additives tested and its concentration are presented in Table 2.III. So far as tested, for the crystallization of cytochrome $bc_1$ complex, these additives did not favorably influence to crystal growth. Sucrose was contained as a stabilizer in the purification procedure after extraction. But the sugar concentration did not affect the crystal growth, although in the absence of sucrose more flaws tend to appear.

**Table 2.III. Effects of additive on crystal growth of cytochrome $bc_1$ complex.**

The additive was added into the protein solution prior to addition of PEG-4000. 660 mM of sucrose was involved in the medium, except for test of sucrose. The other conditions were the same as listed in Table 2.II.

| additive | concentration | result |
|---|---|---|
| sucrose | 0, 165, 330, 660 mM | no effect except for 0 mM |
| 1,2,3-heptanetriol | 1, 2, 3, 4, 5% (w/v) | crystals with many flaws |
| pipecolinic acid | 3% (w/v) | no crystal |
| ethylene-glycol | | |
| $HO(CH_2)_3OH$ | 3% (v/v) | amorphous |
| $HO(CH_2)_4OH$ | 3% (v/v) | amorphous |
| $HO(CH_2)_5OH$ | 3% (w/v) | amorphous |
| $HO(CH_2)_6OH$ | 1, 2, 3, 4, 5% (w/v) | crystals with many flaws<br>crystals with amorphous |
| $HO(CH_2)_7OH$ | 3% (w/v) | amorphous |
| $HO(CH_2)_8OH$ | not dissolved | |
| amino acid | | |
| methionine | 3% (w/v) | no effect |
| proline | 3% (w/v) | no effect |
| organic solvent | | |
| ethanol | 3% (v/v) | crystals with many flaws |
| dimethylsulfoxide | 3% (v/v) | no effect |

It is indicated that small amphiphilic compounds are critical or effective to form large, well-ordered crystals of some membrane proteins (Michel, 1982). These compounds seem to reduce detergent-protein micellar size, enhancing a probability of protein-protein interaction essential for crystal formation (Kühlbrandt, 1988). Alternatively, these reagents may function as stabilizer of membrane proteins solubilized in detergent micelle. It is reported that hexamethylene-glycol and 1,2,3-heptanetriol critically affected the crystallization of membrane proteins containing alkyloxyethylene type detergent or lauryldimethylamine-N-oxide. While, the crystal growth of membrane proteins in the presence of an alkyl-suger type detergent was not affected by these compounds (Kühlbrandt, 1988). In the case of cytochrome $bc_1$ complex in the system containing sucrosemonolaurate, any amphiphiles did not give appreciable results.

*Precipitant* — Ammonium sulfate is one of major precipitants for protein crystallization. Some membrane proteins were crystallized using ammonium sulfate as a precipitant, or in the presence of high level salt, several hundreds mM NaCl besides a precipitant (Kühlbrandt, 1988). However, cytochrome $bc_1$ complex was not crystallized in the system using ammonium sulfate as a precipitant. The enzyme solution containing ammonium sulfate gave rise to phase separation forming oily drops and color change implying denaturation of cytochromes during incubation. This result seems to reflect that the enzyme is unstable in the buffer system at high ionic strength; where the absorbance peaks at 562 nm and 429 nm decreased, indicating denaturation of cytochrome $b$. Moreover, hydrophobic interaction, which is non-specific and unfavorable for well-ordered crystal formation, is strengthened in conditions at high ionic strength. While electrostatic interaction between proteins, which is specific and favorable for crystal formation, is weakened.

PEG, which has no charge, is an another widely used precipitant. Using this compound, cytochrome $bc_1$ complex was crystallized for the first time, as described above. PEG is polydisperse in molecular weight. PEGs with various average molecular weight were commercially purchased, and tested how the molecular weight of PEG

influences crystal formation. Using high molecular weight PEG (6000, 8000, 20000; Extra Pure Reagent), only the clusters of highly mosaic or flawed crystals were obtained. On the other hands, low weight PEG-2000 gave no crystal. According to expectation, the molecular weight of PEG drastically influences crystal formation. PEG-4000, a high purity product for gas chromatography from MERK, gave the best results. Interestingly, only crystals with many flaws formed by PEG-4000, a Extra Pure Reagent. This suggests that purity of PEG may be more effective than size of PEG. Specially purified PEG with other size than PEG-4000 might give the same or preferable results than PEG-4000.

Methylpentanediol was effective for preparation of heavy atom derivative of hydrogenase crystals which were formed in the presence of a precipitant PEG (Prof. Yasuoka, personal communication). However, any crystals could not be obtained yet using this compound as a precipitant instead of PEG,

*Salts concentration* — Although non-ionic compound was used as a precipitant, the ionic strength was important factor for crystal formation of cytochrome $bc_1$ complex, because ionic strength seems to affect the strength of electrostatic force between proteins. Crystallization was carried out at various Tris-HCl concentration as buffer reagent. In Tris buffer system, the concentration of Tris-HCl were optimum at 50 mM for the formation of large crystals. At higher concentration, between 60 mM and 100 mM, no crystal, even microcrystal, was obtained and only amorphous material was formed. At the concentration lower than 40 mM, microcrystals were formed but not any parallelepiped crystals.

*Effects of pH and the species of buffer on the crystallization* — It is expected that the effects of pH on the crystallization are drastic because of the change of charge distribution on the protein surface at different pH. Initially, the conditions were tested in wide range of pH. At various pH, the species of buffer were selected mainly from Good's buffer as listed in Table 2.IV, which also gives results. Since the species of buffer may affect crystal growth, more than 2 species were examined at certain pH. The results show that crystallization of the enzyme seems to be possible in pH range from 7

to 9. At the pH of higher than 9, only amorphous was appeared. On the other hand, at the pH of lower than 6, the enzyme aggregated without a precipitant.

**Table 2.IV. Effects of pH on crystal growth of cytochrome $bc_1$ complex.**

The concentration of all buffer reagent was 50 mM, where the best results was obtained using Tris-HCl buffer. The buffer was replaced by dialysis overnight against a buffer solution containing 660 mM sucrose and 50 mM buffer reagent without detergent, whose micelle did not effectively diffuse through dialysis tube. Another conditions were the same as listed in Table 2.II.

| pH | buffer | PEG-4000 concn. %(w/v) | result |
|---|---|---|---|
| 5 | DMGA | — | aggregate |
| 6 | Bis-Tris | — | aggregate at 20°C |
| | MES | — | aggregate |
| 7 | TES | 1.07 | PS or cloud[a] |
| | HEPES | 1.23 | cloud[a] |
| 8 | Tris | 4.34 | microcrystals |
| | Tricine | 7.84 | microcrystals |
| | HEPPSO | 5.21 | microcrystals |
| 9 | TAPS | 9.06 | microcrystals |
| | CAPSO | 13.5 | cloud[a] |
| 10 | CAPS | 13.5 | PS |
| | CHES | 13.1 | PS |

Abbreviations: Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane CAPS, 3-cyclohexylaminopropanesulfonic acid; CAPSO, 3-N-cyclohexylamino-2-hydroxylpropanesulfonic acid; CHES, 2-(cyclohexylamino)ethane-sulfonic acid; DMGA, dimethylglutaric acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HEPPSO, N-2-hydroxyethylpiperazine-N'-2-hydroxypropane-3-sulfonic acid; MES, 2-(N-morpholino)ethanesufonic acid, monohydrate; PS, phase separation; TAPS, N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; TES, N-Tris-(hydroxymethyl)methyl-2-aminoethanosulfonic acid; Tricine, Tris(hydroxylmethyl)methylglycine.

[a] cloud, amorphous material with birefringence.

Secondary, pH effects using Tris buffer in the range of pH 7 to 9 were investigated. The results are listed in Table 2.V. In many cases, microcrystals deposited and parallelepiped like crystals formed, while at the pH 8.4 tetragonal type crystals were obtained. However, the crystals of this type are not reproducibly formed at present. At lower pH, smaller amount of PEG was required to saturate the protein solution, reflecting moderately low isoelectric point of cytochrome $bc_1$ complex which reacted extremely basic protein, cytochrome *c*. The best result was obtained near pH 8.0. At the other pH, crystals were highly mosaiced and twin with many flaws.

**Table 2.V. Effect of pH on Crystal Growth of Cytochrome $bc_1$ Complex.**

The pH was changed by dialysis overnight against 50 mM Tris-HCl containing 660 mM sucrose at various pH. The other conditions were the same as listed in Table 2.II.

| pH | PEG concn. %(w/v) | crystal form |
|---|---|---|
| 7.2 | 3.57 | needle → parallelepiped(?) |
| 7.4 | 3.42 | needle → parallelepiped(?) |
| 7.6 | 4.98 | needle |
| 7.8 | 4.50 | needle → parallelepiped |
| 8.0 | 6.35 | needle → parallelepiped |
| 8.2 | 6.08 | needle → tetragonalpyramid(?) |
| 8.4 | 7.45 | needle → tetragonalpyramid |
| 8.6 | 9.96 | no crystal (phase separation) |

**Table 2.VI. Crystallization of cytochrome $bc_1$ complex in various buffer system.**

The exchange of buffer species was performed as described in Table 2.V. The buffer concentratrion was 50 mM. The other conditions were the same as listed in Table 2.II.

| buffer species | PEG concn. %(w/v) | crystal form |
|---|---|---|
| POPSO | 3.83 | PS |
| TAPSO | 3.86 | needle |
| EPPS | 5.70 | PS → tetragonalbypyramids |
| TAPS | 6.55 | clouda |
| DIPSO | 4.71 | needle |
| HEPES | 4.12 | needle → parallelepiped |
| TES | 4.46 | needle |
| BES | 4.34 | needle |
| MOPS | 4.16 | needle |
| Tricine | 7.78 | needle |
| Bicine | 5.14 | needle |
| Tris | 3.66 | needle |
| NaPi | 8.7 | PS → hexagonalcolumn |
| KPi | 9.80 | PS → hexagonalcolumn |

Abbreviations: BES, N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; Bicine, N,N-bis(2-hydroxyethyl)glycine; DIPSO, 3-[N,N-bis(2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid; EPPS, N-2-hydroxyethylpiprazine-N'-3-propanesulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MOPS 3-(N-morpholino)propane sulfonic acid; POPSO, piperazine-N,N'-bis(2-hydroxypropane-sulonic acid); PS; phase separation; TAPS, N-tris(hydroxymethyl)methyl-3-amino-propanesulfonic acid; TAPSO, N-tris(hydroxymethyl-2-hydroxy-3-aminopropane-sulfonic acid; TES, N-Tris(hydroxymethyl)-methyl-2-aminoethanosulfonic acid; Tricine, Tris(hydroxylmethyl)methylglycine.

[a] cloud, amorphous material with birefringence.

Finally, the effects of buffer species on the crystallization at pH 8.0 were examined. The buffer species tested and results are listed in Table 2.VI. In the HEPES buffer system, the same crystals as those in the Tris buffer were obtained. But the crystals had many flaws. In the EPPS buffer, crystals in the shape of hexagonal bipyramids were grown in the solution in which phase separation with amorphous material and oily drops occurred. But it is turned out that these crystals were not grown reproducibly. In addition they rapidly decomposed and dissolved within a week. In the phosphate (Pi) buffer, especially KPi buffer, crystals in the shape of hexagonal column were grown reproducibly in solution with amorphous after a week. The largest size of the hexagonal crystal was ca. 0.3 mm × 1 mm, shown in Figure 2.3. This crystal form seems suitable for X-ray experiments with respect to size, shape and stability without any mosaicity investigated under polarized light. The results of X-ray diffraction experiments as described below show that these crystals diffracted to 6.5 Å resolution, which was higher than that given by parallelepiped crystals grown in Tris-HCl buffer system.

### 2.2.5. Hexagonal Crystal in Potassium Phosphate Buffer

*Features in crystallization of hexagonal crystals* — More suitable crystal form, hexagonal one grown in KPi buffer, for X-ray crystallography with respect to resolution and handling was successfully obtained by examining conditions using various buffer system. The hexagonal crystals were grown in protein solution with amorphous, in contrast with the case of monoclinic one. The protein was in three phases in the system; crystals, amorphous and solution. When crystals were grown, protein concentration of solution reduced. Although the protein in solution and amorphous material also decreased, they were not consumed completely. This phenomenon suggested that the range of supersaturation phase, metastable phase in Figure 2.1, was narrow and small in this system, where crystals could grow. It is practically important that PEG concentration just required for forming a few nuclei must be exactly determined to

produce large crystals. Several factors influencing growth of hexagonal crystals were reexamined.

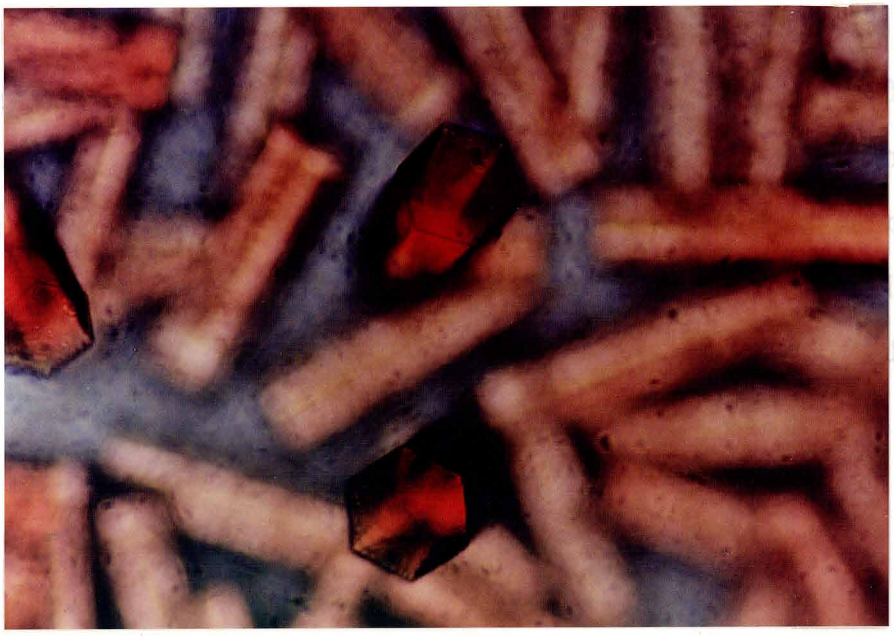


**Figure 2.3. Hexagonal crystals of cytochrome $bc_1$ complex.** The crystallizing conditions were the same to those listed in Table 2.II, except for use of 50 mM KPi buffer as buffer system. PEG-4000 concentration was 8.4% (w/v). The overall dimension of the area in the photograph is 1.4 × 2.1 mm.

*Protein concentration* — As in the case of Tris system, the protein concentration was critical factor for hexagonal crystals of cytochrome $bc_1$ complex. At the lower protein concentration, the higher concentration of precipitant was required to give rise to supersaturation. As a result, no crystal was obtained at less than 300 μM of protein concentration as in the case of parallelepiped crystals in Tris-HCl system. Consequently, crystallization was performed at 350 μM of protein concentration.

*Effects of buffer concentration* — Formation of hexagonal crystals depends critically on KPi concentration. An instance of the relationship between the concentration of KPi and the shape of the crystals is shown in Figure 2.4. At the KPi concentration lower than the optimum by only a few mM, many small crystals were deposited as shown in Figure 2.4 (lower right). At higher KPi concentration by a few mM, only amorphous material were appeared when the incubation started. Even if a few

crystals were grown in mass of amorphous material, a large hole, like cave, was produced at the rapid growing end of the crystal (Fig. 2.4, upper right) or clusters of needle like crystals were formed (Fig. 2.4, upper left). Between these concentration, there is optimum concentration where several satisfactory crystals were deposited in mass of amorphous material (Fig. 2.4, lower left). Additionally, the optimum concentration was different from preparation to preparation, so that the concentration should be determined from each preparation batch. In the optimum conditions, large and well-ordered crystals applicable for crystallographic analysis were reproducibly obtained.

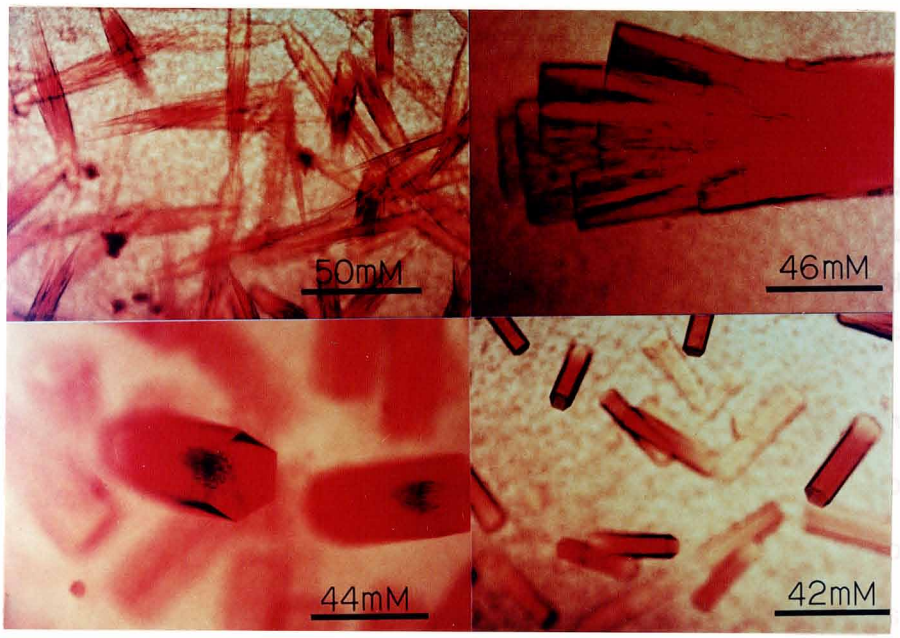



**Figure 2.4. Effects of KPi concentration on hexagonal crystal growth.** The hexagonal crystals grew in various concentration, represented, of KPi buffer pH 8.0. PEG concentrations were 9.5% for 50 mM; 8.4% for 46 mM; 44 mM for 8.0% for 44 mM; 7.2% for 42 mM. The other conditions were the same to those listed in Table 2.II. The bars represent 0.5 mm.

### 2.2.6. Trial for Improving Resolution

By examination of crystallization conditions, more suitable crystal form, hexagonal crystal form, was successfully obtained in KPi buffer system than the

parallelepiped one. This crystal form diffracted X-rays to higher resolution, 6.5 Å, than the parallelepiped form. However, this resolution is not high enough for elucidation of the reaction mechanism of the enzyme; in order to identify stereochemistry of redox centers and the position of amino acid residues, at least 3 Å resolution, so-called atomic resolution, is required.

*Crystallization of antimycin, myxothiazol or cytochrome* c *bound form* — In general, an enzyme takes a rigid conformation and is stabilized by binding the substrates, its analogues or specific inhibitors. Therefore, it is expected that crystals of these complexes diffract X-rays to higher solution than the crystals of unmodified enzyme. Crystallization of the enzyme bound specific inhibitors, antimycin A or myxothiazol and cytochrome *c*, a substrate, were tried.

Antimycin A and myxothiazol are hydrophobic antibiotics, so that these inhibitors must be solubilized in organic solvent to introduce into the enzyme solution. In general, an organic solvent affects crystal growth, especially for membrane proteins. The effects of organic solvent used to introduce the inhibitor into enzyme solutions on crystal growth of cytochrome $bc_1$ complex were tested (Table 2.III). Ethanol or dimethylsulfoxide (DMSO) are conventionally used to introduce the inhibitors. Ethanol, added to the enzyme solution in Tris buffer system at 3% (v/v), affected the crystal growth; the parallelepiped crystals tended toward twin. On the other hand, DMSO apparently have no effect on the crystal growth for both crystal forms, although smaller amount of PEG was required to saturate the solution than that in the absence.

Each of antibiotic, antimycin A and myxothiazol was dissolved into DMSO at ca. 20 mM. Each solution was added into the enzyme solution (3% (v/v)) at more than 2 equivalents to cytochrome $c_1$ prior to addition of PEG. It was confirmed that the amount of 2 equivalents of inhibitor was enough to form the inhibitor-enzyme complex by a spectroscopic analysis; the binding of the inhibitor blue-shifted α-band peak of cytochrome *b* by 2 nm (not shown)(Von Jagow & Engel, 1981). The enzyme-inhibitor complexes, with antimycin A or myxothiazol, were crystallized in both crystal forms in almost the same conditions as those of the free enzyme in the presence of DMSO. The

crystals of these inhibitors complexes were isomorphous to the crystal of the free enzyme. However, they diffracted X-rays to as high resolution as the crystals of the free enzyme did.

Tight complex with the enzyme and horse heart cytochrome *c*, which is one of substrates, showed the solubility quite different from that of free enzyme. Additionally, since cytochrome *c* is extremely basic protein, drastic change in charge distribution will occur when the enzyme forms the complex with cytochrome *c*. The protein complex behaved as if it was an another protein. Crystal of the enzyme-cytochrome *c* complex was not obtained in the similar conditions to those for free enzyme, and entirely new conditions should be surveyed for this complex.

*Recrystallization* — As an attempt to improve resolution, recrystallization was carried out. It was confirmed that crystallization resulted in removal of contaminated peptides, restrictedly proteolyzed subunits and slightly denatured enzymes (see Chapter-3). Even if the resolution was not improved, diffraction data with high quality will be collected using re-crystallized crystals.

Crystallized cytochrome $bc_1$ complex was easily obtained as follows. By concentrating the enzyme in 50 mM Tris-HCl (pH 8.0) to ca. 1 mM after exchange of detergent using ultrafiltration apparatus at 4 °C, the large amount of microcrystals (plate like) was deposited without any amorphous material. The resulting suspension was centrifuged to separate microcrystals from the mother liquor. The precipitated microcrystals were dissolved again into the same medium before crystallization, followed by dialysis against appropriate buffer system. The resulting enzyme solution was used for recrystallization.

Crystals were obtained in the same conditions as those for the hexagonal crystals as described above. As shown in Figure 2.5, the crystals were more transparent than the previous crystals and the caves at the ends in the previous crystals were disappeared. In addition, inconsistency in the optimum buffer concentration from batch to batch was almost eliminated by recrystallization. However, many nuclei were readily

formed from this enzyme solution, giving 'microcrystals showers'. Thus, micro- or macro-seeding technique may provide better conditions.

These crystals are expected to diffract X-rays to higher resolution. Some big crystals, so far obtained form the crystalized enzyme sample, lasted for only a week; the edge of the crystals became less clear with concomitant loss of the transparency, followed by dissolution.

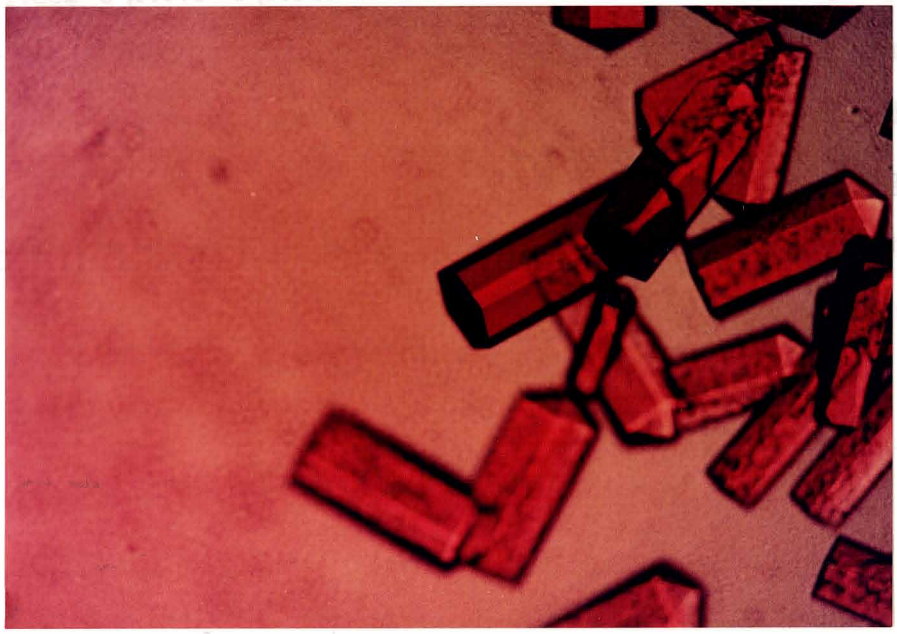


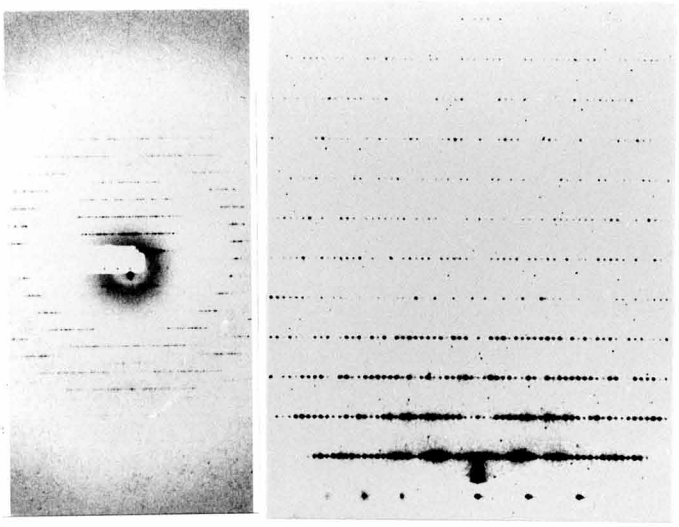
**Figure 2.5. The cytochrome $bc_1$ complex crystals by recrystallization.** The crystalline preparation was dialyzed against 40 mM KPi pH 8.0 buffer containing 660 mM sucrose overnight. The enzyme solution was diluted to 34 mM KPi and 350 μM protein just prior to crystallization experiment. The crystals grew from the protein solution containing 8.4% of PEG. The other conditions were the same to those listed in Table 2.II. The overall dimension of the area in the photograph is 1.4 × 2.1 mm.

### 2.2.7. X-ray Diffraction Experiments

A single crystal was mounted in a quartz capillary just before diffraction experiments, minimizing any mechanical contact. The diffraction experiments were done using a Weissenberg camera for macromolecular crystals (Sakabe, 1983) with synchrotron radiation at the Photon Factory (BL6A2), Tsukuba. The temperature was

kept at 20±2 °C during the experiment. The synchrotron radiation was monochromatized and focused by a cylindrically bent asymmetric-cut Si(111) monochrometer. The X-ray wavelength used was 1.040 or 1.000 Å, the collimator size 0.1 mm, and the radius of the cassette 859.5 mm.

Two crystal forms have been characterized; parallelepiped form in Tris-HCl buffer and hexagonal form in KPi buffer. The crystals in Tris-HCl are monoclinic, space group $P2_1$ with unit-cell constants of $a$=196Å, $b$=179Å, $c$=253Å and $\beta$=97°. Assuming that two dimers (four functional units) were present in an asymmetric unit, $V_m$ value (Matthews, 1968) is 4.4 Å$^3$/dalton. The crystals in KPi buffer are hexagonal, space group $P6_1$ or $P6_5$, with unit cell constants of $a$=$b$=131 Å, $c$=718 Å, and $V_m$ is 3.9 Å$^3$/dalton for one dimer (two functional units) in an asymmetric unit. They diffract X-rays to 6.5 Å resolution. (Fig. 2.6).



X-Ray Diffraction Pattern of the Hexagonal Form of Cytochrome $bc_1$ Complex

**Figure 2.6. Weissenberg photograph of the hexagonal crystal of cytochrome $bc_1$ complex.** The oscillation range was 3.75° around the $c$ axis, and the exposure time was 45 s. The left panel represents whole IP. The right one shows central area indicating separated spots along $c^*$ axis.

**Table 2.VII. Summary of conditions and results of X-ray diffraction experiments.**

| | monoclinic form | hexagonal form | | |
|---|---|---|---|---|
| | | crystal 1 | crystal 2 | crystal 3 |
| rotation axis | *b* | *c* | *c* | *c* |
| wavelength (Å) | 1.04 | 1.04 | 1.00 | 1.00 |
| oscillation angle ω(°/IP) | 5.2 | 6.3 | 3.75 | 5.25 |
| exposure time (s/IP) | 83 | 100.8 | 45.0 | 52.5 |
| # of IPs | 18 | 11 | 15 | 12 |
| resolution (Å) | —[a] | 40-10 | 40-8 | 40-8 |
| # of collected reflections | —[a] | 6,881 | 13,470 | 11,604 |
| # of unique reflections | —[a] | 2,891 | 5,672 | 5,030 |
| % to possible reflections | —[a] | 69.0 | 78.3 | 60.3 |
| $R_{merge}$ (%) | —[a] | 9.4 | 12.3 | 13.5 |

[a] The data process was not carried out for monoclinic crystal due to extreme mosaicity and twin crystal.

The monoclinic crystals were very fragile and ready to flaw by mechanical touch, and most of them were twin. Therefore, collection of diffraction data was carried out using hexagonal crystals.

Intensity data were collected at BL6A2 using Weissenberg camera for macromolecular crystals. After several exposures the crystal was translated to avoid deterioration of diffraction pattern. The diffraction intensities were recorded on the imaging plates (40 × 20 cm), and read out by a Fuji film BA-100. The diffraction patterns on the imaging plates were processed by WEIS program system (Higashi, 1989). The experimental conditions and results are summarized in Table 2.VII.

The hexagonal crystals were moderately stable against X-rays so that full data could be collected from one crystal. Due to large cell dimension along the *c* axis, focused SR beam and large cassette were requisite to resolve the diffraction spots along the *c** axis. The values of $R_{merge}$ indicate that these data would be usable for phasing by isomorphous replacement method. But the length of *c* axis was unstable (ca. ±0.7 %) , that presumably came from slight difference in the crystallization conditions, probably the concentration of KPi.

## 2.3. DISCUSSION

*The enzyme source* — Cytochrome $bc_1$ complex, which is an integral membrane protein composed of different 11 subunits, was successfully crystallized using non-ionic detergent, sucrosemonolaurate, and PEG. This enzyme is widely distributed in living organisms from gram-positive and negative bacteria to all eukaryotic cells of higher plant and animals. For achievement of protein crystallization for X-ray diffraction technique, selection of source organism is important. In the case of this enzyme, molecular weight and subunit compositions are diverse very much, that is, bacterial enzyme with ca. 120k consists of only 3~4 subunits (Yang & Trumpower, 1986), while mitochondrial one with ca. 250k consists of 9~11 subunits (Hatefi, 1985). In this work, the enzyme from bovine heart was used, since not only bovine heart enzyme, mammalian mitochondrial enzyme, has been most extensively studied but also it has large molecular size, probably possessing wide hydrophilic surface. Growth of three-dimensional crystal of type II was required protein-protein interaction rather than micelle-micelle or micelle-protein interaction (Kühlbrandt, 1988). Hence, it is expected that higher protein-protein interaction is possible for larger membrane proteins than for smaller one. Additionally, a larger membrane protein is expected to possess wider hydrophilic surface relative to hydrophobic membrane spanning region. Therefore, it is speculated that a larger membrane protein might be more advantageous for

crystallization. In fact, three groups have succeeded to crystallize the enzyme from bovine heart independently (Yue *et al.*, 1991; Kubota *et al.*, 1991; Berry *et al.*, 1992), but crystallization of bacterial enzyme has not been reported. A similar fact is present in the case of cytochrome *c* oxidase (Yoshikawa *et al.*, 1988). These facts may reflect not only wider hydrophilic surface of mitochondrial enzyme than bacterial one but stability of the eukaryotic enzyme relative to the prokaryotic enzyme. Additionally, bovine heart is uniform as source material in every individual, while bacteria may be variable at every culture. Reproducibility, stability and high yield of preparation are important and fundamental for crystallization.

*Preparation* — In order to crystallize protein, the preparation have to be obtained with high yield, purity and reproducibility. In this work, classical method was used for purification of cytochrome $bc_1$ complex from bovine heart, which consists of only two processes of fractionation by salts (Hatefi *et al.*, 1961; Rieske, 1967). By this method with some modification, it takes only 2 days to purify the enzyme preparation from bovine heart and 4 days to obtain the crystalline preparation. The reduced time for the preparation compared with that of the original method has improved the shape of crystals and the tendency toward nucleation and crystallization. It is emphasized that this preparation method is faster than the preparation including any chromatography. On the another view, although co-purified peptides were removed completely as judged by SDS-polyacrylamido-gel-electrophoresis, an adsorption chromatography might result in heterogeneous preparation with respect to conformation or subunits composition, especially for membrane proteins composed of multi-subunits like this enzyme. Such heterogeneity will banefully influence to crystallization. Thus, special care should be paid to obtain a preparation for crystallization using any chromatography.

*Choice of detergents* — Sucrose was present in the preparative buffer solution during all procedure for purpose of stabilization. The alkyl-suger type detergents, oligo-saccharide derivatives, were tested in this work. Some alkyl-oxyethylene type detergents were used for the crystallization of other membrane proteins (Yoshikawa *et*

*al.*, 1991; Garavito *et al.*, 1984). In the case of cytochrome $bc_1$ complex, however, testing the detergents needs very large amount of the sample, because the method of replacement of each detergent must be examined. In addition, the sample solubilized with another detergents may behave like another proteins with respect to solubility, sensitivity against a reagent, stability, and so on. Thus, crystallization conditions of the sample solubilized with the other detergents will be reexamined *de novo*.

*Methods of crystallization* — Batchwise method was the best for crystallization of cytochrome $bc_1$ complex. Batchwise method has advantage for searching crystallization conditions, because the conditions will not change during crystal growth, except the concentration of the protein. While vapor diffusion method gives rise to denaturation by liquid surface effects, in the case of cytochrome $bc_1$ complex. In the vapor diffusion method, the conditions can not be known when crystals appears and grows.

Decrease in a protein concentration due to crystal growth of the protein inhibits the crystal growth in the case of batchwise method. In order to obtain a few large crystals in one batch, nucleation should be satisfactorily controlled. Seeding technique could be one of the solutions of nucleation problem. Improvement of the crystallization conditions applying seeding method is now in progress.

The material of crystallization vessel was one of critical factors for crystallization of this enzyme because surface of the vessel will often provides many nucleation points. Using a glassware vessel, crystals often grew from glass surface. It was difficult to mount these crystals attached to glass surface. Since formation of many nuclei did not prevent growth of large monoclinic crystals, which grew in mass of microcrystals, a glassware is rather suitable for the monoclinic crystals. On the contrary, to obtain large hexagonal crystals, formation of lesser nucleation is better. Siliconized hole slide, in which the glass surface has coated, might be suitable for crystallization of hexagonal one. Or microdialysis method might give a small number of nucleation, since the parallelepiped crystal was not formed by this method.

*Reproducibility* — The monoclinic crystals in Tris-HCl buffer were grown from different preparation batch in the same way. On the other hand, the growth rate and the size of the hexagonal crystal in KPi buffer system was different from preparation to preparation. Crystals bored on the rapid growing crystal face for certain preparation batches, while any holes did not appear at all from another preparation batches. In order to reduce such difference in preparation batches, recrystallization was effective; bored crystals were merely grown from the crystalline preparation. Preparative crystallization may make the preparation more uniform.

Additionally, it is suggested that the sample stored during 1~2 weeks in ice bath after dialysis against KPi buffer for crystallization gave better results with respect to reproducibility of crystal growth. Such maturation effects might be due to slow detergent-protein micellar interactions, to make the solution homogeneous. This indicates that fresh sample is not always suitable for crystallization of special membrane proteins.

*Improvement of resolution* — The hexagonal crystals were successfully obtained by using K-Pi buffer instead of Tris-HCl; they were easier to handle and diffracted X-rays to higher resolution than the monoclinic one. The resolution of 6.5 Å attained by the hexagonal crystals will be enough to resolve the subunit-subunit arrangement as well as the shape of whole enzyme and the membrane spanning region. However, this resolution is far from atomic resolution and still insufficient for elucidation of the reaction mechanism of the enzyme. The hexagonal crystal has smaller $V_m$ value than that of the monoclinic one, indicating closely packing in crystal lattice. This result suggests that protein-protein interaction to form crystal is variable by small change of the environment. The $V_m$ value of the hexagonal crystal suggests high solvent content in the crystal compared to crystals of small globular proteins. High solvent content and flexible protein-protein interaction may allow miss packing of protein molecule, giving low resolution. Alternatively, the reason for low resolution might be due to heterogeneity in compositions of phospholipids specially bound to each enzyme

molecule (Chapter-3). Michel (1982) pointed out that loosely attached phospholipids seemed to prevent well-ordered crystallization.

In general, an enzyme takes a rigid conformation and is stabilized by binding the substrate or specific inhibitor. Therefore, a crystal of specific inhibitor bound form might diffract to higher resolution. However, crystals of antimycin A or myxothiazol bound form diffracted to the resolution as high as that of the inhibitor-free enzyme. Also, these crystals were isomorphous to that of the inhibitor free enzyme. This suggests that drastic conformational changes in enzyme molecule will not be induced by binding inhibitor. On the other hand, cytochrome *c* bound form did not crystallize in the similar conditions for the free enzyme. Preparation of such crystals of substrate- and inhibitor-enzyme complexes is one of the most important subjects for not only higher resolution analysis but identification of inhibitor or substrate binding site on the three dimensional structure. Additionally, inhibitor analogues attaching to heavy atom clusters will be useful for preparation of heavy atom derivatives.

Acknowledgments: I would like to thank Professor Noriyoshi Sakabe and Dr. Atsushi Nakagawa, Photon Factory, National Laboratory for High Energy Physics, Tsukuba for the helpful advice to X-ray diffraction experiments. I am deeply grateful to Professor Tomitake Tsukihara, for his helpful and encouraging advice to crystallization experiments.

# Chapter-3.
# Characteristics of the Crystalline Cytochrome $bc_1$ Complex

## 3.1. INTRODUCTION

Mitochondrial cytochrome $bc_1$ complex is an integral membrane protein composed of multi-subunits, which catalyzes the electron transfer from ubiquinol to cytochrome *c* coupling with proton translocation across mitochondrial inner membrane (Hatefi, 1985).

This enzyme is composed of 11 different polypeptides (Weiss, 1987), and the amino acid sequences of all subunits were determined. The molecular weight of the functional unit estimated from the amino acid sequences is 230k, assuming that it contains one mole of each subunit. This estimation is consistent with heme content to protein, 4.1 μmol cytochrome $c_1$/g protein (Rieske, 1976). However, from electron microscopic analysis using *Neurospora* enzyme, Karlsson *et al.* (1983) indicated that water soluble subcomplex consisted of subunit I and II contained two molecules of subunit II. If it is true, total molecular weight of the enzyme would be calculate to be ca. 280k. These results are inconsistent in terms of subunit stoichiometry and heme content of the enzyme. Such problem will be solved thorough purification of the enzyme in native form. However, purification of multisubunit membrane protein in homogeneous form is very difficult; for such proteins, removal of contaminated heme peptides, which will cause overestimation of heme content, and slightly denatured species was often accompanied by the deletion of intrinsic components of the preparation purified using conventional purification technique. Crystallization is one of the most effective techniques to purify such enzyme preparation. Considering that the crystalline preparation will be a homogeneous and highly purified sample, the crystalline preparation should be characterized as typical cytochrome $bc_1$ complex preparation.

Cytochrome $bc_1$ complex from bovine heart mitochondria was readily crystallized by concentrating of the final product with ultrafiltration apparatus in the presence of non-ionic detergent, sucrosemonolaurate, as described in Chapter-2. In order to confirm the integrity of the crystal as cytochrome $bc_1$ complex, spectra, enzyme properties and composition of the crystalline preparation were investigated. In this chapter, I will describe the characterization of the crystalline cytochrome $bc_1$ complex; it has electron transfer and proton pumping activity, and all 11 subunits and prosthetic groups are involved in the crystalline preparation. These results indicate that the crystal is of cytochrome $bc_1$ complex. Furthermore, it has been proven that all these components are intrinsic for the enzyme. These analyses on the crystalline cytochrome $bc_1$ complex from bovine heart provide additional evidence on the composition and characteristics of prosthetic groups in this enzyme integral membrane protein; cytochromes in the crystalline preparation reveal high extinction coefficients, and spectroscopic features of cytochromes and iron-sulfur center suggest an asymmetric dimeric nature of the enzyme.

## 3.2. MATERIALS AND METHODS

Ubiquinone-2 ($Q_2$)[1] was kindly supplied by Eisai Co. Ltd., Tokyo. Ubiquinol-2 ($Q_2H_2$) was prepared by the method of Rieske (1967). Horse heart cytochrome *c* was a product of Sigma (Type VI). Sucrosemonolaurate (>99% mono-ester pure) was a product of MITSUBISHI-KASEI FOOD Co. Ltd. Antimycin A and myxothiazol were purchased from Sigma. Polyethyleneglycol (PEG)-4000 was Extra Pure Reagent of WAKO Co.Ltd. $HNO_3$, $HClO_4$ and $H_2O_2$ were Specially Prepared Reagents for

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[1] Abbreviations: EPR, Electron Paramagnetic Resonance; PEG, Polyethyleneglycol; SDS-PAGE, SDS-polyacrylamido gel electrophoresis; $Q_2$, ubiquinone-2; $Q_{10}$, ubiquinone-10 ; $Q_2H_2$, ubiquinol-2

harmful metal analysis of NACALAI TESQUE. Other reagents were of the highest grade commercially available.

The crystalline preparation was obtained by concentrating the enzyme solution as described in Chapter-2. The preparation was dissolved into 50 mM Tris-HCl pH 8.0 and 660 mM sucrose (buffered sucrose) containing 0.5% sucrosemonolaurate, and stored in ice bath.

Heme content in the preparation was determined as pyridine hemochrome. Because the system contains both protoheme and heme C, contribution of each heme in the absorbance difference of pyridine hemochromes at 555.5 nm minus 535 nm and 549.5 nm minus at 535 nm were used to calculate the content of each heme as reported by Takaichi and Morita (1981). These extinction coefficients were listed in Table 3.I. The validity of these values was confirmed independently by this work.

**Table 3.I. The redox difference-difference millimolar extinction coefficients between two wavelengths for the pyridine hemochromes of protoheme and heme C (Takaichi & Morita, 1981).**

Sperm whale skeletal muscle myoglobin and horse heart cytochrome *c* were used as standard materials for protoheme and heme C, respectively. The concentrations of each heme were determined as pyridine hemochrome using redox difference millimolar extinction coefficients, 30.0 at 557 nm for protoheme and 19.1 at 550 nm for heme C. The extinction coefficients listed were obtained from absorbance differences at 555.5 minus 535 nm and 549.5 nm minus 535 nm in pyridine hemochromes of protoheme and heme C and the amounts of each heme. These values were verified in this work by re-examination of above process.

| | protoheme | heme C |
|---|---|---|
| $\Delta(\Delta\varepsilon^{red\text{-}ox})_{555.5\text{-}535}$ | 24.0 | 6.46 |
| $\Delta(\Delta\varepsilon^{red\text{-}ox})_{549.5\text{-}535}$ | 10.9 | 22.0 |

Ubiquinone-10 ($Q_{10}$) contained in the preparation was extracted with cyclohexane as reported by Redfearn (1967). The quantity of $Q_{10}$ was estimated by

spectroscopic method using the extinction coefficients, $\Delta\varepsilon^{red\text{-}ox}{}_{275} = 12.25\ mM^{-1}cm^{-1}$. Ubiquinone was reduced by $NaBH_4$ to measure its absorbance at UV region.

Phosphorus assay was carried out by the slightly modified Bartlett's ultramicro method (Bartlett, 1959); wet ashing was carried out using $HClO_4$, instead of $H_2SO_4$ plus $H_2O_2$, for at least 48 hrs. $K_2HPO_4$ was used as a standard.

The content of protein was determined with a slightly modified Biuret method using Bovine Serum Albumin as a standard material (Gornall *et al*, 1949) and with amino acid analysis (Spackmen *et al*, 1958) with an IRICA model A-5500 amino acid analyzer.

SDS-polyacrylamido gel electrophoresis (SDS-PAGE) was performed as described by Schägger *et al.* (1986).

Absorption spectra were measured by double beam spectrophotometers equipped with double monochrometer of Hitachi 3200 and Shimazu UVPC-3100.

*Content of metals by atomic absorption analysis* — Content of metals, especially of Fe atom, was measured by atomic absorption analysis using HITACHI Polarized Zeeman Atomic Absorption Photometer Z-6100 and Atomic Absorption & Flame Emission Spectrophotometer AA-845 (NIPPON JARREL-ASH). Iron Standard Solution (1000 ppm) for Atomic Absorption Analysis from NACALAI TESQUE was used as an iron standard. Protein solution was ashed as follows. 0.75 ml conc. $HNO_3$ were added to up to 350 μl of the protein solution to be analyzed, followed by heating by micro-burner until precipitate of denatured protein was disappeared. Addition of 25μl of $H_2O_2$ resulted in change of color from brown to yellow. Resulting solution was evaporated to a volume of less than 50 μl by heating. 0.75 ml of conc. $HClO_4$ were added and the solution was heated. After the color was lost, in order to distill out residual $HClO_4$, the evaporation of the solution to less than 50 μl was repeated by three times by heating, followed by addition of 250 μl water. Volume of the solution was adjusted to 5 ml by addition of 0.05 N $HNO_3$ using volumetric-flask. The solution thus obtained was applied to atomic absorption analysis (Einarsdottir & Caughey, 1985).

*Electron paramagnetic resonance spectroscopy* — The Electron Paramagnetic Resonance (EPR) spectra measurements were curried out at X-band (9.23 GHz) microwave frequency by using a Varian X-band cavity with a home-built EPR spectrometer with 100-kHz field modulation. An immersion double Dewar flask and/or an Oxford flow cryostat (ESR-900) were used for the measurements at liquid helium temperature. The enzyme at a concentration of 110 μM (cytochrome $c_1$) was aerobically reduced with 5 mM ascorbate for 30 min or anaerobically with 50 mM dithionite for 20 min, followed by loading into EPR sample tubes. For another conditions of measurement, see figure legend.

*Redox titration* — The fully oxidized cytochrome $bc_1$ complex was prepared by aerobic oxidation in the presence of 0.5% mole equivalent of cytochrome *c* oxidase and cytochrome *c*, prior to degassing. Cytochrome *c* oxidase was a kind gift from Itoh, K. & Yoshikawa, S. (Himeji Institute of Technology). Concentration of cytochrome *c* oxidase was determined by the absorbance difference in the fully reduced form at 605 nm minus 630 nm using extinction coefficient 23.3 $mM^{-1}cm^{-1}$ (Yoshikawa *et al.* unpublished data). Concentration of cytochrome *c* was determined by the absorbance difference in reduced minus oxidized form at 550 nm using redox extinction coefficient 18.5 $mM^{-1}cm^{-1}$. All the anaerobic experiments in this study were carried out with the method of Burleigh *et al.* (1969). The dithionite solution was standardized anaerobically using ferricyanide as a standard material, and kept anaerobic for at least 2 weeks without any change in the concentration. The dithionite concentration was 3~4 mM in terms of reducing equivalent. The fully oxidized enzyme under strictly anaerobic conditions in a Thumberg type cuvette was titrated with the dithionite solution introduced through a port of the cuvette with a Hamilton gas tight syringe. The syringe is tight enough for keeping the enzyme solution anaerobic during the titration. The absorption spectrum was measured with a Hitachi-3401 recording spectrophotometer at room temperature.

*Electron transfer activity* — The enzymic electron transfer activity of the enzyme, which was dispersed using sucrosemonolaurate instead of Tween-20, was measured as previously described (Kubota *et al.*, 1992).

*Preparation of cytochrome* $bc_1$ *complex liposome* — The enzyme was reconstituted into phospholipid vesicles by removing the detergents using Bio-Beads (Penniston *et al.*, 1988) as follows. 240 mg of asolection was dispersed with sonication into 2 ml of 0.5 % sucrosemonolaurate solution in the buffered sucrose. 0.1 ml of the enzyme solution (final 12.9 μM; ca 6 mg) was added into the resulting lipid suspension. Then, Bio-Beads, which was pre-treated with methanol followed by replacement with deionized water, were added and slowly agitated at 4 °C for 2 days. After removal of Bio-Beads with Muromakku filter, the reconstituted vesicle was stored in liquid nitrogen. The yield of reconstitution was only about 10 % to the total enzyme added to the phospholipid suspension. Substantial enzyme was adsorbed by Bio-Beads.

The proton translocation was measured by the method of Papa *et al.* (1983). Proton release was monitored as the change of pH with a complex glass pH electrode (Beckman no. 39532). $Q_2H_2$ was used as a reductant. Other experimental conditions are described in the legends to Figure 3.5 and Table 3.IV.

## 3.3. RESULTS

*Components of crystalline cytochrome* $bc_1$ *complex* — Chemical composition of the crystalline preparation of cytochrome $bc_1$ complex is listed in Table 3.II.

Heme content was estimated as pyridine hemochrome. Heme extraction technique was not applied to separate protoheme from protein component containing heme C. Concentrations of pyridine hemochromes in the solution to be analyzed containing both protoheme and heme C were directly estimated as described in Material and Methods. Heme C in the crystalline preparation was estimated to be 3.3 nmol/mg protein. Protein concentration estimated by both Biuret method and amino acid analysis gave consistent results. This value estimated (3.3 nmol/mg) was appreciably small compared with that reported by Rieske *et al.* (1967), 4.1 nmol/mg protein. This result suggests a higher molecular weight (predicted to 300k) of the crystalline enzyme

complex. From pyridine hemochrome analysis, 2.26±0.098 moles of protoheme were contained to one mole of heme C. This result indicates that 2 equivalents of cytochrome *b* are involved to one equivalent of cytochrome $c_1$ in the crystalline preparation, which is consistent with the results as reported by other groups (Salerno *et al.*, 1989).

**Table 3.II. Composition of the crystalline cytochrome $bc_1$ complex.**

| | content (nmol/mg protein) | ratio to cytochrome $c_1$ (mol/mol) |
|---|---|---|
| Fe[a] | 16[b] | 5.1 ± 0.23[c] |
| protoheme[d] | 7.5[b] | 2.26 ± 0.098[e] |
| heme C[d] | 3.3 ± 0.2 | 1 |
| non-heme iron[f] | 6.6 | 1.9 ± 0.24 |
| ubiquinone[g] | — | 0.13 ~ 0.16 |
| phospholipid[h] | — | 27 ~ 32 |

[a] from atomic absorption analysis
[b] calculated by the value in the right colomn and heme C content to mg protein.
[c] the mean value and the standerd deviation in 5 experiments.
[d] estimated as pyridine hemochromogen.
[e] the mean value and the standerd deviation in 9 experiments.
[f] calculated by subtracting heme content from total iron content.
[g] estimated by absorbance at 275 nm after extraction with organic solvent.
[h] estimated as phosphorous atom.

The crystalline enzyme contains 5.1 ± 0.23 iron atoms to cytochrome $c_1$, but no other metal was detected by atomic absorption analysis. This value agreed with that of reported by other group (Rieske, 1976). Iron atom adsorbed in enzyme surface seems scarcely present in the crystalline preparation, since washing with EDTA (1 mM) by dialysis overnight did not affect the iron content. Non-heme iron was calculated by subtracting of heme content from total iron content; existence of 2 non-heme irons to

cytochrome $c_1$ is consistent with the presence of one 2Fe-2S cluster in a functional unit. While, the parent enzyme preparation before crystallization contained about 6~7 iron atoms, suggesting presence of iron atoms adsorbed in enzyme surface and/or co-purified peptides.

$Q_{10}$ was almost stoichiometrically contained in the present preparation before exchange of the detergents as well as in various preparation of cytochrome $bc_1$ complex (Yu *et al.*, 1978; Fleischer *et al.*, 1961; Rieske, 1976). However, the crystalline preparation contained only residual amount of ubiquinone, as shown in Table 3.II.

Phospholipid content in the crystalline preparation was estimated as phosphorous atom content (Bartlett, 1959). It was reported by Schägger *et al.* (1990) that phospholipid is necessary for enzymic activity as well as $Q_{10}$. They reported that the cytochrome $bc_1$ preparation involved 8~9 moles of cardiolipin, 10~20 moles of phosphatidylethanolamine and 5~10 moles of phosphatidylcholine per mole of cytochrome $c_1$. On the basis of this report, the preparation will contain 30~50 moles of phosphorous atoms per mole of cytochrome $c_1$. The crystalline preparation contained 27~32 moles of phosphorous atoms. This value was almost the smallest value in that range, suggesting that process of exchange of the detergents and/or crystallization removed non-specific phospholipids adhered to the enzyme surface.

*Visible absorption spectrum* — The UV-visible absorbance spectrum of the crystalline preparation are shown in Figure 3.1. The spectrum of the crystalline sample was almost identical to that of fully oxidized form of the Rieske's preparation before the crystallization, although the small peak at 553 nm is observed due to slight reduction of cytochrome $c_1$. The ratio of absorbance of Soret-band to that at 280 nm in the crystalline preparation, almost 1.0, was higher than that of the parent preparation, 0.9~0.8. This suggests high heme content against protein component and/or high extinction coefficients of both cytochromes in the crystalline enzyme. Fully reduced form was obtained by addition of excess amount of solid dithionite. The spectrum shows typical features of cytochrome $bc_1$ complex; peak at 562 nm and shoulder at 553 nm of $\alpha$-band, and peak at 429 nm and shoulder at 416 nm of Soret-band. The peak at

562 nm and 429 nm are assigned to cytochrome *b* and the shoulder at 553 nm and 416 nm to cytochrome $c_1$.

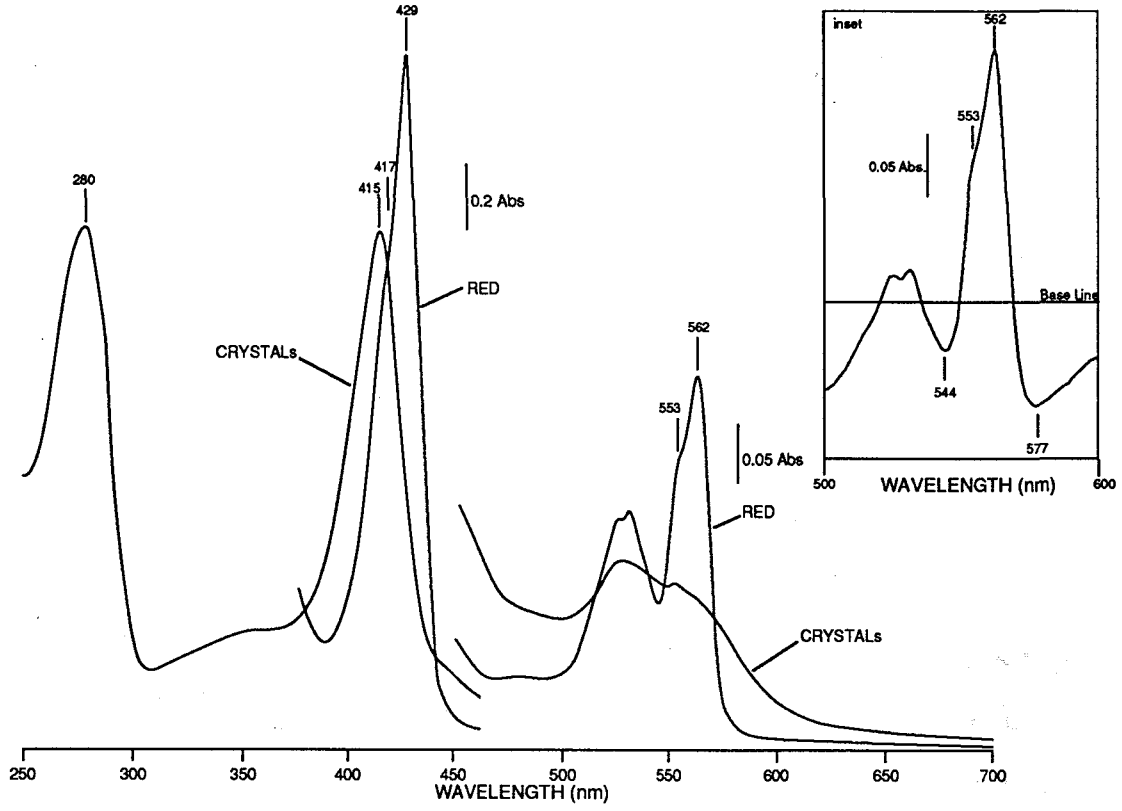


**Figure 3.1. The UV-visible absorption spectra of the cytochrome $bc_1$ complex and the redox difference spectrum (inset).** The crystalline preparation was diluted into buffered sucrose pH 8.0 containing 0.5% sucrosemonolaurate. CRYSTALs represents the crystalline enzyme at 4.0 μM cytochrome $c_1$ (estimated by new extinction coefficient, see text) as prepared and RED represents dithionite-reduced form. Inset shows the redox difference spectrum (reduced form minus oxidized form). The enzyme was fully oxidized with slight exess amount of ferricyanide.

Inset of Figure 3.1 shows the difference spectrum of reduced form minus oxidized form which was prepared by addition of small amount of ferricyanide. Conventionally, concentrations of both cytochromes were estimated on the basis of the absorbance difference between 554 nm and 544 nm in the redox difference spectrum, using the difference extinction coefficient of 19 $mM^{-1}cm^{-1}$ for cytochrome $c_1$ and the difference between 562 nm and 577 nm, using 20 $mM^{-1}cm^{-1}$ for cytochrome *b* (Hatefi

*et al.*, 1962). The molar ratio of cytochrome *b* to cytochrome $c_1$ obtained by these extinction coefficients was calculated to be 1.9~2.0 in the crystalline preparation, which was higher than that in the sample before crystallization (1.8~1.9) or that in mother liquor of crystalline preparation (1.6~1.7) and in a preparation stored for more than 3 weeks after purification (1.6~1.7). This indicates that the crystalline preparation keeps cytochrome *b* intact, which is readily lost by a treatment such as repeated precipitation, exposure to harsh detergent and storage in ice bath for more than 3 weeks. In other words, crystallization may be one of the most effective purification removing slightly denatured species.

The extinction coefficients for both hemes were estimated using the redox difference spectrum of the crystalline preparation and heme contents estimated as pyridine hemochrome. The values estimated based on 7 determinations were $36 \pm 1.1$ $mM^{-1}cm^{-1}$ for cytochrome $c_1$ and $31.4 \pm 0.83$ $mM^{-1}cm^{-1}$ for cytochrome *b*. These were significantly larger by 1.5~1.9 times than the respective values of 19 and 20 reported by Hatefi *et al.* (1962). Considering that cytochromes, especially cytochrome *b*, are readily denatured during purification, this result also suggests that the structures of the cytochromes in the crystalline preparation are more intrinsic than those in the conventional preparation. New extinction coefficients were determined by the absorbance differences at each wavelength in fully reduced form and amount of hemes from pyridine hemochrome to be $\Delta\varepsilon_{553\text{-}545} = 33.0 \pm 0.72$ $mM^{-1}cm^{-1}$ for cytochrome $c_1$ and $\Delta\varepsilon_{562\text{-}575} = 34.6 \pm 0.82$ $mM^{-1}cm^{-1}$ for cytochrome *b*. The experimental accuracy (2 × S.D./ the mean value) was less than 5%. The method of the determination of cytochromes concentration using only fully reduced spectrum is more convenient than the conventional method where the redox difference spectrum is necessary. These values was verified by the heme content, iron content and redox titration with dithionite as described bellow.

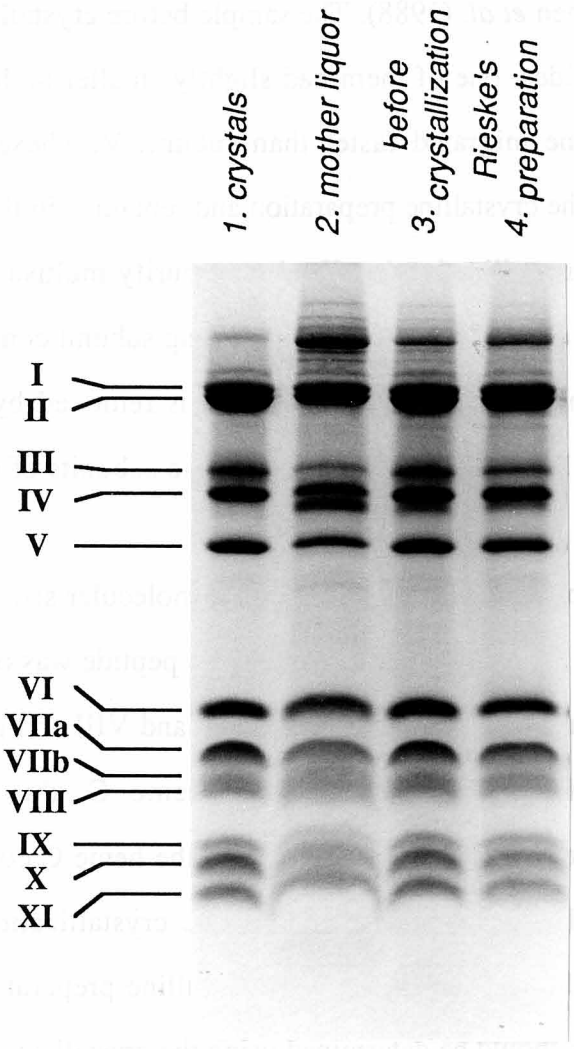


**Figure 3.2. Subunit composition of the crystalline cytochrome $bc_1$ complex.** SDS-PAGE system was carried out as described by Schögger *et al.* (1986) Lane 1, the crystalline preparation; lane 2, the mother liquor for crystallization; lane 3, the preparation before crystallization; lane 4, Rieske's preparation before the exchange of the detergents.

*SDS-PAGE* — The SDS-PAGE system, where all subunits of cytochrome $bc_1$ complex can be separated, has been established by Schägger *et al.* (1986). This SDS-PAGE system was applied to the crystalline preparation as well as the mother liquor from crystals, the preparation whose detergents were exchanged with sucrosemonolaurate before crystallization and Rieske's preparation (Fig. 3.2).

Crystalline preparation (lane 1) contains 11 subunits as reported by Schägger *et al.* (1986) and González-Halphen *et al.* (1988). The sample before crystallization included a few co-purified polypeptides; one of them had slightly smaller molecular size than subunit IV and another one migrated faster than subunit V. These peptides were completely removed from the crystalline preparation and remained in the mother liquor. This result indicates that crystallization is effective to purify multisubunit membrane protein, which is generally difficult to purify with keeping subunit composition innate. In other words, the fact that none of 11 polypeptides is removed by crystallization strongly suggests that all 11 polypeptides are the intrinsic subunits of cytochrome $bc_1$ complex.

Contaminated polypeptide with slightly smaller molecular size than subunit IV was probably a restrictedly digested cytochrome $c_1$. This peptide was often co-purified with cytochrome $c_1$ subcomplex containing subunits IV and VIII and hardly separated from the subcomplex. If this peptide contained heme C, this will cause an overestimation of heme content in the enzyme. In fact, the heme C content relative to protoheme in the mother liquor and the sample before crystallization estimated by absorption spectrum were higher than that in the crystalline preparation. Thus, exact content of prosthetic groups should be determined using the crystalline preparation.

*EPR spectroscopy* — EPR spectra of Rieske-iron sulfur cluster in the crystalline cytochrome $bc_1$ complex, whose signal appear near g=2, were measured under two reduction conditions, aerobically ascorbate reduction (Fig. 3.3A), and anaerobically dithionite reduction (Fig. 3.3B). The crystalline sample reduced with dithionite shows typical rhombic $S$=1/2 line-shape of Rieske type iron-sulfur center: $g_z$=2.02, $g_y$=1.89 and $g_x$=1.76 (Fig. 3.3B). This is very similar to the line-shape of broader type, which was observed by De Vries *et al.* (1979; 1983) in the $Q_{10}$-depleted enzyme or in the $Q_{10}$-containing enzyme at low redox potential. Thus, since the crystalline preparation involved only residual $Q_{10}$ and the sample was anaerobically reduced with excess amount of dithionite, the sharper signal characterized with $g_x$=1.81 was not detected in this experiment. This result shows the existence of Rieske-type iron-sulfur center in the

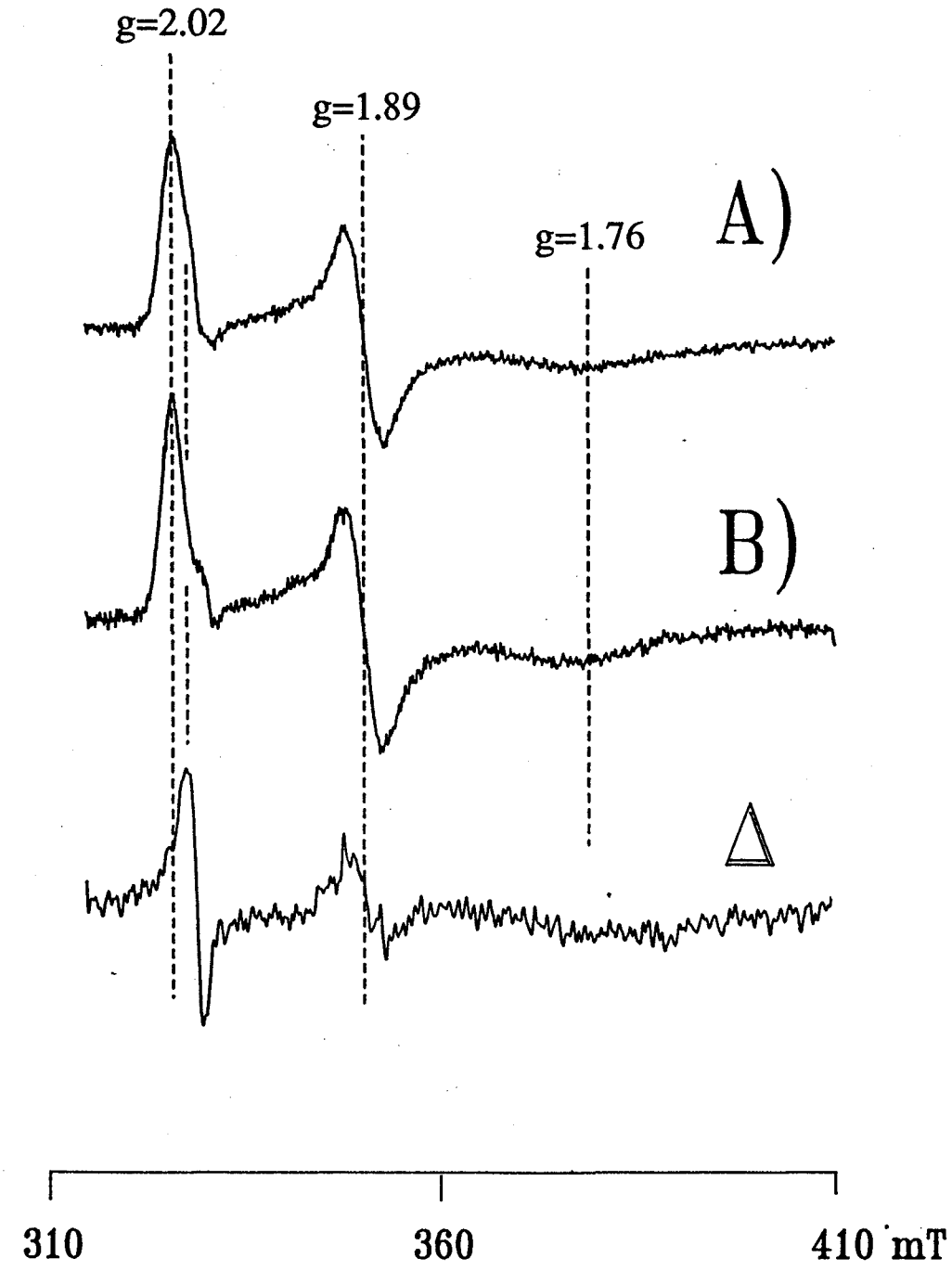


**Figure 3.3. EPR spectra of Rieske iron-sulfur center in the crystalline cytochrome $bc_1$ complex.** The reduction of the sample was performed as described in Materials and Methods. EPR conditions: Frequency, 9227.1 MHz; tempareture, 15K; micromave power, 5mW; modulation amplitude, 0.5 mT; scanning rate, 40 mT/min. A) ascorbate reduced form; B) dithionite reduced form; Δ) difference spectrum A) minus B) multiplied by appropriate factor.

crystalline enzyme. While, in ascorbate reduced form, there is a shoulder on the high-field side of the $g_z$ at 2.02, indicating the presence of a second species with $g_z$=2.01. This is clearly shown in the difference spectrum between ascorbate reduced form minus dithionite reduced form (Fig. 3.3Δ). Such species was observed as the major one in the $Q_{10}$-containing preparation before crystallization aerobically reduced with ascorbate (not shown). However, in the case of the crystalline preparation containing only residual $Q_{10}$, this is a minor species. De Vries *et al.* (1979) suggested that the enzyme exist in an asymmetric dimer based on the fact that the relative amount of any of the two species in the enzyme had never exceeded 50% to cytochrome $c_1$ under any conditions used. Thus, the crystalline preparation would also be an asymmetric dimer.

The EPR signal of ferric cytochromes in low-field susceptibly reflects the heme environment (de Vries *et al.*, 1979; Salerno *et al.*, 1989). The slightly denatured sample in oxidized form, whose absorption maximum at 562 nm was slightly low and slightly blue shifted by ca. 0.5 nm, showed $g$=6 signal, indicating the presence of high spin heme (not shown). The crystalline preparation did not show any $g$=6 signal as well as the preparation before crystallization, suggesting that little perturbation of cytochrome *b* occurred during the crystallization and dissolving of the crystals.

*Redox titration* — Redox titration of the crystalline preparation with dithionite solution was examined by monitoring the spectral change in visible region with spectrophotometer. The crystalline preparation was slightly reduced before titration. Prior to titration the enzyme was aerobically oxidized with catalytic amounts of cytochrome *c* and cytochrome *c* oxidase, the amount of which were 2.5% redox equivalent to cytochrome $c_1$. The change of absorbance spectrum was shown in Figure 3.4A. Reducing reagent was added by 1 redox equivalent. The increase of absorbance at 553 nm reveals reduction of cytochrome $c_1$. Only cytochrome $c_1$ and probably Rieske's iron-sulfur center, whose redox state was hardly identified from absorption spectrum, were reduced by 2 equivalents of reductant, while cytochrome *b* was scarcely reduced. This is clearly shown in the difference spectrum (Fig. 3.4B, spectra 1 and 2), spectral change by every addition of one equivalent of reductant; although cytochrome *b* was

slightly reduced by the second addition of the reductant (shoulder at 562 nm in spectrum 2) and remaining cytochrome $c_1$ was reduced by third addition (shoulder at 553 nm in spectrum 3), cytochrome $c_1$ was almost completely reduced within initial 2 equivalents of reductant. The fact that 2 redox equivalents were enough to reduce cytochrome $c_1$ and probably iron-sulfur center supports the heme content in the enzyme estimated by pyridine hemochrome analysis and iron content from atomic absorption analysis.

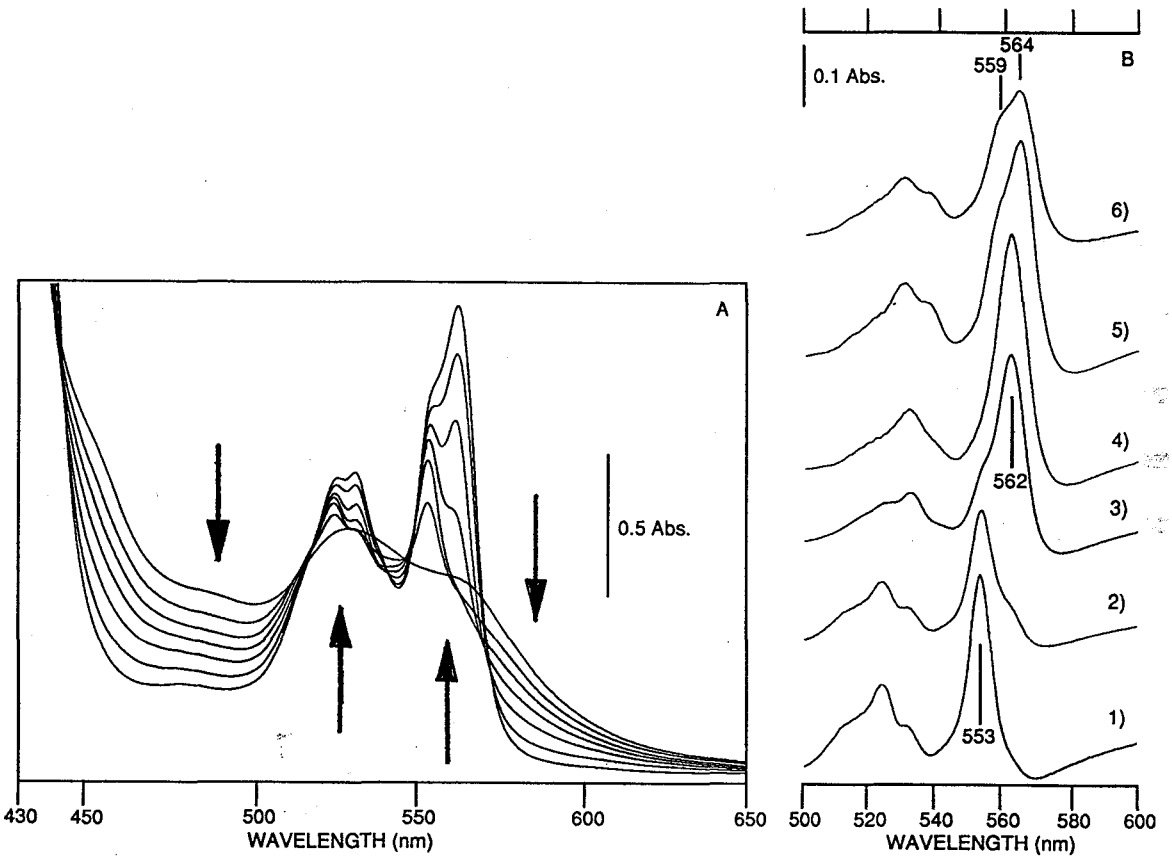


**Figure 3.4. Redox titration of the crystalline cytochrome $bc_1$ complex.** The 12.8 μM sample in 3.65 ml of buffered sucrose pH 8.0 was fully oxidized by 65 nM cytochrome *c* and 67 nM cytochrome *c* oxidase prior to degassing. The volume of the solution was decreased to 2.4 ml, final concentration of the enzyme, 19.8 μM during degassing. The dithionite solution (4.62 mM as reducing equivalent) was added by 10 μl, 1.03 reducing equivalent. Volume of dithionite solution added was 0, 10, 20, 30, 40, 50 and 60 μl. The all spectra were normalized due to dilution for addition of dithionite solution. (A) the absolute spectra; (B) the difference spectra indicating the spectral change due to every addition of reductant; spectrum 1, difference of 10 μl - 0 μl; spectrum 2, 20 μl - 10 μl; spectrum 3, 30 μl - 20 μl; spectrum 4, 40 μl - 30 μl; spectrum 5, 50 μl - 40 μl; spectrum 6, 60 μl - 50 μl. The direction of spectral change was represented by arrows in (A).

In contrast to that cytochrome $c_1$ and iron-sulfur center (high potential centers) were stoichiometrically reduced by dithionite solution, reduction of cytochromes *b* (low potential centers) did not occur stoichiometrically. Full reduction of cytochromes *b* was attained by further 4 equivalents of the reductant, that is, total of 6 redox equivalents were required to reduce them fully (Fig. 3.4A). One of cytochrome *b* components having a absorption maximum at 562 nm was first reduced by 2 equivalents of reductants (Fig. 3.4B, spectra 3 and 4), and the other two components with absorption maxima at 564 nm and 559 nm were second reduced by additional 2 equivalents (Figure 3.4B, spectra 5 and 6). These spectral changes were not explained by reduction of single species with a peak at 564 nm and a shoulder at 559 nm, because the shape of the spectra were different form each other. These species seemed to correspond to cytochromes $b_{566}$ and $b_{560}$, since the position of the peak and the shoulder in these difference spectra are close to those shown by Salerno *et al.* (1989). Although such titration experiment indicated that at least 3 components of cytochrome *b* are present in the enzyme, the molar ratio of these cytochromes *b* components could not be clarified. This result also suggests that the enzyme behaves like asymmetric dimer; it can not be explained with a monomeric or a symmetrical dimeric nature of the enzyme complex.

*Electron transfer activity* — The electron transfer from $Q_2H_2$, an analogue of reduced $Q_{10}$, to ferricytochrome *c* mediated by the crystalline preparation was investigated in steady state conditions (Table 3.III). The detailed feature of the reaction and conditions will be described in Chapter-4. The crystalline preparation as well as the preparation before crystallization possessed the electron transfer activity as high as the preparation by other group (Degli Esposti *et al.*, 1986).

$Q_{10}$ was almost stoichiometrically contained in various preparation of cytochrome $bc_1$ complex (Yu *et al.*, 1978; Fleischer *et al.*, 1961; Rieske, 1976). Yu *et al.* (1978) reported that depletion of $Q_{10}$ by treatment with organic solvent or by repeated precipitation in the presence of cholate made the preparation inactive and that the enzymic activity of $Q_{10}$-depleted enzyme was restored by reconstitution of $Q_{10}$ and phospholipid. These results imply an intrinsic function of $Q_{10}$ in enzymic activity.

Contrary, the present crystalline preparation included only residual $Q_{10}$, 0.16 mole per cytochrome $c_1$, as described above. In addition, $Q_{10}$-depleted enzyme was as active as $Q_{10}$-reconstituted one (see Chapter-4). These contradictory results may come from difference in $Q_{10}$-depletion methods. Kinetical aspects will be discussed in Chapter-4.

**Table 3.III. The electron transfer activity of the crystalline cytochrome *bc*1 complex.**

The enzymic activity was measured at 20 °C under the steady-state conditions containing 10 μM of both substrates as previously described (Kubota *et al.*, 1992; Chapter-4).

| preparation | activity ($s^{-1}$) |
|---|---|
| crystalline | 680 |
| before crystallization | 630 |
| preparation by Degli Esposti *et al.* | 170-500[a] |

[a] From Degli Esposti *et al.*, 1986.

*Proton pump activity* — The crystalline cytochrome $bc_1$ complex was introduced by removal of the detergent with Bio-Beads into phospholipid vesicle in both 'right-side out' and 'in-side out' orientations (Penniston *et al.*, 1988). One substrate of the enzyme is hydrophilic protein, cytochrome *c*, which can attach the enzyme with 'right-side out' only. Thus, in the system involving cytochrome *c*, only 'right-side out' enzyme display the activity (Papa *et al.*, 1983). In the presence of potassium ion and valinomycin, membrane potential was collapsed, maximizing the proton translocation.

The proton pump activity of the crystalline enzyme was measured with two systems. One was 'oxidant pulse experiment' (Papa *et al.*, 1983) that started with addition of oxidant, ferricyanide, in the presence of excess amount (30 μM) of reducing substrate, $Q_2H_2$, and catalytic amount (0.5 μM) of cytochrome *c*. The proton release

was monitored with pH electrode. The pH traces were given in Figure 3.5. The $H^+/e^-$ ratio was obtained from the extent of $H^+$ release and the amount of ferricyanide added by the following technique generally used in other laboratories (Papa *et al.*, 1983). Addition of $Q_2H_2$ dispersed in slightly acidic ethanol (>6 mM HCl) into reaction mixture containing the enzyme vesicle caused large decrease of pH. After pH poised, 5 μM (final concentration) of ferricyanide was added to start the reaction. The proton release occurred, to an H+/e- ratio of 2.3. In the presence of nigericin, which collapses transmembrane ΔpH, the $H^+/e^-$ ratio was 1.3. The proton release investigated in the presence of nigericin was interpreted as scalar proton release accompanied by oxidation of $Q_2H_2$. Net vectorial proton translocation activity was calculated to be 1.0 of $H^+/e^-$ ratio (Table IV(A)) by subtracting the proton release in the presence of nigericin from that in the absence of antibiotic. The proton translocation activity was almost completely inhibited by antimycin A; it is supported that the proton release in the presence of antimycin A, 1.3 $H^+/e^-$, was due to scalar proton release accompanied by non-enzymic oxidation of $Q_2H_2$ with ferricyanide.

In the other system, the reaction was started with addition of reductant (reductant pulse), 10 μM of $Q_2H_2$, in the presence of 8 μM of ferricytochrome *c* (Papa *et al.*, 1983). The $H^+/e^-$ ratio was calculated from initial rate of increase of proton concentration monitored with pH electrode and reduction of cytochrome *c* measured by the absorbance change at 550nm. The result was shown in Table IV, B. The traces of both pH and absorption changes were not shown. Non-enzymic reduction of cytochrome *c* was corrected using initial rates in the presence of antimycin A. The $H^+/e^-$ ratio was estimated to be ca. 3.3, which is surprisingly larger than that predicted. The result indicated qualitatively that at least the crystalline preparation possessed a proton translocating activity. However, response of pH electrode was too slow to analyze the initial rate quantitatively.

According to the proton motive Q-cycle mechanism, $Q_{10}$ functions as vector for proton translocation. However, as described above, the crystalline preparation does not contain $Q_{10}$ stoichiometrically. Consequently, $Q_2H_2$ could function as not only one of

substrates but the proton vector, suggesting that tightly bound $Q_{10}$ may not intrinsic for enzymic function.

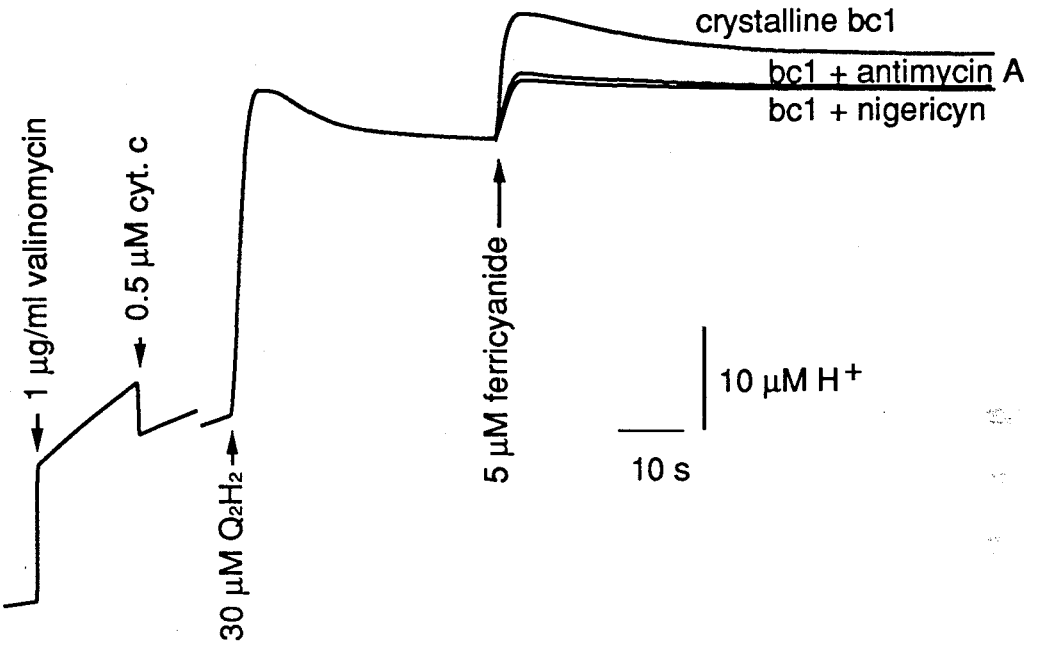


**Figure 3.5. Proton translocation catalyzed by the crystalline cytochrome $bc_1$ complex.** The cytochrome $bc_1$ complex vesicle (0.2 mg/ml) prepared as described in Materials and Methods was dispersed into reaction medium, pH 6.5, containing 150 µM KCl, 5 mM $MgCl_2$ and 1mM $NaN_3$. In the presence of 1 mg/ml valinomycin, 0.8 µM cytochrome *c* and 30 µM $Q_2H_2$, the reaction was started by the addition of 5 µM ferricyanide. Where indicated 1 µg/ml nigericin or 25 µM antimycin was present. The pH change was calibrated by the pH change with addition of final 10 µM HCl after every reaction.

**Table 3.IV. Proton translocation coupled with electron transfer from $Q_2H_2$ to cytochrome *c* catalyzed by the crystalline cytochrome $bc_1$ complex.**

**A) oxidant pulse experiment**

The $H^+/e^-$ ratio was obtained from the extent of $H^+$ release and the amount of ferricyanide added. See the legend to Figure 3.5 for experimental conditions.

| | $H^+/e$ | |
|---|---|---|
| $bc_1$ | 2.3 | total proton release |
| —) $bc_1$ + nigericin | 1.3 | scaler proton release |
| | 1.0 | vectorial proton translocation |

**B) reductant pulse experiment**

The assay medium at pH 6.5 was the same to the oxidant pulse experiment in the presence of 0.05 mg enzyme in liposome and 0.8 μM cytochrome *c*. The reaction was started by addition of 10 μM $Q_2H_2$. The rates of proton release and reduction of cytochrome *c*, were monitored by pH meter and the absorbance change at 550 nm using spectrophotometer. The reduction of cytochrome *c* was estimated using redox difference extinction coefficient, $\Delta\varepsilon^{red\text{-}ox}_{550} = 18.5\ mM^{-1}cm^{-1}$. Antimycin A concentration was 25 μM.

| | nmol $H^+$/min/mg | nmol $e^-$/min/mg | $H^+/e^-$ |
|---|---|---|---|
| $bc_1$ | 10300 | 2000 | |
| —) $bc_1$ + antimycin A | 5400 | 470 | |
| | 4900 | 1500 | 3.3 |

## 3.4. DISCUSSION

*Integrity of the crystals as cytochrome* $bc_1$ *complex* — Primary and fundamental characteristics of the crystalline preparation obtained by concentration method were investigated in order to confirm the integrity of the crystal obtained as cytochrome $bc_1$ complex. The dithionite-reduced preparation showed peak at 562 nm and shoulder at 553 nm in α-band and peak at 429 nm and shoulder at 416 nm in Soret-band (Fig. 3.1), typical visible-Soret absorption spectrum of fully reduced form, indicating the presence of the complete set of cytochrome components as reported earlier (Yu *et al.*, 1974). EPR signal with $g$= 2.02, 1.89 and 1.76 (Fig. 3.3) indicates the presence of Rieske-type [2Fe-2S] cluster (de Vries *et al.*, 1979). The stoichiometry of heme was determined to be heme C : protoheme : non-heme iron = 1 : 2 : 2 from pyridine hemochrome and iron content from atomic absorption analysis. These spectroscopic and chemical analyses indicate the crystalline preparation possesses 4 redox centers in a functional unit. The enzymic activity of the crystalline preparation was also investigated. The electron transfer activity of the preparation in terms of molecular activity, 680 $s^{-1}$, from $Q_2H_2$ to ferricytochrome *c* was as high as the preparation before crystallization, 630 $s^{-1}$. The preparation showed a proton pump activity with $H^+/e^-$ ratio, 1 (oxidant pulse experiment). The enzyme did not lose the enzymic activities during crystallization. In order to confirm subunit composition, SDS-PAGE was carried out. The crystalline preparation gave a similar pattern to the sample before crystallization and that as reported by Schägger *et al.* (1986). Thus, all bands which did not change in the relative intensity between before and after the crystallization were the intrinsic subunits for the enzyme; all 11 polypeptides reported by Schägger *et al.* (1986) were the subunits of the enzyme.

In conclusion, the present crystals are of cytochrome $bc_1$ complex. In other words, all components in the crystalline preparation are intrinsic for the enzyme.

The hexagonal crystals diffracted X-rays to 6.5 Å resolution (Chapter-2). One reason why the crystals diffract to so low resolution may be due to heterogeneity of lipid composition or $Q_{10}$ in the preparation. The phospholipid content was estimated to be ca. 30 in terms of phosphorous atom per cytochrome $c_1$, and it is unlikely that these lipids are attached to every enzyme molecule in the same way. Non-stoichiometric $Q_{10}$ may cause conformational heterogeneity. Such $Q_{10}$ and phospholipids may prevent the enzyme from forming well-ordered crystals. In order to produce crystals diffracting X-rays to higher resolution, thorough removal of $Q_{10}$ or phospholipids should be carried out, although this treatment will often cause denaturation.

*Crystallization as purification procedure* — SDS-PAGE pattern of the crystalline preparation compared with those of the mother liquor and the sample before crystallization clearly shows that some of contaminated bands were completely removed by crystallization; the co-purified peptides were concentrated into mother liquor (Fig. 3.2). The same finding appears in absorption spectra; the crystalline preparation had a higher absorbance ratio at the Soret-band peak to at 280 nm than the parent sample. Additionally, absorbance intensities of the crystalline preparation at 562 and 429 nm assigned to cytochrome *b* were significantly higher than those of the parent, probably due to removal of a restrictedly digested cytochrome $c_1$ peptide and a species having slightly denatured cytochrome *b*. Moreover, comparison of the iron contents in the preparation between before and after crystallization suggested that crystallization is effective to eliminate contaminated iron atoms in solution and/or on the protein surface. These results indicate that crystallization is one of the most adequate purification procedure for multisubunit membrane proteins. Especially, this technique is effective to separate a similar species such as a slightly denatured enzyme molecule, which is removed with difficulty by a chromatography.

Assuming that cytochrome $bc_1$ complex composed of one molecule of all 11 subunits, the total molecular weight of the enzyme complex was calculated to be 230k. Based on this molecular weight, 4.3 nmol of heme C (one mole/functional unit) per mg protein would be contained, which is consistent with the value reported by Rieske

(1967). Heme C content in the crystalline preparation was measured to be 3.3 nmol per mg protein, immediately indicating that the crystalline preparation contained some contaminated peptides even after crystallization. However, this is inconsistent with the fact that the ratio of Soret-band intensity to the absorbance at 280 nm in the absorption spectrum of oxidized form of the present crystalline preparation is almost identical with the reported value (Yu *et al.*, 1974). Alternative explanation for low heme C content in the crystalline preparation is that more than one molecule are contained in the functional unit for some subunits, and extinction coefficients of hemes are relatively high. Karlsson *et al.* (1983) reported that they obtained the subunits I and II subcomplex having stoichiometries of subunit I:subunit II = 1:2 or 2:2. The stoichiometries were judged by the molecular size from sedimentation equilibrium technique using *Neurospora* enzyme. If the bovine heart enzyme contained 2 molecules of subunit I and/or II, total molecular weight of the enzyme would be about 280k~310k, to be calculated to 3.2~3.6 nmol heme C/mg protein. This value is consistent with the resulting value for the crystalline preparation within experimental accuracy (Table 3.II), suggesting possibility of existence of some subunits at more than one stoichiometry. While, the molecular size of bovine heart enzyme was reported to be 250kDa from sedimentation equilibrium (Weiss & Kolb, 1979; Tzagoloff *et al.*, 1965). At present, the subunit stoichiometry and the molecular weight can not be determined definitely for cytochrome $bc_1$ complex. Determination of three-dimensional structure from X-ray crystallography will solve this problem unambiguously.

*Characteristics* — SDS-PAGE (Fig. 3.2) clearly indicates that contaminated polypeptides were removed by crystallization, while 11 polypeptides which were regarded as subunits of the enzyme are completely retained in crystalline preparation. In other words, all of the 11 polypeptides are intrinsic subunits of cytochrome $bc_1$ complex.

The crystalline preparation shows typical absorption spectra of cytochrome $bc_1$ complex; Soret-band intensity in oxidized form is nearly equal to the intensity at 280 nm, protein absorption (Fig. 3.1). However, the crystalline preparation had low heme

content. Thus, the molecular extinction coefficients of cytochromes in the crystalline preparation was re-examined in the fully reduced form as follows; $\Delta\varepsilon_{553\text{-}545} = 33.0 \pm 0.72$ $mM^{-1}cm^{-1}$ for cytochrome $c_1$, $\Delta\varepsilon_{562\text{-}575} = 34.6 \pm 0.82$ $mM^{-1}cm^{-1}$ for cytochrome *b*. These values are higher than any values reported so far; by 1.7~1.8 times higher than the value reported by Hatefi *et al.* (1962) and by 1.2~1.5 times than those reported by Rieske *et al.* (1967), Ljungdahl *et al.*, (1987) and Yu *et al.*, (1974). Any values reported previously will result in overestimation for cytochrome $c_1$ and underestimation for cytochrome *b*. The values obtained in the present work were verified by heme contents estimated from pyridine hemochrome, iron content from atomic absorption analysis and redox titration by dithionite solution. Thermodynamic and physicochemical properties and transient kinetics of the enzyme should be re-examined quantitatively using new extinction coefficients and the crystalline preparation as typical preparation of cytochrome $bc_1$ complex.

Although the crystalline preparation included only residual amount of $Q_{10}$, the preparation possessed the electron transfer activity as well as proton pump activity. Additionally, the crystalline preparation was as active as the $Q_{10}$-containing enzyme and the $Q_{10}$-reconstituted enzyme, as described in Chapter-4, indicating that $Q_{10}$ was not indispensable for the activity of the enzyme, in contrast to the result reported by Yu *et al.*, (1978) that deletion of $Q_{10}$ by wash with organic solvent or repeated precipitation with ammonium sulfate in the presence of cholate made the enzyme inactive. On the other hand, phospholipid content in the crystalline preparation was as many as in the preparation before crystallization, suggesting tightly bound phospholipids with important role for the enzymic activity, probably stabilization of the protein conformation. Schägger *et al.* (1990) reported an essential role of phospholipids using the enzyme treated by phospholipase. Loss of the enzymic activity for $Q_{10}$-deleted enzyme reported by Yu *et al.* may be due to removal of phospholipid.

EPR signal of Rieske type iron-sulfur cluster reveals that at least two types of clusters are present in the enzyme. Additionally, the redox titration experiment indicates existence of third component of cytochrome *b* characterized with α-peak maximum at

559 nm and lower redox potential. This component is distinct from cytochrome $b_{566}$ and $b_{562}$, consistent with detailed EPR analysis (De Vries *et al.*, 1979). Salerno *et al.* (1989) suggested from redox titration analysis of cytochromes with EPR spectroscopy that cytochrome $b_{560}$ was not a component of cytochrome $bc_1$ complex but that of complex II co-purified in the enzyme preparation. However, the presence of cytochrome $b_{560}$ in the crystalline preparation indicates that this is the intrinsic component of cytochrome $bc_1$ complex. The present results suggest an asymmetric dimeric nature of cytochrome $bc_1$ complex. Salerno *et al.* (1989) pointed out that heterogeneity in spectroscopic behavior of cytochromes, which was obvious in submitochondrial particle or succinate-cytochrome *c* reductase, was often lost during purification. The spectroscopic feature in the crystalline preparation arises probably from asymmetric dimer. It is speculated that asymmetry might be produced not by heterogeneity in subunit composition of each monomer, but an interaction between monomers, forming so-called hetro-diprotomer. In addition, it is highly probable that dimeric species are packed both in two crystal forms of cytochrome $bc_1$ complex (Chapter-2). These results suggest dimeric nature of the enzyme.

Acknowledgment: I am deeply indebted to Professor Hajime Hirata, Himeji Institute of Technology, Hyogo, for the reconstitution of protein liposome and the measurement of the proton pump activity. I am also deeply indebted to Dr. Hiroshi Hori, Faculty of Basic Technology, Osaka University, for the measurement of EPR spectrum. I would like to thank Dr. Shigeru Tamiya, Central Workshop, Osaka University, for the atomic absorption analysis.

# Chapter-4.
# Kinetic Mechanism of Cytochrome $bc_1$ Complex

## 4.1. INTRODUCTION

Mitochondrial ubiquinol: ferricytochrome *c* oxidoreductase (E.C. 1.10.2.2) is the middle component of the respiratory chain, which catalyzes the transfer of electrons from ubiquinol to cytochrome *c*, coupled with proton translocation across the inner mitochondrial membrane (Hatefi, 1985; Rieske, 1976). Extensive studies have been performed on this enzyme because of its important role in energy coupling during oxidative phosphorylation at site 2. As a result, several mechanisms have been proposed for this electron transfer within the molecule, coupled with proton translocation (Mitchell, 1976; Crofts *et al.*, 1983; Slater, 1983; Wikström & Krab, 1986).

It has been proved that extensive analysis of the initial steady-state kinetics for an enzyme system involving multiple substrates and products provides highly unique information about the reaction mechanism, such as the order of substrate binding and product release, and the number of intermediate enzyme species (Cleland, 1963). However, only a few preliminary reports on the initial steady-state kinetics for this enzyme have appeared, and the conclusions drawn have been inconsistent, for example, a ping-pong mechanism (Kauten *et al.*, 1987) *versus* a sequential one (Degli Esposti & Lenaz, 1982a), and linear Lineweaver-Burk plots (Nalecz & Azzi, 1985) versus non-linear ones (Degli Esposti & Lenaz, 1983b).

This chapter deals with the steady-state kinetics for the bovine heart enzyme system involving ubiquinol-2 ($Q_2H_2$)[1] and ferricytochrome *c* (cytochrome $c^{3+}$) as the

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[1] Abbreviations: $Q_2$, ubiquinone-2; $Q_2H_2$, ubiquinol-2; $Q_{10}$, ubiquinone-10; $Q_{10}H_2$, ubiquinol-10.

substrates. The present results indicate Hexa-Uni ping-pong mechanism involving a strong repulsive interaction between the two substrate binding sites and the irreversible release of one of the products, ubiquinone-2 ($Q_2$).

## 4.2. MATERIALS AND METHODS

$Q_2$ was kindly donated by Eisai Co., Ltd., Tokyo. $Q_2H_2$ was prepared by the method of Rieske (1967). Horse heart cytochrome *c* was a product of Sigma (Type VI). Sucrosemonolaurate (>99% monoester pure) was a product of MITSUBISHI-KASEI FOOD Co. Ltd. The enzyme was prepared from fresh bovine heart muscle by the method of Rieske (1967) with slight modifications, as described in Chapter-2. The enzyme used in this kinetical studies was not the crystalline preparation but "Rieske's preparation" in which the detergents were not exchanged. The enzyme preparation was stored in an ice bath at about 200 μM and used within three weeks without any detectable change in enzymic activity.

The enzyme concentration was expressed in terms of the cytochrome $c_1$ concentration determined from the absorbance difference for the fully reduced form (dithionite-reduced form) between 554 nm and 544 nm minus that for the fully oxidized form, using a difference extinction coefficient of 19 $mM^{-1}cm^{-1}$ (Hatefi, 1962). The concentrations of cytochrome *c* and $Q_2H_2$ were estimated using $\Delta\varepsilon^{red\text{-}ox}_{550}$ of 18.5 $mM^{-1}cm^{-1}$ and $\Delta\varepsilon_{290}$ of 4.14 $mM^{-1}cm^{-1}$ in ethanol (Morrison *et al.*, 1982), respectively. The content of ubiquinone-10 ($Q_{10}$) in the enzyme preparation was determined by the method of Redfearn (1967).

The $Q_{10}$-depleted cytochrome $bc_1$ complex was prepared by the method for exchange of the detergents in the preparation (Kubota *et al.*, 1991; see Chapter-2). The $Q_{10}$-depleted cytochrome $bc_1$ complex showed the same absorption spectrum and SDS-polyacrylamido-gel-electrophoresis pattern as before the treatment (see Chapter-3). This

preparation contained about 0.16 equivalents of $Q_{10}$ per cytochrome $c_1$. Reconstitution of $Q_{10}$ was carried out by the method reported by Yu *et al.* (1978).

*The enzymic assay* — The temperature of the reaction mixture in the cell was maintained at 20 ± 0.1°C in a thermostated water-jacketed cell holder with a small magnetic stirrer. The rate of electron transfer from $Q_2H_2$ to cytochrome $c^{3+}$ catalyzed by the enzyme was estimated from the rate of reduction of cytochrome *c* measured at 550 nm in a buffer comprising 55 mM Tris-HCl (pH 7.4), 2 mM $NaN_3$, 0.5 mM Na-EDTA, 0.01% Tween-20 and 20 mM $MgCl_2$, unless otherwise stated. Non-enzymic electron transfer between $Q_2H_2$ and cytochrome *c* was estimated by measuring the linear increase in absorbance at 550 nm for 1 min in the absence of the enzyme. The rate of enzymic reduction was monitored for 2 min after the addition of the enzyme. Then, a slight excess of solid dithionite was added to determine the absorbance level at the fully reduced state of cytochrome *c*. The enzymic reaction rate was estimated by subtracting the non-enzymic reduction, which was less than 1% of the initial rate of the enzymic reaction under the present assay conditions. The enzyme solution for each assay was made up daily by dilution of the stock solution (about 200 μM) to 1 μM with a buffer comprising 50 mM Tris-HCl (pH 8.0), 660 mM sucrose, 1 mM L-histidine and 0.01% Tween-20, and kept in an ice-bath. The enzymic assay measurements were performed within 3 h using the diluted enzyme solution after incubation for 2 h at 0 °C. The specific activity remained constant for the 3-h period. The initial rate data were fitted to the Michaelis-Menten equation with a non-linear least square regression program, kindly written by Prof. T. Tsukihara, Tokushima Univ. The enzymic activity was expressed in terms of the turnover number, $s^{-1}$ (mol of cytochrome *c* reduced/ cytochrome $c_1$/s).

## 4.3. RESULTS

*Enzyme preparation and conditions for the enzyme assay* — The present modification of the preparation method significantly improved the reproducibility as to both the yield and the enzymic activity. The yield was 500 ± 100 mg per 800 g of minced bovine heart muscle. The specific activity was 86 μmol/min/mg protein for the system containing $Q_2H_2$ (25 μM), cytochrome $c^{3+}$ (10 μM) at 20 °C. This preparation contains 1.1 equivalents of $Q_{10}$ to cytochrome $c_1$.

Incubation of the enzyme solution in the presence of Tween-20 at 0 °C for 2 h prior to the reaction, careful temperature control of the reaction mixture and the presence of Tween-20 in the reaction medium, as stated above, were most effective for improving the experimental accuracy. The experimental accuracy was 1.2% (±4.6 $s^{-1}$/393 $s^{-1}$) for the system containing $Q_2H_2$ (25 μM), cytochrome $c^{3+}$ (10 μM) and the enzyme (600 pM), but 10% (±21 $s^{-1}$/210 $s^{-1}$) in the absence of the detergent at room temperature using the enzyme without preincubation. The variance of the specific activity depending on the preparation batch was ± 6% (± 11$s^{-1}$/180$s^{-1}$) with 10 μM $Q_2H_2$ and 10 μM cytochrome $c^{3+}$. The set of data given in each figure in this paper was obtained with using a single batch of the enzyme preparation. The variance of the enzymic activity in each preparation was not large enough to affect the initial rate pattern.

*Stoichiometry* — The stoichiometry of $Q_2H_2$ oxidized versus cytochrome $c^{3+}$ reduced was determined in order to confirm that the observed reduction of cytochrome $c^{3+}$ was due to the electron transfer reaction catalyzed by the enzyme. The assay system contained various concentrations of cytochrome $c^{3+}$ and a constant concentration of $Q_2H_2$, 10 μM. The enzyme concentration (5 nM) was higher than that under the usual assay conditions in order to obtain rapid completion of the reaction. The amount of cytochrome *c* reduced during the reaction was plotted against the total concentration of cytochrome *c* in the reaction mixture, as shown in Figure 4.1. Cytochrome $c^{3+}$ contained initially in the system was completely reduced as long as the cytochrome *c*

concentration was lower than 23 μM. Above that concentration, only 23 μM of the cytochrome $c^{3+}$ initially present was reduced. This result indicates that $Q_2H_2$ is oxidized stoichiometrically by cytochrome $c^{3+}$. The enzymic reduction was completely inhibited by antimycin-A (5 μM).

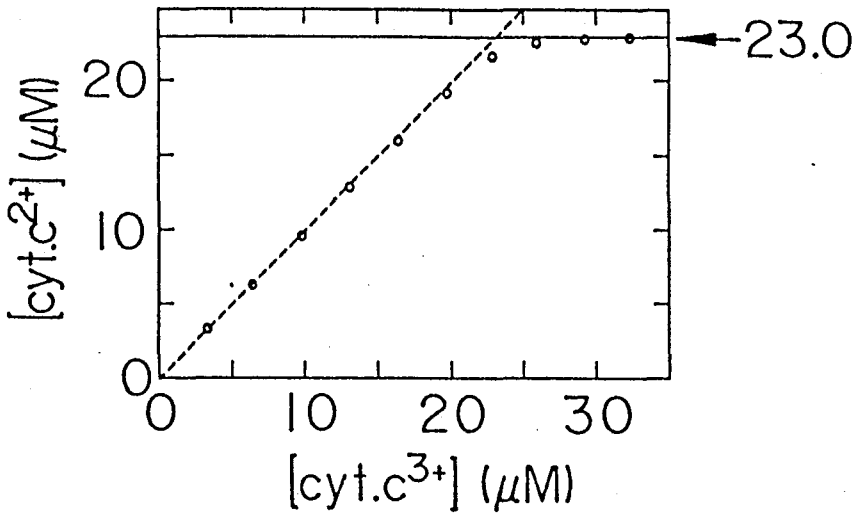



**Figure 4.1. Stoichiometry of the reaction between $Q_2H_2$ and cytochrome $c^{3+}$.** The amounts of cytochrome *c* reduced were plotted against the total concentration of cytochrome $c^{3+}$ at a constant level of 10 μM $Q_2H_2$. The reaction catalyzed by 5 nM enzyme was completed within 5 min. The arrow indicates 23.0 μM, the estimated maximum concentration of cytochrome *c* reduced with $Q_2H_2$ in the system.

*Time course of the enzymic reaction* — The enzymic reduction of cytochrome *c* proceeded as shown typically in Figure 4.2. Each point represents the time-dependent decrease in the fractional concentration of cytochrome $c^{3+}$ (ratio of [cytochrome $c^{3+}$] during the reaction to the initial [cytochrome $c^{3+}$] level). The initial concentrations of cytochrome $c^{3+}$, $Q_2H_2$ and the enzyme were 7.6 μM, 15 μM and 0.6 nM for the open triangles, and 20.7 μM, 19 μM and 1.0 nM for the open circles, respectively. The solid curves are for the first order reaction with respect to the cytochrome $c^{3+}$ concentration. Slight but systematic deviations from the solid curves were obvious from 60 s after initiation of the reaction. The deviations in other determinations performed thus far were smaller than those in the upper trace in Figure 4.2. The initial velocity was estimated from the first-order rate constant multiplied by the initial concentration of

cytochrome $c^{3+}$. The initial rate thus obtained was proportional to the enzyme concentration at any fixed initial concentrations of the two substrates. Therefore, the enzyme concentration was adjusted to provide a reaction rate appropriate for accurate measurement under the present experimental conditions ($t_{1/2}$; 30-300 s).

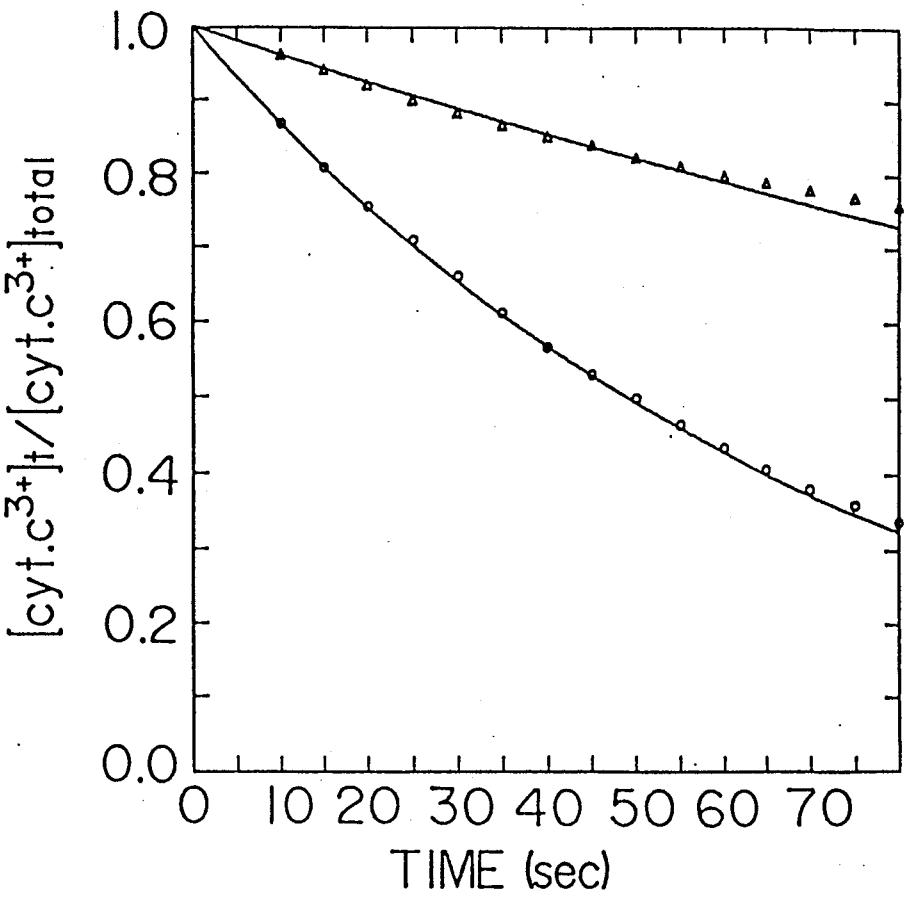



**Figure 4.2. The time course of the electron transfer from $Q_2H_2$ to cytochrome $c^{3+}$ catalyzed by the enzyme.** The ratios of the cytochrome *c* concentration at each time to the initial concentration were plotted against time after initiation of the reaction. Non-enzymic reduction of cytochrome $c^{3+}$ was subtracted from the data. The initial concentrations of cytochrome *c*, $Q_2H_2$ and the enzyme were 7.6 μM, 15 μM and 0.6 nM for open triangles, and 20.7 μM, 19 μM and 1.0 nM for open circles, respectively. The solid curves are the best-fit exponential curves with first-order rate constants of $4.0 \times 10^{-3}$ $s^{-1}$ for the open triangles and $14.2 \times 10^{-3}$ $s^{-1}$ for the open circles. The final level of absorbance to be obtained after completion of the reaction was estimated from the absorbance level obtained on addition of a slight excess of dithionite to the reaction mixture.

The $K_m$ value for cytochrome $c^{3+}$ was decreased from 4 μM to 1 μM without affecting $V_{max}$ by eliminating 20 mM $MgCl_2$ from the reaction mixture, while $K_m$ for $Q_2H_2$ was not effected by $MgCl_2$ (data not shown). These results suggest that $MgCl_2$ affects the binding of cytochrome $c^{3+}$ to the enzyme without affecting any other part of the reaction mechanism. In the present study, $MgCl_2$ was added to the reaction mixture because the $K_m$ value in the absence of $MgCl_2$ was too small for accurate determination under the present experimental conditions.

*$Q_{10}$-depleted cytochrome* $bc_1$ *complex* — The enzymic activity of the $Q_{10}$-depleted cytochrome $bc_1$ complex was measured in the same reaction medium as above except that it contained 0.05% sucrosemonolaurate instead of 0.01% Tween-20. Under these conditions, the activity of the $Q_{10}$-depleted enzyme was 370 $s^{-1}$ in the system containing 10 μM of both substrates, while those for the $Q_{10}$-reconstituted enzyme and the enzyme as prepared, which contained 1.1 equivalent of $Q_{10}$ as described above, were 380 $s^{-1}$ and 350 $s^{-1}$, respectively. These activity values are comparable to the reported value (Degli Esposti *et al.*, 1986). This showed that $Q_{10}$ was not a prerequisite for the enzymic activity, which conflicted with the observation of Yu *et al.* (1978). This discrepancy seems to be due to the difference in the procedures for $Q_{10}$-depletion; only one precipitation with polyethyleneglycol-4000 in the presence of sucrosemonolaurate *versus* several precipitations with ammonium sulfate in the presence of cholate (Yu *et al.*, 1978). The former procedure is milder than the latter, considering the ionic strength sensitivity of this enzyme (Rieske, 1967). It is unlikely that the $Q_{10}$ attached to the enzyme mediates electron transfer from $Q_2H_2$ to the enzyme, as has been suggested.

*Initial steady-state rate pattern* — The dependency of the initial steady-state rate as to the concentration of cytochrome $c^{3+}$ was examined with various levels of $Q_2H_2$. According to Cleland's notation (Cleland, 1963), cytochrome $c^{3+}$ and $Q_2H_2$ are a variable substrate and a changing fixed substrate, respectively. As shown in Figure 4.3, at each level of $Q_2H_2$, the initial rate pattern was a rectangular hyperbolic curve with respect to the substrate concentration. The solid lines are best-fit curves computed as described above. No systematic deviation from the experimental curves was observed.

The computed values of $K_m$ and $V_{max}$ are given in Table 4.I. The slopes of the Lineweaver-Burk plots, $K_m/V_{max}$, shown in the table are independent of the $Q_2H_2$ level within the limits of experimental accuracy, giving a series of parallel Lineweaver-Burk lines, as shown in the inset in Figure 4.3. The initial rate pattern for $Q_2H_2$ and cytochrome $c^{3+}$ as the variable substrate and the changing fixed substrate, respectively, estimated from the solid lines in Figure 4.3, provides a set of linear and parallel Lineweaver-Burk plots. This pattern was confirmed experimentally (data not shown). Therefore, the reciprocal rate is a linear function with respect to the reciprocal concentrations of both substrates, as follows,

$$1/v = K_1/A + K_2/B + 1/V_{max}$$

where $A$ and $B$ are the substrate concentrations, and $K_1$, $K_2$ and $V_{max}$ are the constants independent of $A$ and $B$. This result indicates ping-pong-type binding and release of the substrates and products.

**Table 4.I. Kinetic parameters for ferricytochrome *c* with various levels of $Q_2H_2$.**

The data were obtained with the Michaelis-Menten equation using a non-linear least-square regression program.

| Concentration of $Q_2H_2$ (μM) | $K_m$ (μM) | $V_{max}$ ($s^{-1}$) | $K_m/V_{max}$ (nM•s) |
|---|---|---|---|
| 25 | 7.7 ±0.71 | 450 ±12 | 17 ±1.7 |
| 15 | 5.7 ±0.49 | 322 ± 8.9 | 18 ±1.6 |
| 10 | 4.6 ±0.35 | 266 ± 6.4 | 17 ±1.4 |
| 7 | 3.0 ±0.41 | 167 ± 6.4 | 18 ±2.5 |

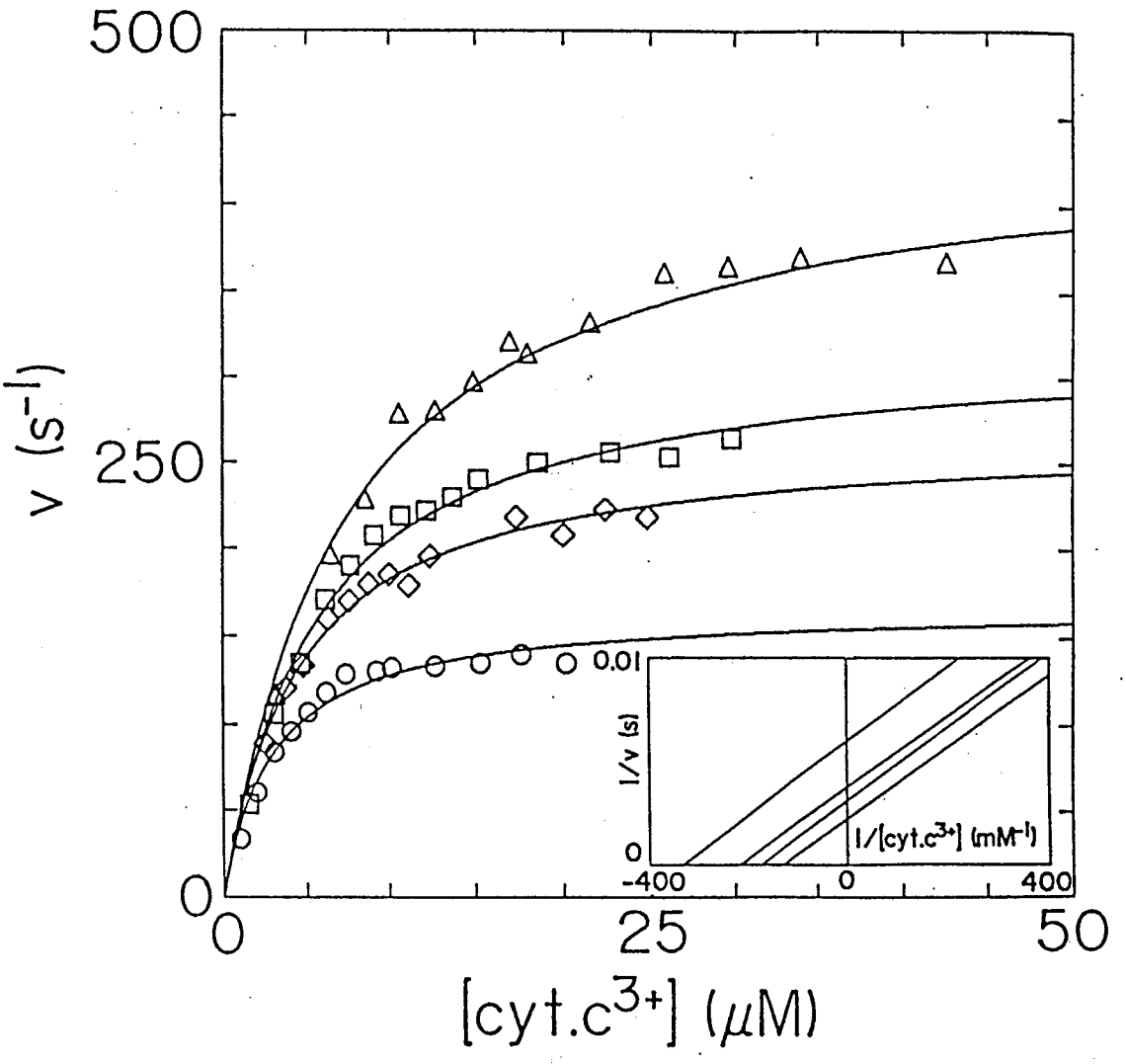



**Figure 4.3. Dependence of the initial rate on the concentration of cytochrome $c^{3+}$ at various fixed levels of $Q_2H_2$.** $Q_2H_2$ was fixed at various levels, as follows; triangles, 25μM; squares, 15 μM; diamonds, 10 μM; circles, 7 μM. Each point shows the initial rate obtained from the first-order rate constant multiplied by the concentration of cytochrome $c^{3+}$. The best-fit curves based on the Michaelis-Menten equation are also given. The Lineweaver-Burk plots in the inset were obtained from the parameters given in Table 4.I.

*Effects of products on the initial steady-state rate pattern* — The effect of a product at a constant level on the initial velocity pattern was examined in order to obtain further evidence for the ping-pong mechanism. The initial rate pattern for the enzyme system containing $Q_2H_2$ and cytochrome $c^{3+}$ as the variable and changing fixed substrates, respectively, in the presence of a constant level of ferrocytochrome *c* (cytochrome $c^{2+}$), 10 μM, as shown in Figure 4.4, gave a series of Michaelis-Menten patterns with the $K_m$ and $V_{max}$ values given in Table 4.II. The computed values of $K_m/V_{max}$ in Table 4.II depend on the level of cytochrome $c^{3+}$, giving the intersecting double reciprocal plots shown in the inset in Figure 4.4. The intersecting pattern in the presence of a product is one of the most important pieces of evidence for a ping-pong mechanism. On the other hand, the other product, $Q_2$, up to 100 μM, showed only a 20% decrease in activity, as shown in Figure 4.5. An increase in $Q_2$ concentration above 50 μM did not affect the enzymic activity. Therefore, the small decrease in activity induced by $Q_2$ up to 40 μM is not due to product inhibition caused by the direct competition of $Q_2$ with one of the substrates. Therefore, this result (absence of product inhibition) indicates the irreversible release of $Q_2$ from the enzyme under turnover conditions.

**Table 4.II. Kinetic parameters for $Q_2H_2$ with various levels of ferricytochrome *c* in the presence of ferrocytochrome *c*.**

The data were obtained as in Table 4.I.

| Concentration of ferricytochrome *c* (μM) | $K_m$ (μM) | $V_{max}$ ($s^{-1}$) | $K_m/V_{max}$ (nM•s) |
|---|---|---|---|
| 30 | 21 ±3.0 | 400 ±32 | 53 ± 8.7 |
| 20 | 19 ±1.3 | 340 ±13 | 56 ± 4.3 |
| 12 | 17 ±1.7 | 260 ±14 | 63 ± 7.2 |
| 7 | 15 ±1.6 | 149 ± 8.5 | 100 ±12 |

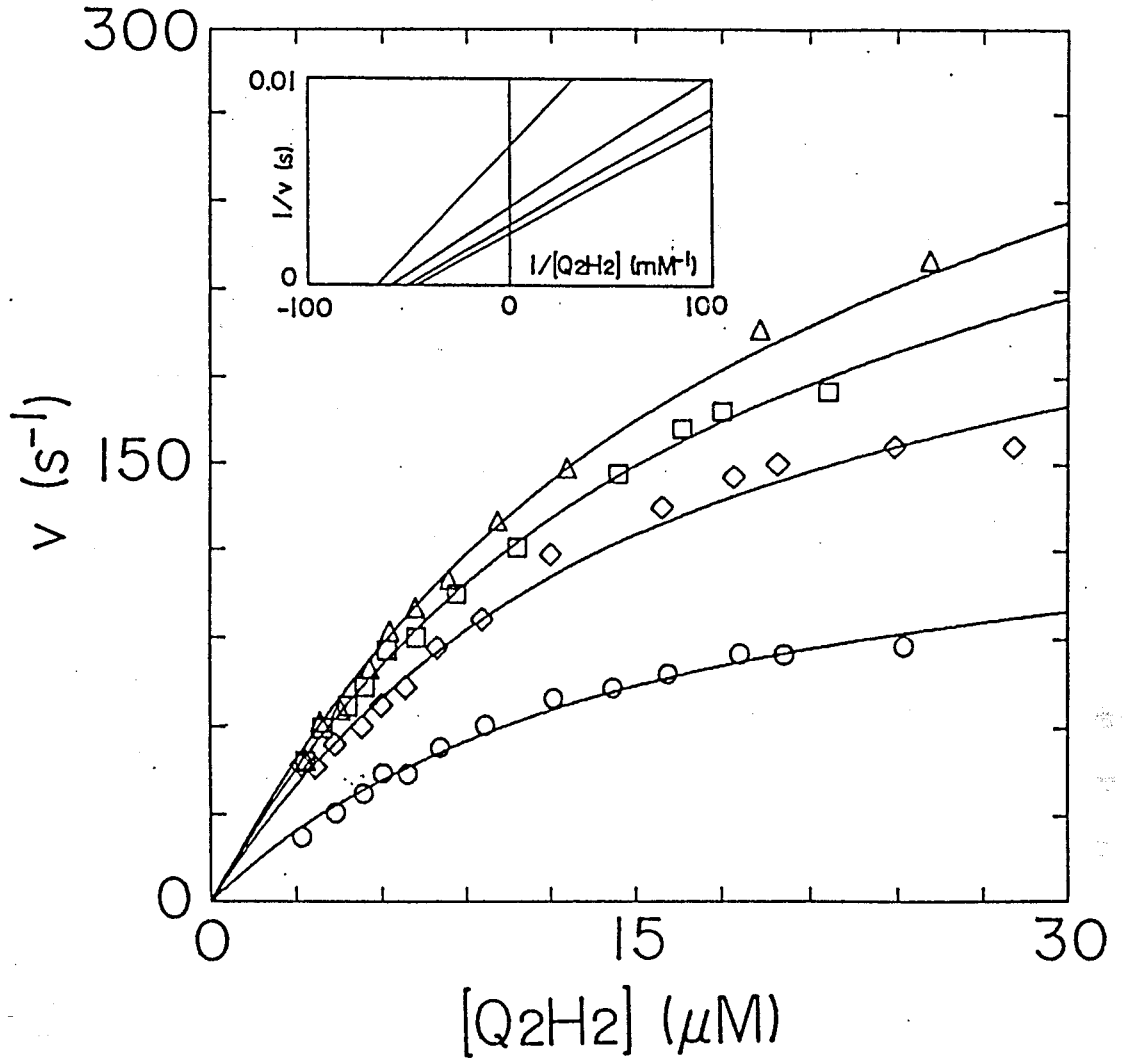



**Figure 4.4. Dependence of the initial rate on the concentration of $Q_2H_2$ in the presence of cytochrome $c^{2+}$.** All measurements were carried out in an assay mixture containing a constant level of 10 μM cytochrome $c^{2+}$. The cytochrome $c^{3+}$ level was fixed at various levels, as follows; triangles, 30μM; squares, 20 μM; diamonds, 12 μM; circles, 7 μM. Each point shows the initial rate value *vs.* the concentration of $Q_2H_2$. Solid curves are the best-fit curves obtained as in Fig. 4.4. The Lineweaver-Burk plots in the inset were obtained using the parameters given in Table 4.II.

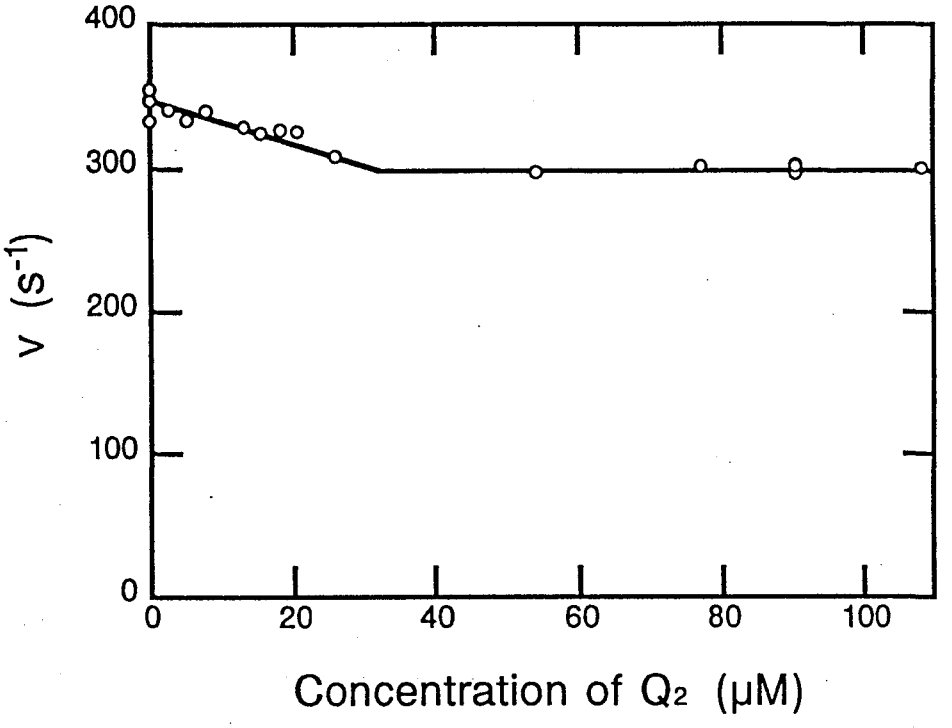



**Figure 4.5. Effect of $Q_2$ on the initial rate. Initial rates were measured at 30 μM $Q_2H_2$ and 25 μM cytochrome $c^{3+}$ in the presence of various concentrations of $Q_2$.** A constant volume of ethanol, 10 μM, containing a constant amount of $Q_2H_2$ and various amounts of $Q_2$ was added to the reaction mixture.

**Table 4.III. Kinetic parameters for $Q_2H_2$ as to the effect of product inhibition of ferrocytochrome *c* with 10 μM ferricytochrome *c*.**

The data were obtained as in Table 4.I.

| Concentration of ferrocytochrome *c* (μM) | $K_m$ (μM) | $V_{max}$ ($s^{-1}$) | $K_m/V_{max}$ (nM•s) |
|---|---|---|---|
| 0 | 11 ±1.4 | 450 ±27 | 26 ±3.4 |
| 5 | 13 ±1.5 | 420 ±24 | 32 ±3.9 |
| 10 | 14 ±1.6 | 370 ±23 | 37 ±4.4 |
| 15 | 14 ±1.9 | 340 ±24 | 42 ±6.3 |

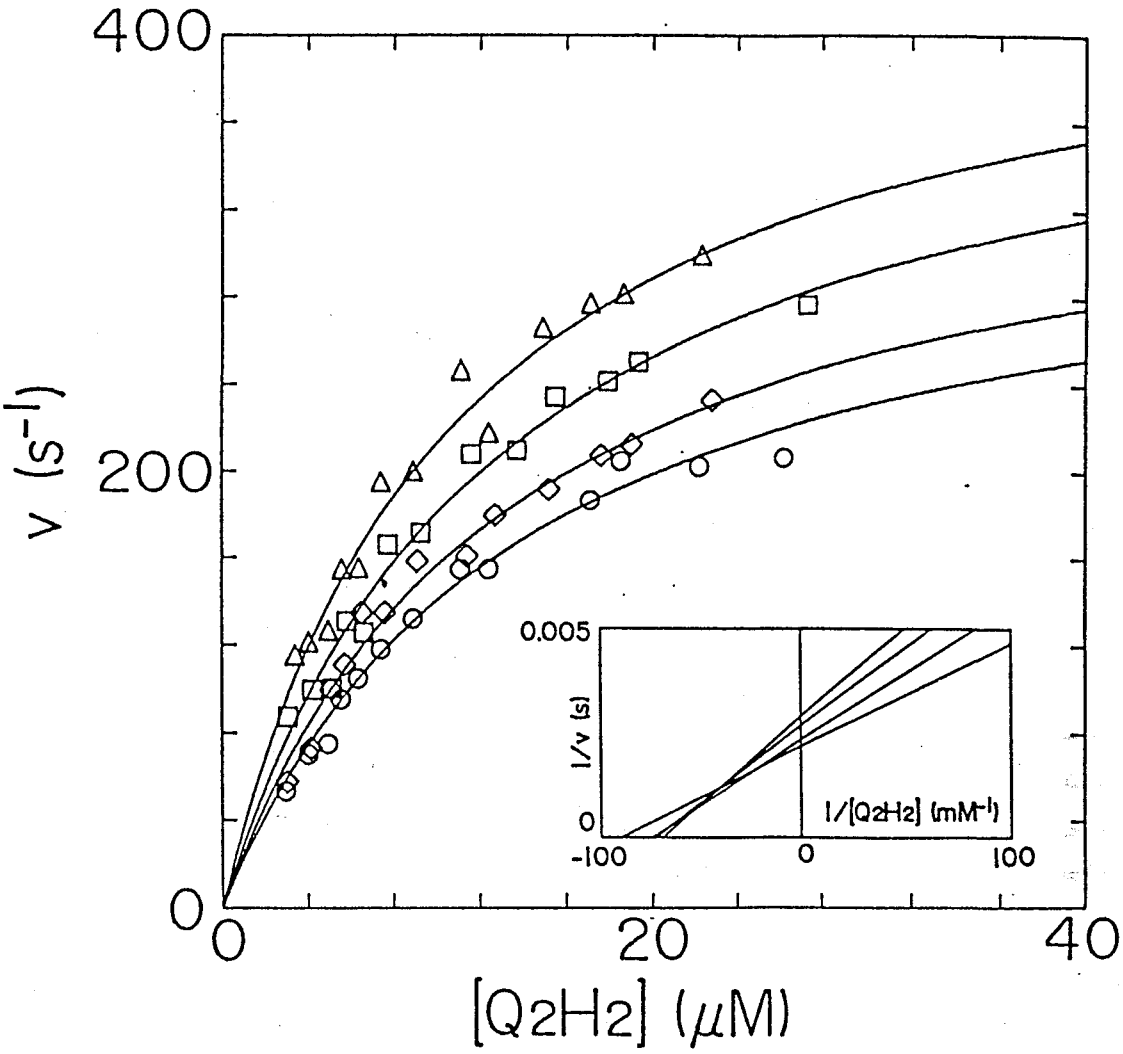
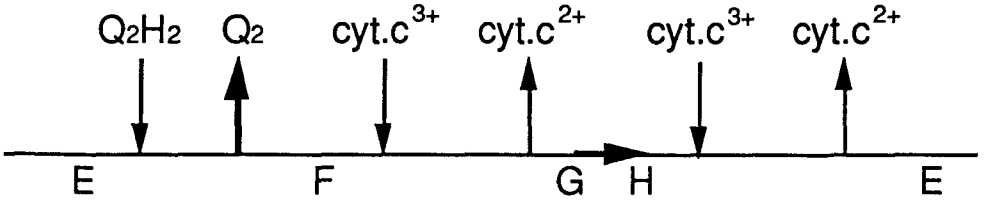



**Figure 4.6. Effect of cytochrome $c^{2+}$ on the initial rate of the reaction system with $Q_2H_2$ as a variable substrate.** The concentration of cytochrome $c^{3+}$ was fixed at 10 μM. The cytochrome $c^{2+}$ concentration was fixed at various levels, as follows; triangles, 0 μM; squares, 5 μM; diamonds, 10 μM; circles, 15 μM. The initial rate is plotted against the concentration of $Q_2H_2$. The best-fit curves are also given. The inset shows Lineweaver-Burk plots calculated using the parameters given in Table 4.III.

*Inhibition patterns of a product, cytochrome $c^{2+}$* — The product inhibition pattern of cytochrome $c^{2+}$ was examined in order to prove the involvement of more than two molecules of cytochrome $c^{3+}$ as a substrate in the enzyme system. The inhibitory effect of cytochrome $c^{2+}$ on the initial rate pattern for the enzyme system with $Q_2H_2$ as

the variable substrate at a fixed concentration of 10 μM cytochrome $c^{3+}$ was examined, as shown in Figure 4.6. The results clearly indicated that cytochrome $c^{2+}$ inhibits the enzymic reaction non-competitively with respect to $Q_2H_2$. This non-competitive inhibitory effect is considered to be due to the non-competitive inhibitory effect of the cytochrome $c^{2+}$ released as the second product against the first binding substrate, $Q_2H_2$, although cytochrome $c^{2+}$ released as the last product competes for E (Scheme 1) with $Q_2H_2$. As shown in the inset in Figure 4.6, the double reciprocal patterns calculated from the best-fit curve to the experimental results in this figure (see Table 4.III. for kinetic parameters) seem to have points of intersection that depend on the concentration of cytochrome $c^{2+}$. However, statistical examination of the experimental accuracy indicated no significant difference in the positions of these intersection points. The effect of cytochrome $c^{2+}$ on the initial rate pattern was examined for a system containing cytochrome $c^{3+}$ as the variable substrate and $Q_2H_2$ (20 μM) as the fixed substrate. As shown in the inset in Figure 4.7, cytochrome $c^{2+}$ inhibited the enzyme reaction with a tendency of non-competitive inhibition against cytochrome $c^{3+}$. This figure preferably indicates that no systematic deviation from Michaelis-Menten kinetics was detected for the initial rate pattern in the presence of cytochrome $c^{2+}$, suggesting an irreversible step between the release of cytochrome $c^{2+}$ as the second product and the binding of cytochrome $c^{3+}$ as the third substrate.

The simplest mechanism derived from all of the above results is a Hexa-Uni ping-pong mechanism in terms of Cleland's notation, as shown below,




(Scheme 1)

where the release of $Q_2$ and the isomerization of G to H presented by the bold arrows are irreversible.

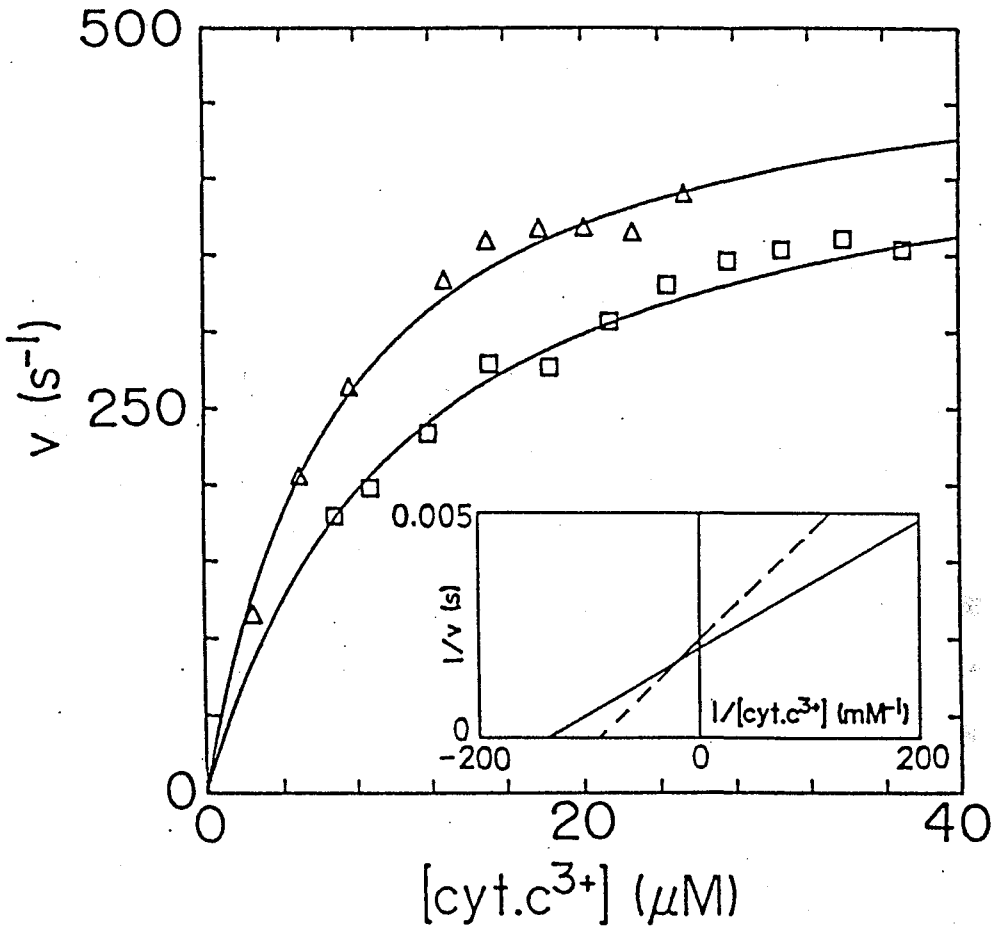
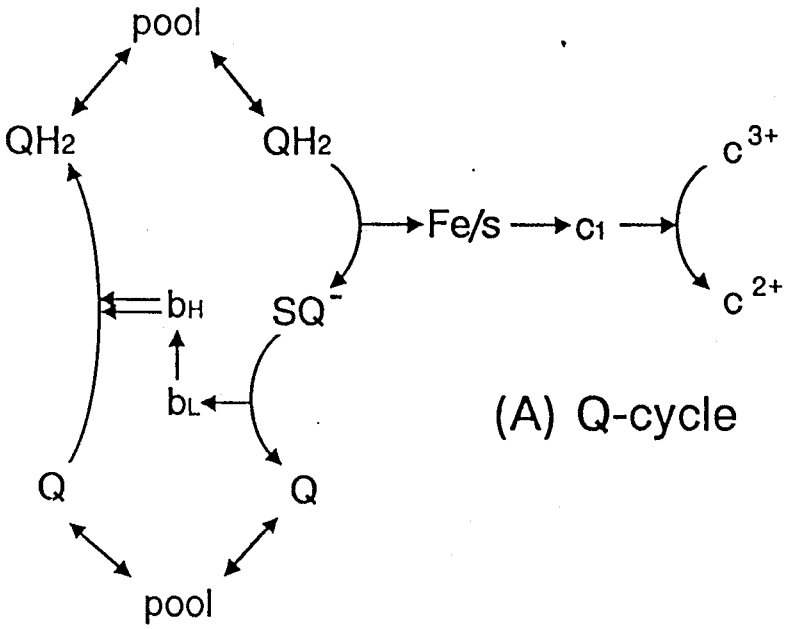



**Figure 4.7. Effect of cytochrome $c^{2+}$ on the initial rate with cytochrome $c^{3+}$ as a variable substrate.** Initial rates were measured at a fixed level of 20 μM $Q_2H_2$ and various levels of cytochrome $c^{3+}$ in the absence or presence of cytochrome $c^{2+}$; triangles, 0 μM; squares, 10 μM. Each symbol shows the initial rate vs. the concentration of cytochrome $c^{3+}$. The Lineweaver-Burk plots in the inset were obtained using $K_m$ and $V_{max}$ values of 7.17 μM and 502 $s^{-1}$ for the solid line, and 10.8 μM and 459.6 $s^{-1}$ for the broken line, respectively.

## 4.4. DISCUSSION

Degli Esposti and Lenaz (1982) reported that the initial steady-state rate of the bovine heart enzyme in the absence of any products gave intersecting double reciprocal plots suggesting a sequential mechanism. However, they determined this by fitting straight lines to the double reciprocal plots assuming constant reliability for each point of the reciprocal rate, regardless of the variable substrate concentration. As is well known, such analysis is likely to produce a misleading conclusion. Kauten et al. (1987) reported, without giving any experimental results, that the yeast enzyme showed ping-pong-type kinetics. Non-linear double reciprocal plots for quinol or quinol derivatives as variable substrates have been reported for the enzyme in its isolated (Nalecz & Azzi, 1985) as well as the membrane-bound form (Degli Esposti & Lenaz, 1982). However, the non-linearity was proposed based on double reciprocal plots without any statistical analysis. Therefore, more extensive analyses of the initial steady-state kinetics, with careful consideration to experimental accuracy, are required in order to establish the steady-state properties of this enzyme system.

Non-linear least-square fitting of the Michaelis-Menten equation to the present experimental results was quite successful, as shown in Figures 4.3, 4.4, 4.6 and 4.7. No systematic deviation from the equation was detectable. Scheme 1 is the simplest mechanism explaining the present results, and indicates that the second substrate, cytochrome $c^{3+}$, reacts with the two-electron-reduced form after the first product, Q, has been released from the enzyme. This suggests a strong negative cooperativity between the $Q_2H_2$ and cytochrome $c^{3+}$ binding sites, since the fairly hydrophobic molecule, $Q_2H_2$, is unlikely to compete for a single site with a highly hydrophilic protein, cytochrome $c^{3+}$.



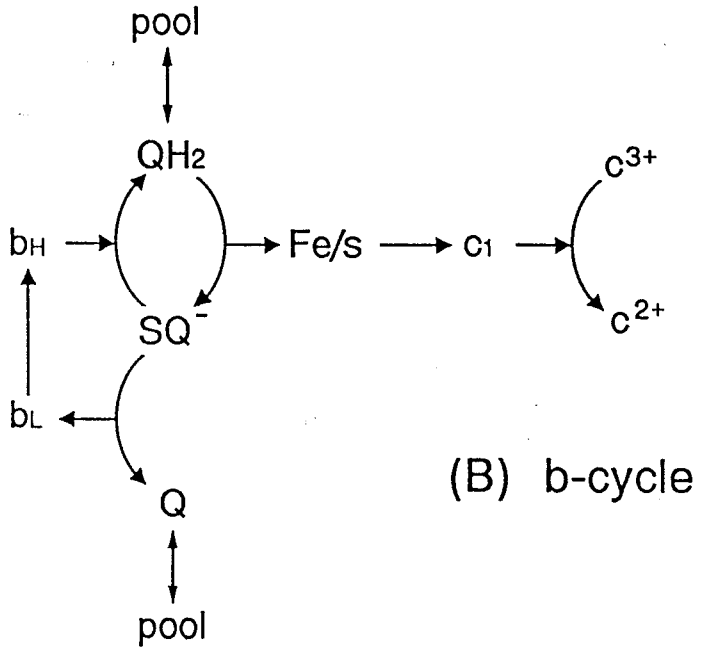
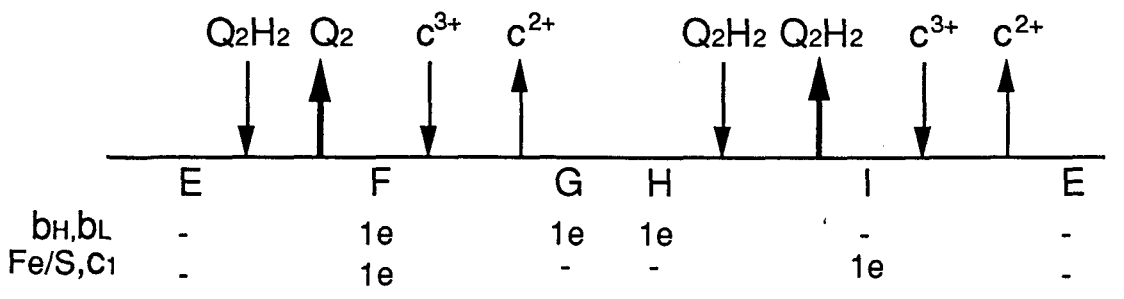




**Figure 4.8. The Q-cycle (A) and the semiquinone-cycle (B) mechanism for electron transfer through the $bc_1$ complex.** The Q-cycle (double turnover mechanism) was modified from Crofts et al. (1983). The semiquinone-cycle (b-cycle) proposed by Wikström and Krab (1986) is shown without the concept of center-*i* and center-*o*. $b_L$, low-potential heme $b_{566}$; $b_H$, high-potential heme $b_{562}$; $c_1$, cytochrome $c_1$; c, cytochrome *c*; Fe/S, Rieske iron-sulfur center; pool, ubiquinone pool in the membrane.

The $Q_{10}$-depleted cytochrome $bc_1$ complex showed comparable enzymic activity to that of the untreated enzyme, which contained one equivalent of $Q_{10}$ per cytochrome $c_1$. This result suggests that $Q_{10}$ is not a prerequisite for the enzymic activity, that is, $Q_2H_2$ in a bulk solution functions as a direct substrate (a quinol-pool). Perhaps $Q_{10}$ produced on the oxidation of ubiquinol-10 ($Q_{10}H_2$) at the active site is released irreversibly from the ubiquinone binding site like $Q_2$. However, the $Q_{10}$ released from the active site is likely to stay on the enzyme surface because of its hydrophobicity. Thus, this result strongly suggests that $Q_2H_2$ binds to the same site as that which accepts $Q_{10}H_2$. In other words, the electron transfer reaction within the enzyme after it has received electrons from the substrate is likely to be identical regardless of the quinol structure, $Q_{10}H_2$, in the membrane or, $Q_2H_2$, in a solution.

As is well known, two mechanisms, the Q-cycle (double-turnover mechanism) (Crofts et al., 1983) and the semiquinone-cycle (Wikström & Krab, 1986), have been proposed for interpretation of the cyclic electron transfer reactions within the enzyme, as given schematically in Figure 4.8. The present steady-state kinetic results can be used to evaluate these proposals and provide new insights in to the reaction mechanism.

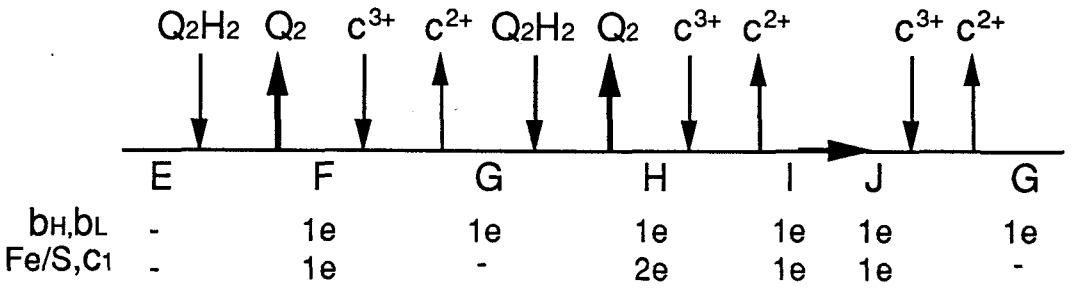
The absence of any significant effect of $Q_2$ on the enzyme reaction under turnover conditions (Fig. 4.5) strongly conflicts with the Q-cycle mechanism (Fig. 4.8A), since in this mechanism, $Q_2$ must bind to the enzyme as a substrate to accomplish the catalytic cycle. Thus, the present results support the semiquinone-cycle rather than the Q-cycle. One of the simplest schemes which explains the present kinetic results as well as the semiquinone-cycle mechanism is as follows;




(Scheme 2)

where the bold arrows denote irreversible reaction steps and the regular arrows, reversible steps. The possible distribution of reducing equivalents in each enzyme form is also given in the scheme. Each substrate (or product)-bound form is not given for the sake of simplicity. The rate equation for this scheme is given in the Appendix. The irreversible release of $Q_2$ was proven experimentally in the present study. Reversible isomerization from G to H is required for the non-competitive inhibition of cytochrome $c^{2+}$ against $Q_2H_2$, as shown in Figure 4.6. If the second $Q_2H_2$ binds to G without forming H, cytochrome $c^{2+}$ would compete with $Q_2H_2$ for G as well as E, leading to competitive inhibition against $Q_2H_2$. The release of $Q_2H_2$ must be irreversible in order to provide the parallel double reciprocal plots in the absence of any product (Fig. 4.3) and to give linear double reciprocal plots for the initial rate versus the concentration of cytochrome $c^{3+}$ as the variable substrate in the presence of a constant level of cytochrome $c^{2+}$ (Fig. 4.7). This means that the affinity of $Q_2H_2$ is rigorously controlled by the electron distribution within the enzyme, as in the case of the enzyme species, H versus I. The second $Q_2H_2$ to bind to H causes an electron left on the low potential centers from the first $Q_2H_2$ to migrate to the high potential centers.

Furthermore, another unique scheme was deduced from the semiquinone-cycle, as follows,




(Scheme 3)

which does not include the binding of two molecules of $Q_2H_2$ under turnover conditions, but requires initial activation of the enzyme from E to G. In this mechanism,

the enzyme form, G, in which one molecule of cytochrome *b* is reduced, is an active enzyme form that receives electrons from $Q_2H_2$ under turnover conditions (see the Appendix for the rate equation). The fully oxidized enzyme, E, and the two-electron-reduced species, F, do not participate in the catalytic cycle. In the pre-steady-state, G is formed from E via F. No kinetic information on the initial activation can be obtained from the results of initial steady-state analysis. The formation of H from G may be described as follows; $Q_2H_2$ bound to G only reduces Fe/S, forming the semiquinoid form of $Q_2$ (ubisemiquinone-2). Then, this ubisemiquinone-2 is reduced to $Q_2H_2$ with an electron from one of the cytochromes *b*. The second formed $Q_2H_2$, without release, reduces the enzyme form in which one of the high-potential redox centers is reduced, giving H in which one electron equivalent is at one of the low-potential centers and two equivalents are at the high-potential centers, as shown in the scheme. This mechanism also requires a strong control of $Q_2H_2$ binding by the oxidation state of the enzyme, that is, one electron equivalent at the high potential centers in the intermediate producing the second formed $Q_2H_2$ prevents the release of the $Q_2H_2$ from the enzyme. The isomerization of I to J in Scheme 3 is required for linear double reciprocal plots for the initial rate versus the concentration of cytochrome $c^{3+}$ as the variable substrate in the presence of a constant level of cytochrome $c^{2+}$ (Fig. 4.7). Tsai et al. (1983) reported a rapid equilibrium in the electron transfer reaction between Fe/S and cytochrome $c_1$, based on the results of stopped-flow kinetic analysis of the ferricyanide oxidation of the ascorbate-reduced enzyme in which only cytochrome $c_1$ and the Fe/S cluster were reduced. Preliminary stopped-flow analysis of the reaction between cytochrome $c^{2+}$ and the fully oxidized enzyme (to be published elsewhere) confirmed the reversible electron transfer between the two redox centers. The irreversible isomerization of I to J in this scheme, which possibly corresponds to the electron transfer from the Fe/S site to cytochrome $c_1$, apparently conflicts with these stopped-flow data. However, one electron equivalent is at one of the cytochromes *b* in I and J, while both cytochromes *b* were oxidized in the enzyme species in the above stopped-flow experiments. Therefore,

the reverse electron transfer (isomerization of J to I) is likely to be prevented by the reduced cytochrome *b*.

In conclusion, the simplest schemes which explain the experimental results obtained so far are Schemes 2 and 3, which indicate rigorous mechanisms of control through the electron distribution within the enzyme molecule for the binding of ubiquinone or ubiquinol to the enzyme (in Schemes 2 and 3), and for the electron transfer reaction between the Fe/S cluster and cytochrome $c_1$ (in Scheme 3). Both schemes involve a strong repulsive interaction between the two substrate binding sites, for ubiquinone and cytochrome *c*.

# Chapter-5
# Conclusion and Summary

Cytochrome $bc_1$ complex (ubiquinol:ferricytochrome *c* oxidoreductase, E.C. 1.10.2.2) from bovine heart mitochondria was crystallized by a batchwise method from protein solution containing sucrosemonolaurate using polyethyleneglycol-4000 as a precipitant. Two crystal forms were obtained from different buffer systems; the parallelepiped crystals were grown in Tris-HCl buffer system to a size of approximately 1 mm × 1 mm × 1 mm, which belonged monoclinic, space group $P2_1$ with unit-cell constants of $a$ = 196 Å, $b$ = 179 Å, $c$ = 253 Å, and $\beta$ = 97°, while the hexagonal crystals (0.3 mm × 1 mm) were grown in potassium-phosphate buffer system, which belonged hexagonal, $P6_1$ or $P6_5$ with unit-cell dimensions of $a = b$ = 131 Å, $c$ = 718 Å. The monoclinic and hexagonal crystals diffracted X-rays to 7.5 Å and 6.5 Å resolution, respectively. The hexagonal crystals were applied to collection of X-ray diffraction data, due to higher resolution and stability against change of the environment compared with the monoclinic crystals. The crystal growth of this type depended critically on the concentration of potassium-phosphate buffer, and this dependency was different from each preparation. Recrystallization was not only a satisfactory approach for such problem, also resulted in forming better crystals under microscopic observation. X-ray crystallographic analysis using the hexagonal crystals is expected to provide three-dimensional information for subunit arrangement and membrane spanning region as well as the shape of whole enzyme molecule. Also, trial for improving resolution is in progress.

Characteristics of the crystalline preparation was investigated in order to confirm the integrity of the crystals as cytochrome $bc_1$ complex. The crystalline protein showed the enzymic activity of electron transfer from ubiquinol-2 to cytochrome *c* and proton translocation using a reconstituted protein liposome. The subunit composition of

the crystalline enzyme were identical to that reported by other group [Schägger *et al.* (1986). *Methods Enzymol.*, **126**, 224-237.]; all 11 polypeptides were contained in the crystalline preparation. Spectroscopic analyses indicated that the preparation contained four redox centers. These results indicate that the crystals are of cytochrome $bc_1$ complex. Furthermore, it has been proven that all these components are intrinsic for the enzyme. Contaminated polypeptides, iron atoms non-specifically contained to the enzyme preparation and slightly denatured enzyme species were effectively removed by crystallization. While, spectroscopic features which were readily lost during conventional purification were retained in the crystalline preparation. These suggest that crystallization is the most suitable technique for purification of multisubunit membrane proteins, like this enzyme, with keeping its intrinsic structural and functional features. The characteristics of the crystalline enzyme as a typical preparation of cytochrome $bc_1$ complex were more extensively examined; the preparation possesses high extinction coefficients for both hemes, and the results of redox titration and EPR analysis suggest an asymmetrical dimeric nature of the enzyme, so-called diprotomer composed of two functional units.

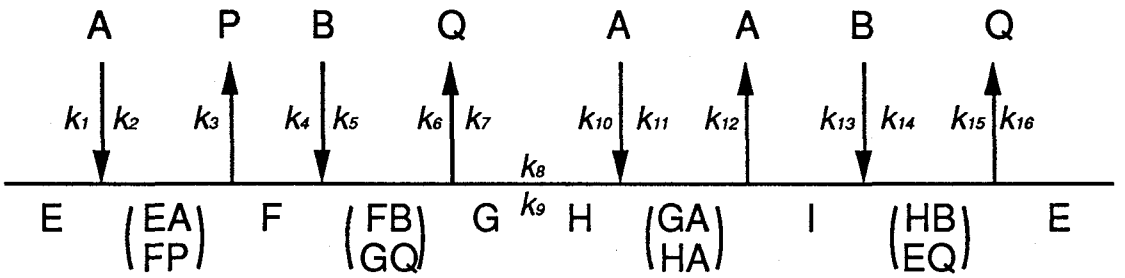
The electron transfer from ubiquinol-2 to ferricytochrome *c* mediated by ubiquinol:cytochrome *c* oxidoreductase (cytochrome $bc_1$ complex) purified from bovine heart mitochondria, which contained one equivalent of ubiquinone-10 ($Q_{10}$), was investigated under initial steady-state conditions. The $Q_{10}$-depleted enzyme was as active as the $Q_{10}$-containing one. Double reciprocal plots for the initial steady-state rate versus one of the two substrates at various fixed levels of the other substrate gave parallel straight lines in the absence of any product. Intersecting straight lines were obtained in the presence of a constant level of one of the products, ferrocytochrome *c*. The other product, ubiquinone-2, did not show any significant effect on the enzymic reaction. Ferrocytochrome *c* non-competitively inhibited the enzymic reaction against either ubiquinol-2 or ferricytochrome *c*. These results indicate a Hexa-Uni ping-pong mechanism with one ubiquinol-2 and two ferricytochrome *c* molecules as the substrates,

which involves the irreversible release of ubiquinone-2 as the first product and the irreversible isomerization between the release of the first ferrocytochrome *c* and the binding of the second ferricytochrome *c*. Considering the cyclic electron transfer reaction mechanism, this scheme suggests that the binding of quinone or quinol to the enzyme and electron transfer between the iron-sulfur center and cytochrome $c_1$ are rigorously controlled by the electron distribution within the enzyme. Molecular architecture around these redox centers at atomic level is required for understanding the molecular mechanism of these electron transfer and their controls.

# APPENDIX

Each scheme is presented according to Cleland's notation [Cleland, W. W. (1963). *Biochim. Biophys. Acta.* **67**, 104-137.]; *A*, ubiquinol-2; *B*, ferricytochrome *c*; *P*, ubiquinone-2; *Q*, ferrocytochrome *c*; *E*, *F*, *G*, *H*, and *I*, enzyme species; $k_n$, rate constant.

Scheme 2:




Rate equation:

$$\frac{Et}{v} = (K1\frac{1}{AB} + K2\frac{1}{A} + K3\frac{1}{B} + K4)Q + K5\frac{1}{A} + K6\frac{1}{B} + K7$$

where $A$, $B$, $P$ and $Q$ are the concentration of the reactants represented by the corresponding letters, and $K$n are constants defined as follows;

$$K1 = \frac{k12k14k16 + k3k14k16}{k1k3k13k15} + \frac{k5k7k9k11 + k5k7k10k12}{k4k6k8k10k12},$$

$$K2 = \frac{k7k9k12 + k7k9k11}{k6k8k10k12} + \frac{k2k16 + k3k16}{k1k3k15},$$

$$K3 = \frac{k5k7}{k4k6k8},$$

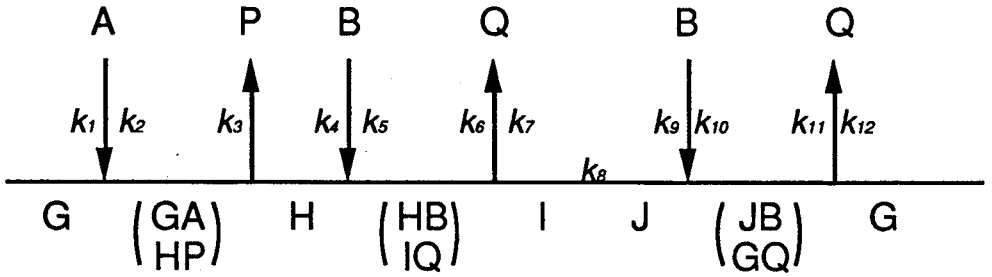
$$K4 = \frac{k7}{k6k8},$$

$$K5 = \frac{k8k11 + k9k11}{k8k10k12} + \frac{k2 + k3}{k1k3} + \frac{1}{k10},$$

$$K6 = \frac{k5 + k6}{k4k6} + \frac{k14 + k15}{k13k15} + \frac{k9}{k8k10},$$

$$K7 = \frac{1}{k3} + \frac{1}{k6} + \frac{1}{k8} + \frac{1}{k12} + \frac{1}{k15}.$$

Scheme 3:




Rate equation:

$$\frac{Et}{v} = (K1\frac{1}{AB} + K2\frac{1}{A} + K3\frac{1}{B} + K4)Q + K5\frac{1}{A} + K6\frac{1}{B} + K7$$

where Kn are defined as follows;

$$K1 = \frac{k2k10k12 + k3k10k12}{k1k3k9k11},$$

$$K2 = \frac{k2k12 + k3k12}{k1k3k11},$$

$$K3 = \frac{k5k7}{k4k6k8},$$

$$K4 = \frac{k7}{k6k8},$$

$$K5 = \frac{k2 + k3}{k1k3},$$

$$K6 = \frac{k5 + k6}{k4k6} + \frac{k10 + k11}{k9k11},$$

$$K7 = \frac{1}{k3} + \frac{1}{k6} + \frac{1}{k8} + \frac{1}{k11}.$$

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