CROSSTALK BETWEEN CYTOCHROME BC1 COMPLEX AND MITOCHONDRIAL MATRIX ENZYMES

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ENZYMES

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TABLE OF CONTENTS

Chapter Page

Chapter Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

1.1 Introduction of cytochrome bc_1 complex

1.1.1 Mitochondrial Electron transfer chain

Mitochondria, also known as the "cellular power plants", generate more than 90% of energy required for eukaryotic organisms, through a process called oxidative phosphorylation. This process is carried out in the inner membrane of mitochondria and by a series of complexes. They are: Complex I (NADH-Q Oxidoreductase), Complex II (Succinate- Q Oxidoreductase), Complex III (ubiquinol-Cyt c Oxidoreductase), Complex IV (cytochrome c oxidase) and Complex V (ATP synthase) (Fig.1-1). The first four complexes are electron transfer complexes. They catalyze sequential electron transfer from NADH or succinate to ubiquinone, then from ubiquinol to cytochrome *c* (cyt *c*) and finally from cyt *c* to molecular oxygen respectively. The electrons transferred through Complex I, III and IV is with concomitant translocation of protons across the inner mitochondrial membrane to generate the proton gradient and membrane potential for ATP synthesis by Complex V.

All the five complexes have been isolated and purified from submitochondrial particle preparation. Purified Complex I contains 45 protein subunits with 10 redox centers: 1 FMN and 9 iron sulfur clusters. Complex II contains 4 protein subunits with 1 FAD, 3 FeS clusters and 1 heme b_{560}. Complex III contains 11 proteins subunits with two *b*-type hemes (b_H and b_L) , one *c*-type heme (c_1), and one [2Fe2S] cluster. Complex IV contains 13 protein subunits with a-type hemes (a and a_3) and two Copper centers (Cu_A and Cu_B). Complex V, the ATPase, contains 16 protein subunits but does not contain any redox centers.

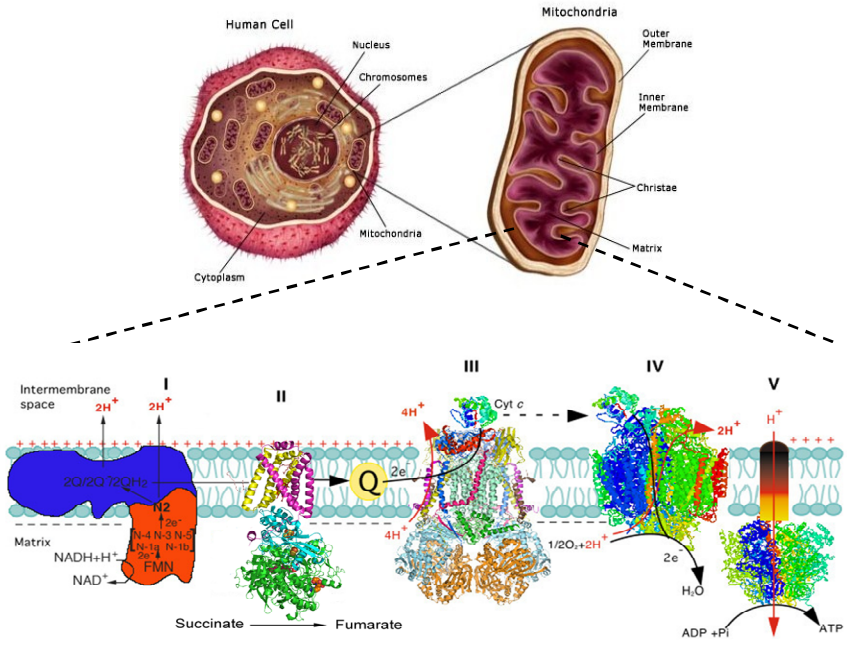


Fig.1-1 Model of the electron transport chain in the mitochondrial inner membrane. (modified) (*1*).

Table 1-1 Properties of Bovine Heart Mitochondiral complexes involved in oxidative phosphorylation. (*2-8*)

Complex	Other names	Subunits	Proton Pumping	Molecular weight (monomer) (kDa)	Prosthetic groups	Midpoint potential (mV)
I	NADH-UQ reductase (NQR)	45	Yes	907	FMN [2Fe2S]N-1a [2Fe2S]N-1b [2Fe2S]N-2 [2Fe2S]N-3,4 [2Fe2S]N-5,6	-245~-335 -375 -250 -150 -250 -260
II	Succinate-UQ reductase (SQR)	4	No	127	FAD [2Fe2S]S-1 [4Fe4S]S-2 [3Fe4S]S-3 b_{560}	-40 0 -270 130 -185
III	UQH_2-cytochrome c reductase (QCR)	11	Yes	248	b_{562} (b_H) b_{566} (b_L) c_1 2Fe2S	+30 -30 230 280
IV	Cytochrome c oxidase (CcO)	13	Yes	204	a Cu_A Cu_B a_3	210 245 340 385
V	ATPase	16	Yes	380	No	No

The most significant progress in the study of mitochondrial oxidative phosphorylation is the success of crystallization of these complexes in the recent years.

1.1.2 Complex III (cytochrome bc_1 complex)

Complex III, also known as cytochrome bc_1 complex, is an essential electron transfer complex. It catalyzes electron transfer from ubiquinol to cyt *c* with translocation of 2 proton/e from matrix to mitochondrial inter membrane space to generate the electrochemical gradient for ATP synthesis. This protein was first isolated from submitochondrial particles (SMP) of beef heart by Dr. Youssef Hatefi in 1970s. And then our group modified the purification procedures to obtain a better preparation. The major steps involved in purification procedures are potassium deoxycholate solubilization, followed by ammonium acetate fractionation, then centrifugation.

Purified bovine Complex III contains 11 protein subunits. Three of them, cyt *b,* cyt c_1 and ISP house two *b* hemes, one c_1 heme and a [2Fe2S] cluster. These three essential subunits are called core subunits and they exist in all cytochrome bc_1 complexes, from *Rhodobacter capsulatus* to bovine mitochondria (*9*). The other 8 subunits containing no redox prosthetic centers are the supernumerary subunits. The number of supernumerary subunits in the bc_1 complex varies from 0 to 8 (Table 1-2, 1-3). The core subunits are essential for catalytic function of the complex whereas the supernumerary subunits are likely involved in complex structure stability or regulating functions.

Table I-2 The number of subunits in bc_1 complexes from different species. (*9*)

Species	Number of subunits	Number of Core subunits	Number of supernumerary subunits
Bovine	11	3	8
Chicken	10	3	7
Potato	10	3	7
Yeast	10	3	7
Rhodobacter sphaeroides	4	3	1
Rhodobacter capsulatus	3	3	0

Table 1-3 Compositions and molecular mass of monomeric bc_1 complexes (*10*)

Subunit	Beef	No. res.	*Yeast*	No. res	*R. sph*	No. res
1	core 1	446	COR 1	431	-	-
2	core 2	439	COR 2	352	-	-
3	cyt. *b*	379	COB	385	cyt *b*	445
4	cyt. c_1	241	CYT1	249	cyt c_1	286
5	ISP	196	RIP1	185	ISP	187
6	QPK	111	QCR7	126	-	-
7	QPC	81	QCR8	93	sub4	124
8	c_1-hinge	78	QCR6	122	-	-
9	ISP precursor	78	-	-	-	-
10	c_1 assoc.	62	QCR9	52	-	-
11	unnamed	56	QCR10	78	-	-
No. res. / monomer		2,167		2,073		1,042
Prosthetic groups (b_L, b_H, c_1, Fe_2S_2)						

Study of mitochondrial cytochrome bc_1 complex was highlighted when the first 3-D structure of bovine bc_1 complex was available in 1997, by a joint effort of our group and Deisenhofer's group (Fig.1-2). The 3-D structure of bc_1 complex was diffracted at X-ray to 2.9 Å resolution and it exists as an intertwined dimer with each monomer containing 11 protein subunits. The head domain of ISP in one monomer is connected to the tail domain of ISP in the other monomer. The structure is 130 Å in width and 155 Å in height. It can be divided into three regions: matrix, trans-membrane, and the inter-membrane space regions (see Fig. 1-2).

The matrix region of the molecule occupies more than one-half of the molecular mass and extends 75 Å from the transmembrane helices. Subunits I, II, VI, IX, C-terminal part of ISP and the N-terminal of subunit VII are in this region. The transmembrane region (42 Å) in each monomer consists of 13 transmembrane helices, 8 of which are from cytochrome *b*, 5 from cytochrome c_1, ISP, subunit VII, X, and XI, respectively. The cytochrome *b* houses two *b* type hemes, b_L and b_H; and two Q-binding pockets, Q_N and Q_P in the transmembrane region. The intermembrane space region extends 38 Å, housing cytochrome c_1 and ISP functional domains, as well as subunit VIII (*11*).

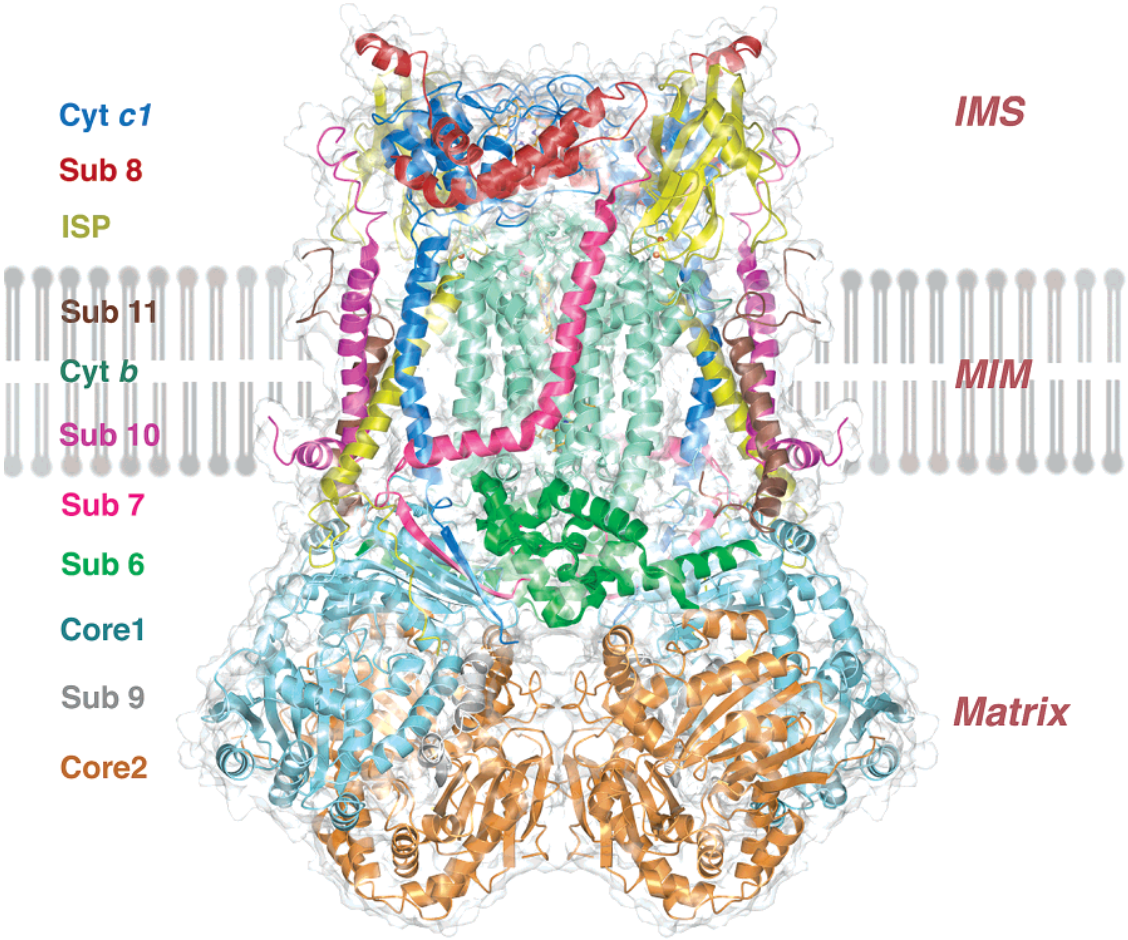


Fig.1-2 Structure of the dimeric bovine mitochondrial cytochrome bc_1 complex shown in ribbon form. The color code for each subunit is given on the side bar. The boundary of mitochondrial inner membrane is delineated by two parallel lines. The mitochondrial inter-membrane space (IMS), the transmembrane (MIM, mitochondrial inner-membrane) and the mitochondrial matrix parts (Matrix) are as labeled. (*10*)

The distance between heme irons in b_L, b_H, c_1, and the iron-sulfur cluster in the bc_1 complex is shown as Fig.1-3. It is 21 Å from heme b_L to b_H, 27 Å from heme b_L to [2Fe2S] cluster and 31 Å from the [2Fe2S] cluster to heme c_1. Low electron density region shown in the inter-membrane part of bc_1 complex represents the ISP head domain in the structure (*11*). The observation that the distance between heme c_1 and [2Fe2S] is too far for an effective electron transfer, together with the finding that the electron density for the ISP head domain is particularly poor, indicate the movement of ISP. This movement was also further substantiated by its different positions when bc_1 complex binds to different inhibitors (*12*).

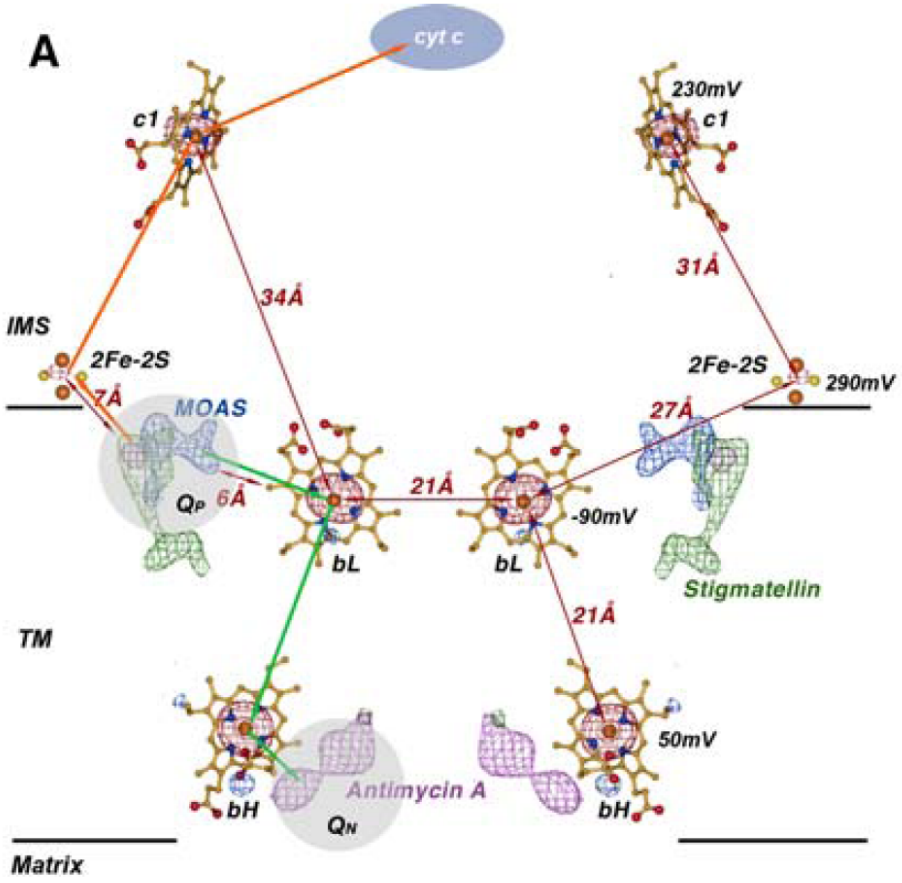


Fig.1-3 Distance between the redox prosthetic groups in the bovine mitochondrial cytochrome bc_1 complexes. (*10*)

1.1.3 Q-cycle mechanism

The "protonmotive Q cycle" mechanism is the most popular hypothesis for the electron and proton transfer in the cytochrome bc_1 complex. It was first proposed by Dr. Peter Mitchell and then modified by Dr. Trumpower(*13-21*). The main feature of this mechanism is the presence of the Q binding sites: Q oxidative (Q_o or Q_P) and Q reductive (Q_i or Q_P) sites, and the bifurcation of electrons from QH_2 at the Q_P site (see Fig.1-4).

In the Q cycle, the reduced Q (quiquinol, QH_2) binds the Q_P pocket, and transfers its electrons either sequentially or concertedly to ISP and heme b_L. With the ISP head domain movement, ISP transfers one electron from ISC (iron sulfur cluster) to the heme c_1 of cyt c_1, and then from heme c_1 to the heme c of cytochrome c. The other electron is transferred from heme b_L to b_H, and then to the Q or SQ at the Q_N site. The catalysis of bc_1 complex couples proton pumping from mitochondrial matrix to intermembrane space to the electron transfer. In the Q_P pocket, with the bifurcation oxidation of QH_2, four protons from two QH_2 are also pumped into intermenbrane space simultaneously. And in the Q_N pocket, one oxidized Q receives two electrons (one each time) from heme b_H totally during the catalysis, and this re-reduced Q uses two protons in mitochondrial matrix to form a new QH_2. Then this new QH_2 goes to the Q_P pocket for next Q cycle. Therefore, the overall reaction catalyzed by bc_1 should be the equation (a) below:

$$QH_2 + 2\ \text{Cyt}\ c^{3+} + 2H^+_{(N)} \rightarrow Q + 2\ \text{Cyt}\ c^{2+} + 4H^+_{(P)}$$

In all, the whole Q cycle pumps four protons into the inter membrane space, use two protons in the matrix, oxidize one ubiquinol to ubiquinone, and reduce two cytochrome c.

There are two hypotheses for bifurcated QH_2 oxidation at the Q_P site. They are the "sequential" (*22-24*) and "concerted" (*25-28*) mechanisms. In the "sequential" mechanism, the first electron from QH_2 is transferred to ISC, and then the ubisemiquinone is produced and used to reduce heme b_L. In the "concerted" mechanism, the two electrons are simultaneously transferred from QH_2 to ISC and heme b_L.

However, the fact that no ubisemiquinone radical can be detected at the Qp site (SQp) (*29, 30*) questions the validity of the sequential oxidation mechanism. Several reasonable explanations for the absence of Q radical have been proposed (*22, 24*) such as antiferomagnetic coupling between SQp and ISC or SQp being fastly oxidized by heme b_L. It was reported that the SQp under abnormal conditions can be detected (*31, 32*), but this has not been confirmed by other investigators. The absence of SQp in a mutant bc_1 complex without heme b_L further confirms that SQp is not involved in QH_2 oxidation at the Qp site (*30*). So far there is no evidence to show that ISC can receive an electron from QH_2 before the other redox components. Therefore, developing a technique for simultaneously determining the reduction rates of ISC and heme b_L is a breakthrough in the mechanistic study of the bc_1 complex (*28*). It is likely that the concerted mechanism can really give a more reliable explanation for the electron transport at the Q_P site so far. This will be more accurate when we can get the bc_1 complex structure including the exact QH_2 binding information at the Q_P site.

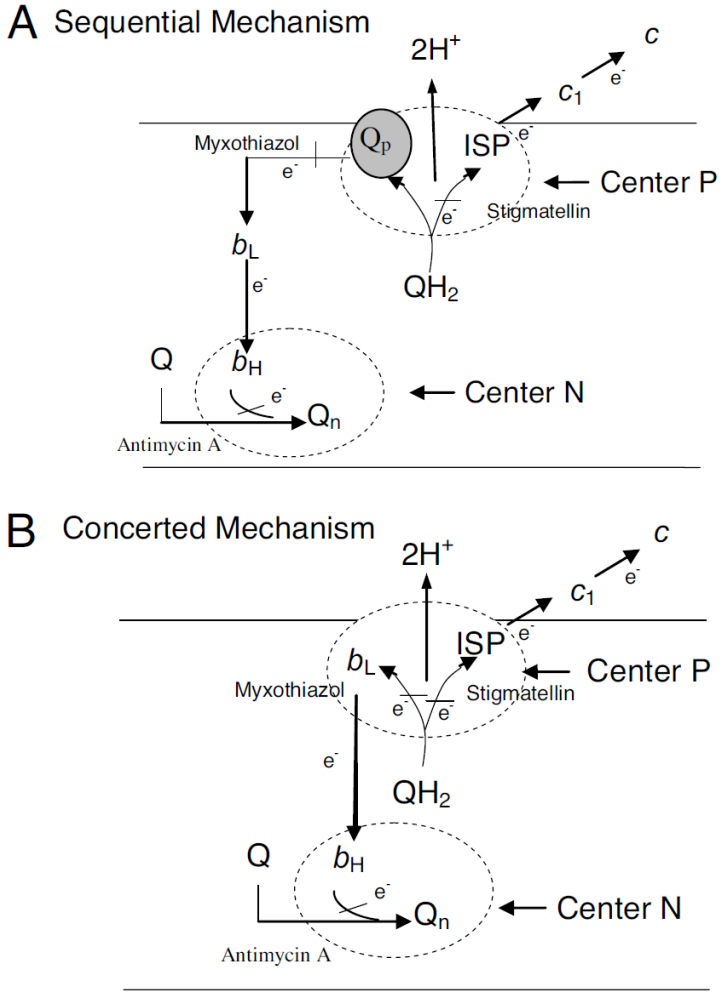


Fig.1-4 The Q-cycle mechanism. (A) Sequential bifurcated oxidation of quinol at Qp site. (B) Concerted bifurcated oxidation of ubiquinol at Qp site. (*33*)

1.1.4 Inhibitors of cytochrome bc_1

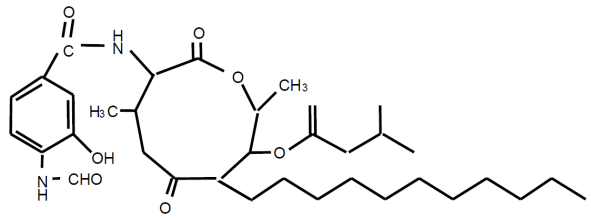
Cytochrome bc_1 inhibitors can be classified into three major classes based on the differences in the binding sites (38-40, Table 2). They are: class I hibitors (also called class P), which bind to Q_P pocket and block ubiquinol oxidation or the electron transfer from ISP to cytochrome c_1; class II (also called class N), which bind to the Q_N site and prohibit the electron transfer from heme b_H to ubiquinone; class III (also called class PN), which bind to both Q_P and Q_N sites and cause all the inhibitions. (*34-36*). Table I-4 and Figure I-5 show these three classes of inhibitors and their chemical structures respectively.

Class I inhibitors can also be sub-classified into three groups: Ia, Ib and Ic. A beta-methoxyacrylate (MOA) group is a typical structural element in class Ia inhibitors. When class Ia inhibitors bind to cytochrome *b*, a red-shift in the α and β-bands of the reduced heme b_L spectrum is observed. Myxothiazol and β-methoxyacrylate stilbene (MOAS), which block electron transfer from ubiquinol to ISP, are typical class Ia inhibitors. A chromone ring system exists in Class Ib inhibitors. The binding of Ib inhibitors results in a remarkable increase of ISP mid-point potential and induce a red-shift of cytochrome b_L spectrum. Stigmatellin, a typical class Ib inhibitor, inhibits the electron transfer from ISP to cytochrome c_1. Class Ic inhibitors are 2-hydroxy quinone analogues. They cause a slight increase in ISP redox potential shift but show no influence on the spectrum of cytochrome b_L. UHDBT is a typical Ic inhibitor. Antimycin A and diuron are two typical inhibitors of class II (N). Class PN includes inhibitors such as NQNO and funiculosin (*37*).

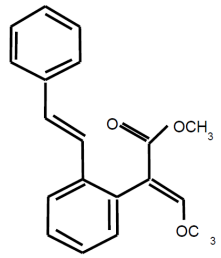
Table 1-4. Classification of cytochrome bc_1 inhibitors.(*37*)

Class	Subgroup	Representative inhibitors
I (Q_P site)	Ia	Myxothiazol, MOAS
	Ib	Stigmatellin
	Ic	UHDBT
II (Q_N site)		Antimycin A, diuron
PN (Q_P and Q_N)		NQNO, funiculosin

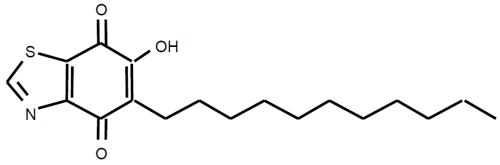
NQNO: 2-n-nonyl-4-hydroxyquinoline N-oxide;
MOAS: methoxyacrylate-stilbene;
UHDBT: 5-undecyl-6-hydroxy-4, 7-dioxobenzothiazole;



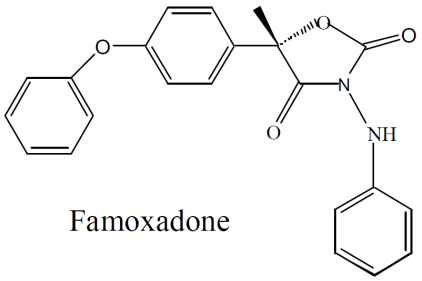
Antimycin A



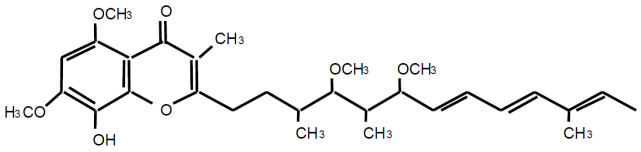
MOA-stilbene



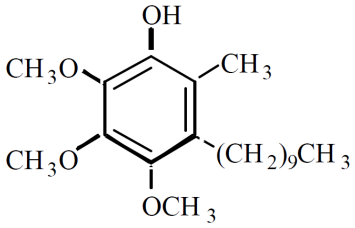
UHDBT



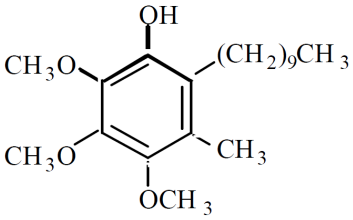
Famoxadone



Stigmatellin



TMDMP



TMMDP

Fig. 1-5. Structure of some representative inhibitors of the cytochrome *bc*$_1$ complex.(*38-40*)

1.1.5 Other functions of mitochondrial bc_1 complex (MPP and Superoxide generating activities)

In addition to electron and proton transfer activity, the bovine bc_1 complex also have mitochondrial processing peptidase (MPP) like activity (*41, 42*). The comparison of core I and core II with α and β subunits of MPP, shows some sequence homology. A sequence of Y91XXE94H95$(X)_{76}$E171(*11*) in core I is similar to the MPP's zinc-binding motif HXXEHX_{76}E on its β-subunit. Further-more, residues K_{300} and R_{301} of core II are structurally close to this zinc-binding motif in core I and may play a role in the active site. Triton X-100 can activate the MPP acivity of bovine bc_1 complex. It disrupts the structural integrity of the complex, and so the binding of the inhibitory polypeptide to core I&II is weakened.(*41*) During the maturation of bc_1 complex, the presequence peptide of ISP is cleaved by core I&II, and then it is bound to them as subunit IX and thus inhibits MPP activity. Since the binding of subunit IX inhibits the MPP activity of core I and core II, they function as a suicide peptidase.

During electron transfer through complex III, superoxide generation is observed. Mitochondrial electron transfer chain is a major source for cellular superoxide production (*43*). Electrons transferring through Complex I and III generate superoxide anion (*44*). In complex I, the electron donors for superoxide generation are the flavin radical and the ubiquinone radical. In bc_1 complex, the ubisemiquinone radical at the Q_P site (*45*) and/or the reduced heme b_{566} (or b_L) (*46*) are believed to be the electron leakage sites for superoxide production during bc_1 complex catalysis.(see Fig. 1-6)

Although $O_2^{\cdot-}$ production through bc_1 complex might result from electron leakage from their normal pathways, the reaction mechanism of $O_2^{\cdot-}$ generation remains elusive. Under normal catalytic conditions, the electrons which leak from bc_1 to form $O_2^{\cdot-}$ account for only a very small amount(*47, 48*). But this $O_2^{\cdot-}$ generating activity increased when there was antimycin or the electron transport chain (ETC) was over reduced (*48*). Our most recent results found that neither SQp nor reduced heme b_L was the site of electron leakage (*30*). O_2 functions as a low potential electron acceptor to be involved in bifurcated oxidation of QH_2 in a hydrophobic environment and in the absence of heme b_L when there is a high protential acceptor, ISP. Protonated superoxide is produced and diffused to a hydrophilic environment (intermembrane space or matrix), and finally becomes $O_2^{\cdot-}$ by deprotonation.

In most kinds of organisms, "oxidative stress" can cause extensive toxicities. In this oxidative stress, the reactive oxygen species (ROS) reacts with cellular components (such as DNA/RNA, proteins, carbohydrates, and unsaturated lipids), generate non-reversible damage to them. The ROS can make the iron-sulfur clusters of complexes I and III inactive, thus restrain electron transport in mitochondria, and limit the mitochondrial respiration (*49*). ROS damages take part in many disease states, such as inflammation, ischemia-reperfusion injury, cancer and aging process. (*50-54*).

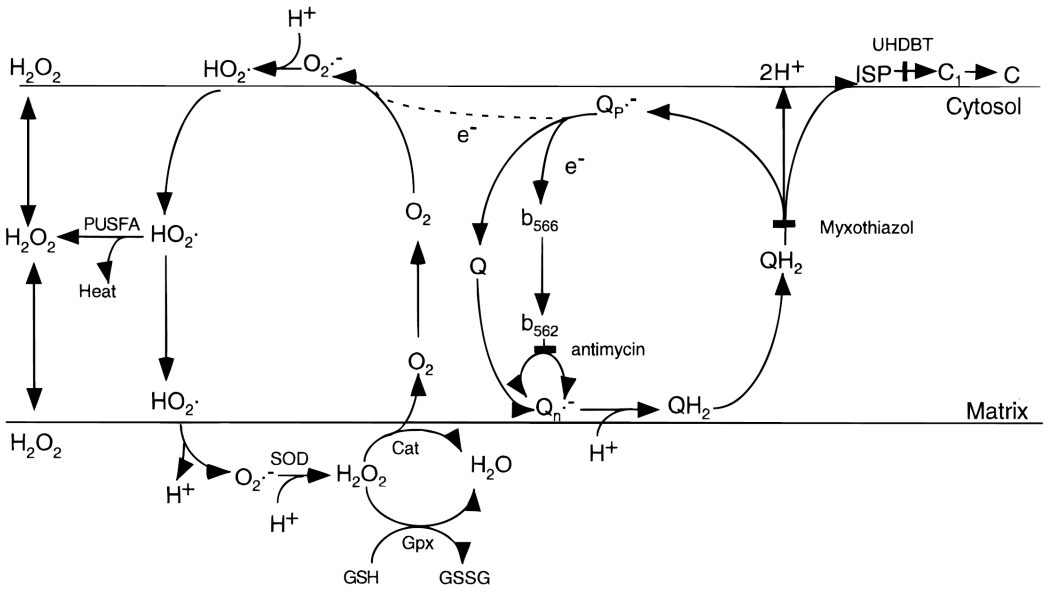


Fig.1-6. Relationship between the membrane potential regulating superoxide cycle and proton motive Q-cycle in the cytochrome *bc*$_1$ region of the respiratory chain. Abbreviations used are: SOD, superoxide dismutase; PUSFA, polyunsaturated fatty acyl groups of lipids; UHDBT, 5-undecyl-6-hydroxyl-4,7- dioxobenzothiazol; Gpx, glutathione peroxidase; Cat, catalase. GSH and GSSG are reduced and oxidized glutathione respectively.(*55*)

1.1.6 Interaction between matrix proteins and electron transport chain

After more than a decade of intensive post 3-D structure studies, only a handful structure/function questions remain unsettled. We anticipate that the next phase of study of the bc_1 complex will be the integration of this complex into a larger bioenergetics scheme that includes the regulation of and interaction with TCA cycle enzymes as well as the redox linked signal transduction (*56, 57*).

So far, no study on the interactions between electron transport chain complexes and soluble matrix enzyme has been conducted. The only enzyme that participates in both the citric acid cycle and electron transport chain is Complex II (*58*). It catalyzes succinate to fumarate with electron transferred to Q. Some other enzymes of TCA cycle are only known to couple with electron transfer chain through their product NADH at complex I (*59*). No direct interaction between them has been reported yet.

We have recently developed a simple precipitation pull-down technique for studying the interaction between bc_1 and matrix enzymes. Three proteins have been pulled down by the bc_1 complex and identified as bemitochondrial malate dehydrogenase (MDH), asparate transaminase and aconitase, based on peptide mass fingerprinting analysis. The study of the interaction between MDH or aconitase and bc_1, its binding sites as well as its roles in regulation of energy metabolism will be the specific aims of our research.

1.2 Introduction to Citric Acid cycle enzymes MDH and ACON

The citric acid cycle is also named as the TCA cycle (tricarboxylic acid cycle) or Krebs cycle. A series of enzyme-catalysed chemical reactions are included in TCA cycle and it is crucial to all living cells using oxygen in cellular respiration. It accounts for the oxidation of acetyl CoA derived from amino acids, carbohydrates and fat acids and generates numerous biosynthetic precursors. In eukaryotes, the citric acid cycle occurs in the mitochondrial matrix region.

The eight enzymes of the citric acid cycle catalyze a series of well-known reactions (shown as Fig. 1-7). Two enzymes, aconitase and malate dehydrogenase, which were co-precipitated with bc_1 complex, will be the focus of this study.

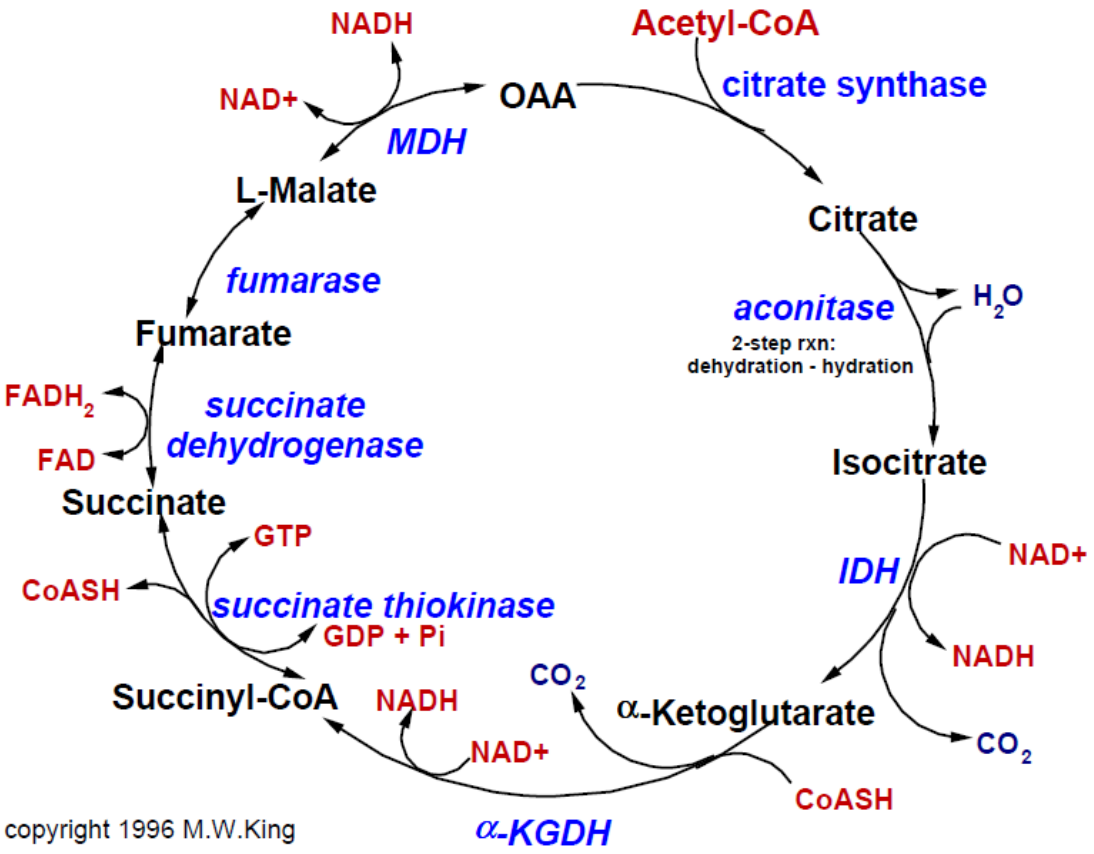


Fig.1-7 Model for citric acid cycle.

1.2.1 Malate dehydrogenase (MDH)

Malate dehydrogenase (EC 1.1.1.37) catalyzes the final reaction of the citric acid cycle, which converts malate into oxaloacetate. The reaction is NAD^+-dependent and oxidizes malate's secondary hydroxyl group to a corresponding ketone(*60*). And this reaction is reversible. There are two types of malate dehydrogenase: one in cytosolic,the other in mitochondria.

The 3-D structure of mitochondrial malate dehydrogenase from porcine heart has already been obtained. And in this MDH crystal structure, it contains four identical subunits in the asymmetric unit of a monoclinic cell.(*61*)

Besides the TCA cycle, MDH also participates in the malate-aspartate shuttle, translocating electrons produced during glycolysis across the mitochondrial inner membrane. There are two forms of malate dehydrogenase in the shuttle system: mitchondrial malate dehydrogenase and cytosolic dehydrogenase. In the cytosol, malate dehydogenase catalize the reaction of oxaloacetate and NADH to produce malate and NAD^+. Then malate will be imported into the mitochondrial matrix by the Malate-alpha-Ketoglutarate antiporter and meanwhile alpha-ketoglutarate be exported from the matrix into the cytosol. Once get into matrix, mitochrondrial malate dehydrogenase will convert malate into oxaloacetate. And NAD^+ is also reduced to NADH.(*62*)

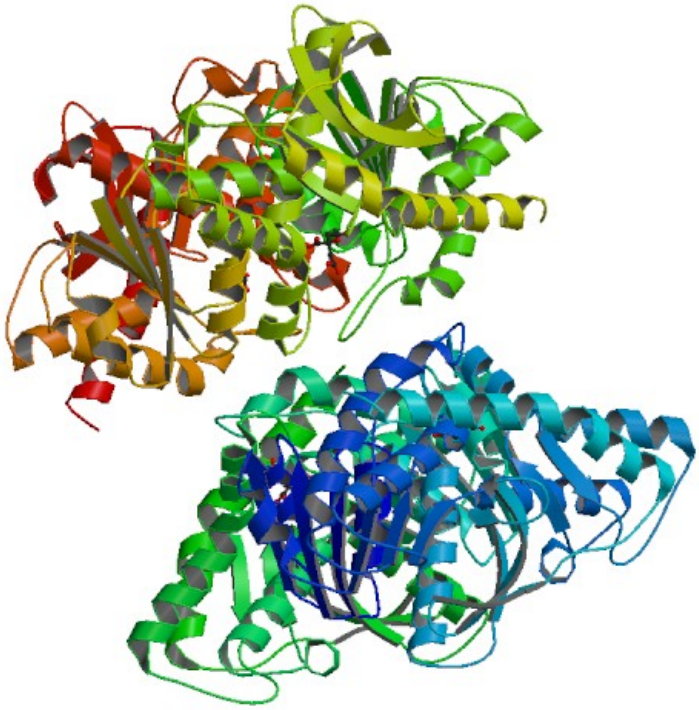


Fig.1-8 Ribbon model of malate dehydrogenase (MDH) dimer from the pig (Sus scrofa) with NAD as balls. (*61*)

Malate dehydrogenase also participated in gluconeogenesis, in which glucose is synthesized from smaller molecules. Malate dehydrogenase reduces the oxaloacetate to malate, which is able to be transported through the inner mitochondrial membrane. Then malate can be oxidized by cytosolic malate dehydrogenase to form oxaloacetate again and phosphoenol pyruvate carboxy kinase (PEPCK) converts oxaloacetate to phosphoenol pyruvate finally. (*63*)

1.2.2 Aconitase (ACON)

Aconitase (EC 4.2.1.3) catalyses the reversible isomerization between citrate and isocitrate via cis-aconitate as the intermediate. It is a non-redox-active process. A covalently bound [4Fe-4S] iron-sulfur cluster is needed for the catalytic activity of aconitase.

Most of the other iron-sulfur proteins which mainly function as electron carriers, but the iron-sulfur cluster of aconitase reacts directly with an enzyme substrate. It has an active $[Fe_4S_4]^{2+}$ cluster, which may change to an inactive $[Fe_3S_4]^{+}$ form. This $[Fe_4S_4]$ centre has been shown to be coordinated with three protein Cys sulfhydryl groups. The labile iron ion of the $[Fe_4S_4]$ cluster is coordinated by water molecules (not Cys) in the active state. Aconitase is inhibited by fluoroacetate and the iron sulfur cluster can be easily oxidized by superoxide (*64*).

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CHAPTER II

CROSSTALK BETWEEN MITOCHONDRIAL MALATE DEHYDROGENASE AND CYTOCHROME BC_1 COMPLEX

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2.1 Abstract

The interactions between the mitochondrial cytochrome bc_1 complex and matrix soluble proteins were studied by a precipitation pull-down technique. Purified, detergent dispersed bc_1 complex was incubated with mitochondrial matrix proteins followed by dialysis in the absence of detergent. The interacting protein(s) was co-precipitated with bc_1 complex upon centrifugation. One of the matrix proteins pulled down by bc_1 complex was identified as mitochondrial malate dehydrogenase (MDH) by MALDI-TOF Mass spectrometry and confirmed by Western blotting with anti-MDH antibody. Using cross linking technique, subunits I, II (core I & II) and V of the bc_1 complex were identified as the interacting sites for MDH. Incubating purified MDH with the detergent dispersed bc_1 complex results in an increase of the activities of both the bc_1 complex and MDH. The effect of the bc_1 complex on the activities of MDH is unidirectional (oxalacetate → malate). These results suggest that the novel crosstalk between citric acid cycle enzymes and electron transfer chain complexes might play a regulatory role in mitochondrial bioenergetics.

2.2 Introduction

The mitochondrial cytochrome bc_1 complex catalyzes electron transfer from ubiquinol to cytochrome *c* with the concomitant translocation of protons across the membrane to generate a proton gradient and membrane potential for ATP synthesis (*1*). Although the structural and functional studies of the mitochondrial bc_1 complex have been intensive, few studies of the interactions between the bc_1 complex and the soluble matrix enzymes have been available. Complex II (succinate-ubiquinone oxidoreductase)

is the only enzyme that participates in both the citric acid cycle and the electron transport chain (*2*). Complex II is composed of a succinate-dehydrogenase, which is a citric acid cycle enzyme, and a membrane anchoring protein fraction, which houses ubiquinone. Complex II catalyzes the oxidation of succinate to fumarate and the reduction of ubiquinone to ubiquinol. Other enzymes of citric acid cycle are known to couple to the electron transfer chain through their product NADH at complex I (*3*).

Structurally the mitochondrial bc_1 complex can be divided into three regions: matrix, trans-membrane, and the inter-membrane space regions. The matrix region contains subunits I (core I), II (core II), VI, IX, and the N-terminal part of subunits V (ISP) and VII (*4, 5*). Although this region contains the major part of the complex mass, it possesses no redox centers; it is not directly involved in electron/proton transfer activity. The functions of subunits in this region, although, mostly unknown, may contribute to a structural or regulatory role of the bc_1 complex. If the interaction between the matrix enzymes and the bc_1 complex exists, this region would be a good choice for the contact.

In the present work, we have observed an increase in bc_1 activity when the bc_1 complex was incubated with the mitochondrial soluble matrix protein fraction, suggesting that an interaction between the electron transfer complex and the matrix enzymes exists. To confirm this suggestion, we developed a technique to pull down the interacting matrix proteins. This technique involves incubation of the purified, detergent dispersed bc_1 complex with the soluble matrix enzymes fraction followed by extensive dialysis and centrifugation to collect the precipitates that contain the matrix enzymes and the bc_1 complex. One of the matrix enzymes pulled down with the bc_1 complex has been identified as MDH. Herein we report the detailed procedures for pulling-down the matrix

enzymes that interact with the cytochrome bc_1 complex and the identification of MDH as one of the matrix enzymes that interacts with bc_1 complex. The effect of the interaction on the activities of the bc_1 complex and MDH, as well as the likely interacting domain, was also investigated.

2.3 Experimental procedures

Chemicals- Cytochrome *c* (horse, type III), sodium cholate, deoxycholate, NADH, oxaloacetate (OAA), Malate, NAD, and bovine MDH were purchased from Sigma. Cross linker, Sulfo-SBED (Sulfosuccinimidyl-2- [6-(biotinamido)-2-(p-azidobenzamido) hexa-noamido] ethyl-1,3′- dithiopropionate) was purchased from Pierce. 2,3-Dimethoxy-5-methyl-6-(10-bromodecyl)-1,4-benzoquinol ($Q_0C_{10}BrH_2$) was prepared as previously reported (*6*). Inhibitor chlorothricin was purchased from A.G. Scientific. Stigmatellin and Antimycin A were purchased from Sigma. The concentrations of stigmatellin and antimycin A were determined spectrophotometrically using millimolar extinction coefficients of 65.5 cm^{-1} mM^{-1} and 4.8 cm^{-1} mM^{-1}, respectively (*7*).

Cytochrome bc_1 complex preparation and activity assay- Bovine heart mitochondrial cytochrome bc_1 complex was prepared as previously reported (*8, 9*). The purified complex contained 9.3 nmol of cytochrome *b* and 4.9 nmol of cytochrome c_1 per milligram of protein. It was dissolved in a TS buffer (50 mM TrisHCl buffer, 0.66 M sucrose, pH 8.0) to a protein concentration of 22 mg/mL and frozen at −80 °C until use. The concentrations of cytochromes *b* and c_1 were determined spectrophotometrically using millimolar extinction coefficients of 28.5 cm^{-1} mM^{-1} (*10*) and 17.5 cm^{-1} mM^{-1} (*11*), respectively.

For activity determination, the cytochrome bc_1 complex was diluted with 50 mM phosphate buffer, pH 7.4, containing 1mM EDTA and 0.01% KDOC to a protein concentration of 0.022 mg/mL. Diluted protein solution (10 μL) was added to 990 μL of an assay mixture (100 mM phosphate buffer, pH 7.4, 1 mM EDTA, 1 mM KCN, 100 μM cytochrome *c* and 25 μM $Q_0C_{10}BrH_2$). Activity was determined by measuring the reduction of cytochrome *c* in a Shimadzu UV-2401PC spectrophotometer at 25 °C using a millimolar extinction coefficient of 18.5 cm^{-1} mM^{-1} (*11*) for calculation. The non-enzymatic oxidation of $Q_0C_{10}BrH_2$ determined under the same conditions in the absence of the enzyme was subtracted from the assay. The bc_1 complex used in these experiments had a specific activity of about 12 μmol cytochrome *c* reduced/min/nmol cytochrome *b*.

Preparation of mitochondrial matrix proteins- Three pounds of fresh, fat and connective tissue free beef heart muscles were cut into small pieces and then homogenized with matrix protein buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM Dithiothreitol (DTT), 0.1mM phenylmethylsulphonyl fluoride (PMSF), 250 mM sucrose, pH 7.4) on ice. The homogenate was centrifuged at 600 ×g for 10 min at 4 ^oC. The supernatant was collected and centrifuged at 8000 ×g for 15 mins at 4 ^oC. The supernatant and light-colored fluffy upper layer of precipitate were discarded. The dark red precipitate was collected, suspended in the 100 mL matrix protein buffer, and centrifuged at 8000 ×g for 15 mins at 4 ^oC. The light-colored supernatant solutions were discarded and the dark red precipitates were suspended in the 50 mL matrix protein buffer. The mitochondria were then broken with sonication for 5 min at 0 ^oC. The

mixture was centrifuged at 100,000 ×g for 2 hours at 4 ^{o}C. The upper floating lipids and precipitates were discarded and the supernatant solution was collected and subjected to centrifugation at 200,000 ×g for 2 hours at 4 ^{o}C. The supernatant (the matrix protein fraction) has a protein concentration of 7.5 mg/mL and was stored at -80 ^{o}C until use. Protein concentration was determined by the Bradford protein assay with crystalline bovine serum albumin (BSA) as the standard.

The pull down experiment- One mg of bc_1 complex was incubated separately with 1 mL 0.28, 0.83 and 2.50 mg/mL of mitochondrial matrix extract on ice for 2 hours. After incubation, the mixtures were dialyzed against 1 L of the matrix protein buffer for 4 hours with one change of buffer at 4 ^{o}C to remove the detergent used for dispersing the cytochrome bc_1 complex. The dialyzed preparations were subjected to centrifugation at 100,000 ×g for 1 hour at 4 ^{o}C. The resulting precipitates were dissolved in the PES buffer (50 mM phosphate buffer, 1 mM EDTA, 0.5% sodium cholate, pH7.4) and analyzed by SDS-PAGE. The matrix protein fraction and the cytochrome bc_1 complex alone were subjected to identical dialysis and centrifugation conditions as those described and used as controls.

MDH Activity assay- A conventional spectrophoto- metric method was used for MDH activity assays (Malate + NAD^+ ↔ Oxaloacetate + NADH + H^+) (*12-14*). The reversed reaction of MDH was assayed using 0.2 mM NADH and 0.2 mM OAA in a 50 mM phosphate buffer (pH 7.4). The forward reaction of MDH was assayed using 2 mM NAD and 2 mM malate in a 50 mM phosphate buffer (pH 7.4). The activity was determined by measuring the decrease or increase of NADH absorbance at 340 nm, in a

Shimadzu UV-2401PC spectrophotometer at 25 °C using a millimolar extinction coefficient of 6.22 cm^{-1} mM^{-1}.

Mass Spectroscopy- Samples were run with SDS-PAGE first. The selected protein bands were sliced from the gel and washed with 50% acetonitrile /25 mM ammonium bicarbonate (pH8.0) three times, for 40 min each. The washed gel slices were dehydrated by acetonitrile. In-gel digestion was carried out with 8 µg/mL of freshly prepared trypsin in 50 mM of ammonium bicarbonate (pH8.0) on ice for half an hour. The final sample was mixed with the light absorbing compound (10 mg/mL of alpha-cyano-4-hydroxycinnamic acid dissolved in 0.3 mL of 50% acetonitrile / 0.1% trifluoroacetic acid (TFA)) and loaded on the MALDI plate to co-crystallized (dried). Data was collected from the MALDI-TOF MS (Voyager-DE^{TM} PRO Biospectrometry Workstation) and the intensities within 10% of the main peak were set as noises and not shown with a peak marker. The monoisotopic masses of the first peaks of the main clusters were collected. The unknown protein was searched within mammalian proteins of MSDB database with software Mascot Daemon, using the tolerance of +100 ppm.

Surface Plasmon Resonance (SPR)- SensíQ from ICX Nomadics is a dual-channel, semi-automated surface plasmon resonance system. 250 µl of MDH (100 µg/mL) were used to bind with channel 1 (channel 2 as control) in the COOH chip until it reached balance at 2000Ru. Various concentrations of bc_1 complex in a dilution buffer (50 mM phosphate buffer, pH 7.5, containing 0.01% KDOC) were injected through both channel 1 and 2. The sample dilution buffer was used as the flowing buffer over the chip all the time except for during the bc_1 complex sample injection time. Data was analyzed by the software Qdat from the same SPR manufacture company.

Cross linking- Sulfo-SBED (Sulfosuccinimidyl -2-[6-(biotinamido)-2-(p-azidobenzamido)hexanoamido] ethyl-1,3´-dithiopropionate) is a trifunctional cross-linking reagent containing a biotin, a sulfonated N-hydroxysuccinimide (Sulfo-NHS) active ester, and a photoactivatable aryl azide. First, the amine-reactive Sulfo-NHS ester of Sulfo-SBED was created on the carboxyl-containing amino acids on the surface of MDH. After dialysis to remove the nonreacted Sulfo-NHS, labeled MDH was mixed with bc_1. The mixture was illuminated with a long-wave UV lamp (365 nm) at a distance of 5 cm for 15 minutes. The disulfide bond in the spacer arm originally attached to the Sulfo-NHS ester was cleaved by incubating it with 100 mM of β-mercaptoethanol. The final biotin-labeled proteins were subjected to Western blotting analysis.

Western blotting- Western blotting was performed with biotin labeled polyclonal rabbit antibodies against bovine mitochondrial MDH. The polypeptides separated by SDS-PAGE gel were transferred to a 45 μm nitrocellulose membrane for immunoblotting. Strepavi- din conjugated to horseradish peroxidase (HRP) was used as the second antibody. Color development was carried out using an HRP color development solution.

2.4 Results and discussion

2.4.1 Mitochondrial soluble matrix proteins increase the activity of cytochrome bc_1 complex.

In searching for possible interactions between the electron transfer complexes and the mitochondrial matrix proteins, we found that the electron transfer activity of mitochondrial cytochrome bc_1 complex increases in the presence of bovine mitochondrial matrix extract (Table 2-1). The involvement of phospholipids in the matrix extract to activate the bc_1 complex was ruled out, as the addition of phospholipase A-treated matrix extract give the same level of bc_1 activity increase as that of untreated matrix extract. However, when the mitochondrial matrix extract was heated to 95 °C and then incubated with the bc_1 complex, no activity increase was observed, suggesting that some proteins in the matrix extract play the activation role. This suggestion was further confirmed by the observation that the proteinase digested matrix extract showed no activation on the activity of bc_1 complex. Since the purified cytochrome bc_1 complex contains no other proteins besides the subunits of the complex, it is likely that some matrix proteins, which are capable of activating the bc_1 complex, might be physically associated with the complex *in vivo* and then dissociated from the bc_1 complex during the purification steps. The use of multiple steps of salt fractionation/ precipitation in the presence of detergents for purification of the bc_1 complex certainly will remove the interacting matrix proteins.

Table 2-1. Effect of mitochondrial matrix extract on the activity of bovine mitochondrial bc_1 complex.

Treatments [a]	Activity (μmol Cyt *c* reduced/min/ nmol Cyt *b*)	Activity (%)[b]
bc_1 + buffer	12.00 ± 0.24	100 ± 2
bc_1 + MP	15.84 ± 0.24	132 ± 2
bc_1 + (MP + PLA_2)	15.60 ± 0.36	130 ± 3
bc_1 + denatured MP	11.88 ± 0.86	99 ± 7

[a] Purified bc_1 complex were incubated with mitochondrial matrix extract (MP) or treated MP for 20 min at 4 ˚C before ubiquinol-cytochrome *c* reductase activity was determined. Buffer, MP preparation buffer as control; (MP + PLA_2), MP was treated with phospholipase A_2 for 30min first; denatured MP, MP was heated at 95 ˚C for 30 min and then centrifuged to discard the precipitate.

[b] 100% activity represents 12 μmol cytochrome *c* reduced/min/ nmol cytochrome *b* at 23 ˚C. N=3, means ± SE.

2.4.2 Identification of MDH as an interacting protein with the bc_1 complex.

Purified cytochrome bc_1 complex is only soluble/dispersed in an aqueous solution in the presence of detergent. It becomes precipitate when the detergent in the system is removed upon dialysis. It is expected that a soluble protein interacting with bc_1 complex will co-precipitate with the complex when the bc_1 complex becomes the precipitate upon dialysis. In this study, we employed the soluble/insoluble properties of the bc_1 complex to pull down protein(s) in the matrix extract that interacts with the complex. The use of the dialysis/precipitation method has advantages over the commonly used antibody/antigen method in pulling-down interacting proteins. The antibody/antigen method depends on the location of the epitopes that the antibodies are against. If the epitopes are located at the surface of the complex where the interacting protein is associated, the antibody will cover the association site and thus cause false negative result. This problem will not be encountered when the dialysis/precipitation method is used, because the surface area in the bc_1 complex will not be covered. Therefore, all the interacting proteins will be pulled- down by using the dialysis/precipitation method.

Aliquots of a given amount of the detergent dispersed bc_1 complex were incubated with various amounts of the matrix extract at 4 ˚C for 2 hours. The mixtures were then dialyzed against the matrix protein buffer for 4 hours with one change of buffer to remove detergent and then centrifuged for 1 hour at 100,000 ×g to recover the precipitates of the bc_1 complex. Fig.2-1-A shows the SDS-PAGE of the recovered precipitates after centrifugation. In addition to the subunits of the cytochrome bc_1 complex, several protein bands are observed and their intensities increased as the amount of matrix protein extract used in the incubation increased.

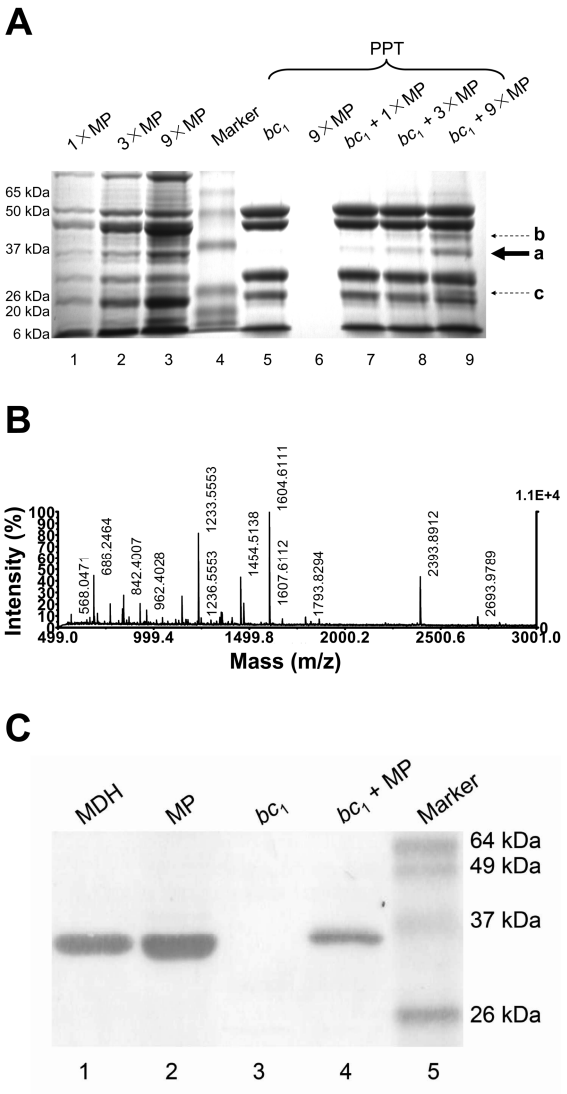

Fig.2-1. Identification of MDH as an interacting protein with the bc_1 complex. ***A.*** SDS PAGE analysis of bc_1 pulled-down proteins from matrix extract. Lane 1~3, various amounts of MP as controls to show the proteins in the matrix extract; lane 4, standard proteins; lane 5~6, bc_1 complex and MP only as controls to show the precipitates after centrifugation; lane7~9, bc_1 complex together with the pulled-down proteins from matrix extract. Solid arrow shows the main matrix protein being pulled down by the bc_1 complex. Dashed arrow shows some other bands being pulled down by the bc_1 complex. MP stands for mitochondrial matrix proteins; 1×, 3× and 9× stand for 0.28, 0.83 and 2.50 mg/mL of MP in the experiment; PPT stands for precipitates. ***B.*** MS spectra of protein sliced from SDS-PAGE gel. Spectrum data were collected from a MALDI-TOF Mass spectrometer. It consisted of a series of multiple charged ions from each fragment on a mass-to-charge (m/z) ratio scale. 100% intensity represents the highest peak. ***C.*** Western blot analysis of bc_1 pull-down matrix proteins with anti-MDH antibody. Lane 1, purified bovine mitochondrial malate dehydrogenase (MDH); lane 2, mitochondrial matrix extract (MP); lane 3, bc_1; lane 4, the precipitate from dialyzed mixture of the bc_1 complex and mitochondrial matrix extract (bc_1+MP); lane 5, standard proteins.

A major protein band, whose band intensity increased as the matrix concentration increased, was cut out from the gel, subjected to in-gel digestion with trypsin, and analyzed by MALDI-TOF mass spectrometry.

This protein was identified as bovine mitochondria MDH based on Peptide Mass Fingerprinting (PMF) analysis (Fig.2-1-B). The identity of the mitochondrial MDH was further confirmed by Western blotting using a polyclonal antibody against the mitochondrial MDH (Fig.2-1-C). As expected, a protein band with an apparent molecular mass of 35 kDa corresponding to mitochondrial malate dehydrogense was detected in the matrix extract and in the bc_1 precipitate fraction as the positive control. The other two minor bands that were indicated by the dotted arrows in Fig.2-1-A were also identified by MS. The larger one was aspartate transaminase, but the smaller one was not identified by MS due to the contaminant of the adjacent band.

2.4.3 Effect of MDH on the activity of the cytochrome bc_1 complex.

As shown in the previous section, the mitochondrial matrix extract has the ability to enhance bc_1 activity. If MDH in the matrix extract is indeed responsible for interaction with the bc_1 complex, one would expect to see an increase of bc_1 activity upon addition of purified MDH. Purified mitochondrial MDH, purchased from Sigma, was incubated with the cytochrome bc_1 complex and the activity of ubiquinol- cytochrome *c* reductase was assayed. As shown in Fig. 2-2, the activity of the bc_1 complex increases as the MDH concentration in the incubation mixture increases. This result indicates that MDH is

indeed one of the major proteins in the matrix extract interacting with bc_1 complex to enhance the complex activity.

2.4.4 Binding between MDH and cytochrome bc_1 complex.

The binding kinetics of MDH to bc_1 was studied by Surface Plasmon Resonance using a SensiQ machine. MDH was bonded to the COOH chip, and different concentrations of bc_1 complex were run through the MDH channel and control channel. Data analysis showed that the K_D for the interaction between bc_1 complex and MDH is about 0.047 μM (Fig.2-3), indicating a strong interaction. The interaction is buffer concentration dependent. At a 100 mM Na^+/K^+ phosphate buffer, the K_D is 0.047μM; at a 50 mM Na^+/K^+ phosphate buffer, the K_D is 0.027μM. The fact that MDH interacts better with the bc_1 complex in a lower buffer concentration than a higher one, indicates that the interaction between MDH and the bc_1 complex is hydrophilic.

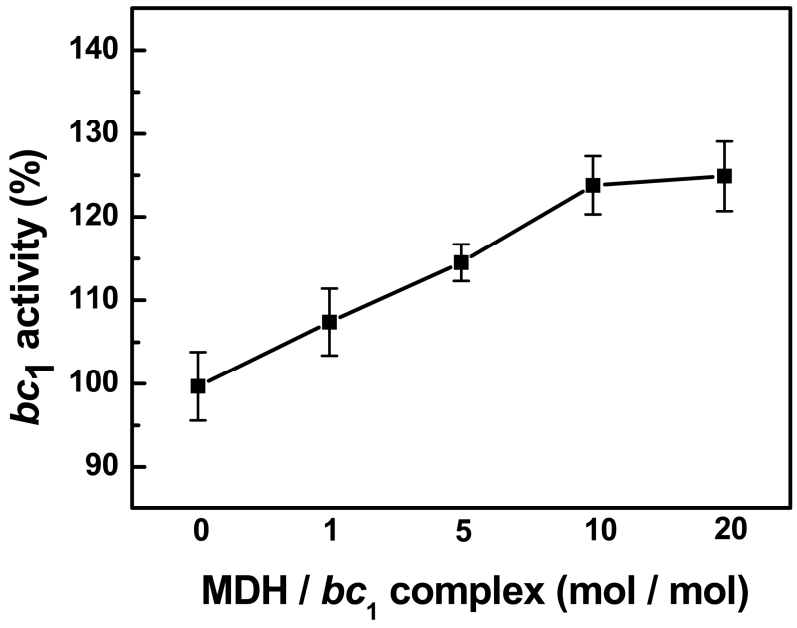

Fig. 2-2. Effect of MDH concentration on the bc_1 complex activity. A given amount of bc_1 complex was added to different amounts of MDH to give the indicated molar ratios. After the mixtures were incubated on ice for 20 min, the bc_1 activity was determined. The activity of bc_1 complex only was used as 100%. N=3, means ± SE.

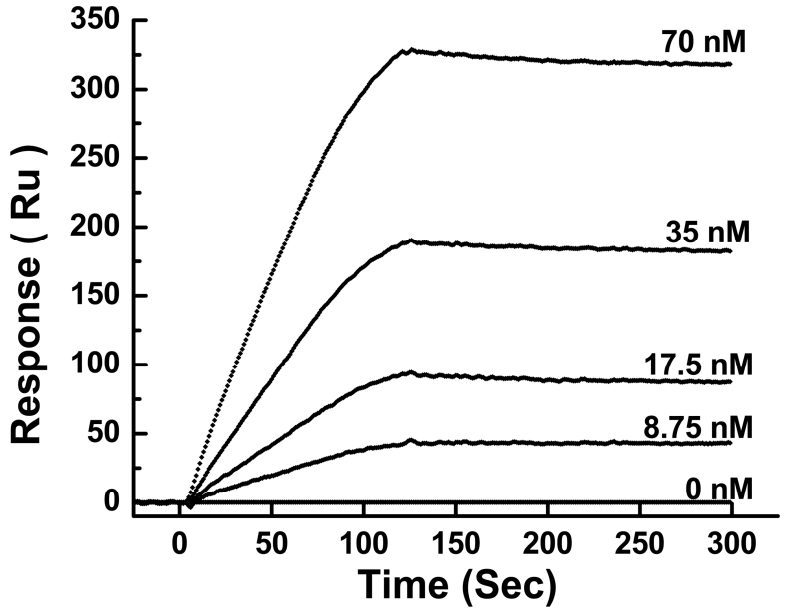

Fig. 2-3. Surface Plasmon Resonance analysis of the interaction between bc_1 complex and MDH. The bc_1 complex was diluted with the flowing buffer to give concentrations of 8.75, 17.5, 35, and 70 nM. The flowing buffer used was 100 mM Na^+/K^+ phosphate buffer, pH 7.4, containing 0.01% KDOC. Aliquots of 100 µL of diluted bc_1 at indicated concentrations were injected into both the control channel and MDH bound channel. The flow rate was held constant at 50 µL/min. Data was analyzed with Qdat software.

2.4.5 Identification of the subunits in bc_1 complex that interact with MDH.

To identify the subunit(s) in the bc_1 complex that interacts with MDH, a biotin labeled transfer cross-linker, Sulfo-SBED, was used as described in the experimental procedures. Fig.2-4-A shows the SDS-PAGE and the Western blotting analysis of Sulfo-SBED labeled MDH photolyzed alone and in the presence of the bc_1 complex followed by β- mercaptoethanol treatment. The strepavidin horseradish peroxide conjugate was used to probe proteins having a transferred biotin tag. Four protein bands, with apparent molecular masses of 53, 51, 35, and 21 kDa were shown in the SDS-PAGE part around 15~60 kDa region. The detection of the transferred biotin tag in MDH itself is expected in the Western blotting as the 35 kDa band, because MDH works as a dimer. The other three bands in the Western blotting had the same molecular masses as Core I, II and ISP. The band of Cyt c_1, which showed in this SDS-PAGE, and the band of Cyt *b*, which did not show clearly in the SDS-PAGE, were not found in the Western blotting. Other small subunits which are smaller than 15 kDa were not detected by the Western blotting (not shown in this range of the SDS-PAGE). The identification of Core I, II and ISP in the bc_1 complex as the interaction partner for MDH is in line with the 3-D structure of the bc_1 complex (shown as Fig.2-4-B).

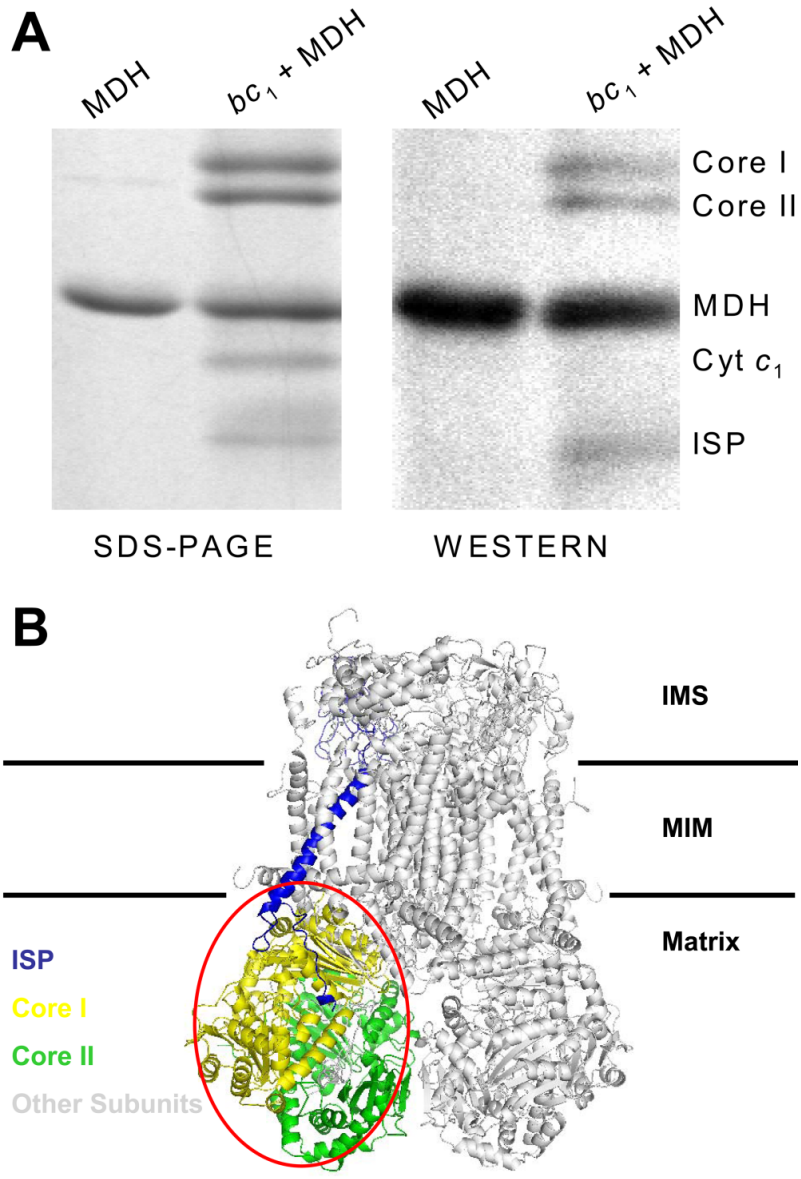

Fig. 2-4. SDS-PAGE and Western blotting of sulfo-SBED cross linked MDH and bc_1. ***A.*** Ten µg of MDH in 100 µl of 50 mM Na^+/K^+ phosphate buffer, pH 7.4 was incubated with sulfo-SBED for 2 hrs on ice in the dark then dialyzed against 500 mL of a 50 mM Na^+/K^+ phosphate buffer, pH 7.4, in the dark to remove any un-reacted sulfo-SBED. The chemically labeled MDH was then incubated without and with 30 µg of bc_1 in 200µl of a 50 mM Na^+/K^+ phosphate buffer, pH 7.4, on ice for 1 hr. After incubation, the mixtures were photolyzed with a long wavelength UV light (365 nm) at a distance of 5 cm for 15 minutes. The photolyzed samples were treated with ß-ME and subjected to SDS-PAGE and Western blotting. The biotin tags were probed with avidin-horseradish peroxidase conjugate. ***B.*** The interaction partners for MDH were shown in the 3-D structure of the bc_1 complex.

Core I and II account for a major portion of the matrix part of the bc_1 complex. Subunits VI, IX, the N-terminal parts of subunit VII and V (ISP), and the C-terminal part of subunit IV (cytochrome c_1) are also part of it. Since the mitochondrial MDH is an enzyme of the citric acid cycle and is located in the matrix, interaction of MDH with the bc_1 complex through Core I and II in the matrix region is expected. Furthermore, the involvement of Core I and II in the interaction with MDH is in line with the finding of the interaction being hydrophilic, since MDH and Core I & II of the complex are covered with charged amino acid residues. Although ISP was mainly in the inter-membrane space, the N terminus was still exposed in the matrix, therefore, MDH could bind with this N terminus as shown in Fig. 2-4-B. Because ISP plays an important role in the electron transfer within the bc_1 complex, this interaction indicates that MDH would have a chance to affect the electron transfer through ISP or ISP could transfer some signal to MDH through this crowbar-like N terminus.

2.4.6 Cytochrome bc_1 complex increases the activity of MDH unidirectionally (OAA to malate).

Since the binding of bc_1 complex with MDH enhances the bc_1 activity, it was of interest to see whether or not this binding could affect the MDH activity. MDH catalyses not only the oxidation of malate to oxalacetate (forward reaction) but also the reduction of oxalacetate to malate (reverse reaction); both activities were investigated. When MDH was incubated with varying concentrations of bc_1 complex, no increase in activity of MDH of the forward reaction was observed (see Fig. 2-5-A). On the other hand, the

reverse reaction activity of MDH increased as the bc_1 concentration increased (see Fig. 2-5-B). A maximum activation of 140% was observed in the reversed reaction. The activation was found to be ionic strength dependent. When the ionic strength increased, the extent of activity enhancement decreased (see Fig. 2-6). This confirmed that the interaction was hydrophilic.

The activity of bc_1 complex can be affected by its inhibitors such as stigmatellin, antimycin A, or zinc ion (*15-17*). When MDH was incubated with bc_1 complex treated with its inhibitors, only a partial enhancement of MDH reversed activity was observed (Table 2-2). Similarly, when bc_1 complex was incubated with chlorothricin-treated MDH (*18*), no enhancement of bc_1 activity was observed. These results indicated that only active cytochrome bc_1 complex and MDH can have an effect on each other's activity upon their interaction.

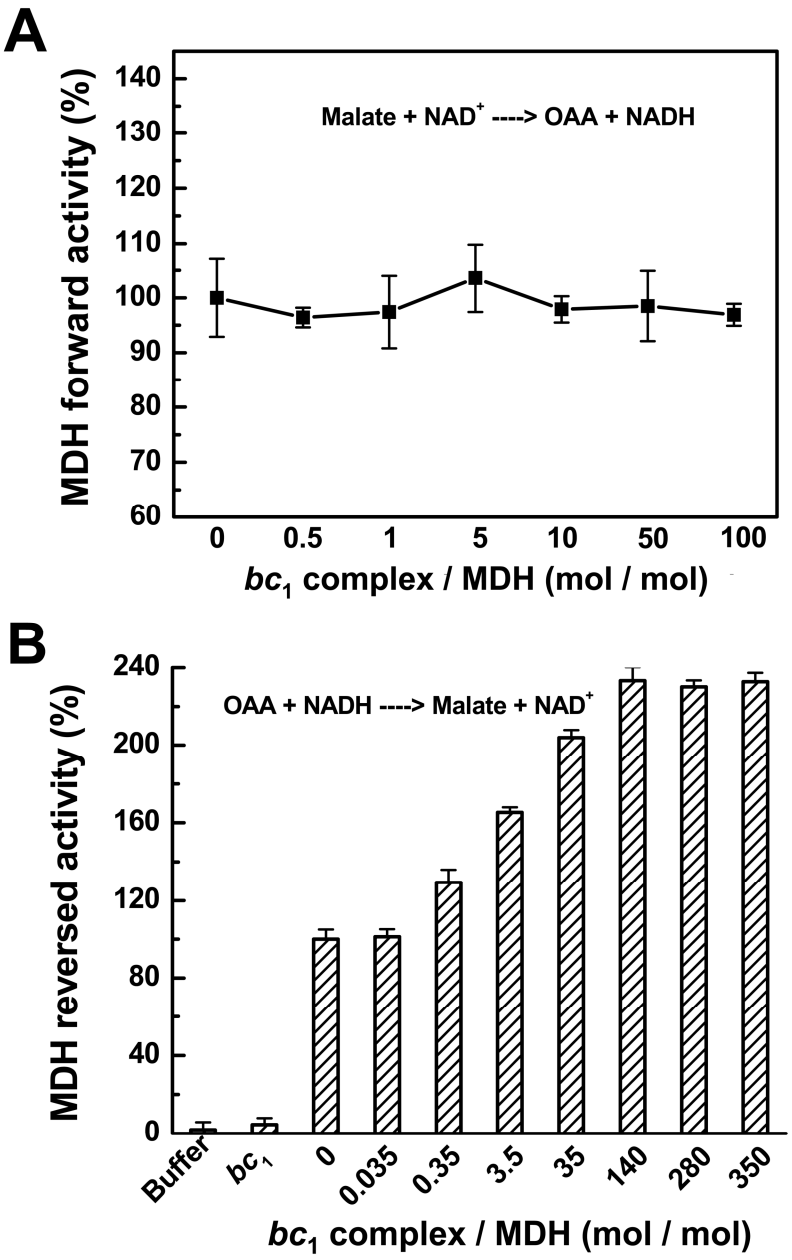

Fig. 2-5. Effect of bc_1 complex on the activity of forward reaction (from malate to oxalacetate) and reversed reaction (from oxalacetate to malate) of MDH. Varying amounts of bc_1 complexes were incubated on ice for 20 minutes with a given amount of MDH to give indicated molar ratios. The mixtures were then subjected to assay for MDH forward (***A***) and reversed (***B***) activities. The activity of MDH in the absence of bc_1 was used as 100%. N=3, means ± SE.

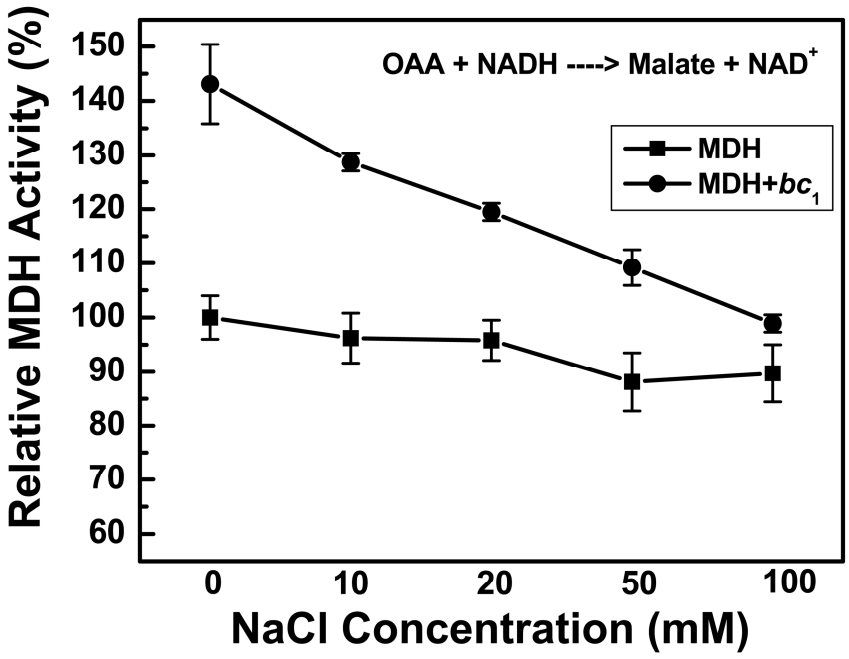

Fig. 2-6. Effect of ionic strength on reverse MDH activity enhancement by bc_1. MDH only or together with bc_1 complex in 100 mM Na^+/K^+ phosphate buffer, pH 7.5, was incubated with indicated concentrations of NaCl on ice for 30 minutes before MDH activity (the reverse reaction) was determined. The activity of MDH only was used as 100%. N=4, means ± SD.

Table 2-2. Effect of MDH activity upon interaction with intact and the inhibitor treated bc_1 complex.

Treatments [a]	Activity (mmol /min/mg)	Enhancement (%) [b]
MDH + Buffer [c]	1.77 ± 0.06	0 ± 3
MDH + Inhibitor [d]	1.75 ± 0.07	-1 ± 4
MDH + bc_1	2.46 ± 0.11	39 ± 6
MDH + bc_1 + QH_2 + cyt *c*	2.49 ± 0.13	41 ± 7
MDH + (bc_1 + Stigmatelin)	2.05 ± 0.08	16 ± 5
MDH + (bc_1 + Antimycin A)	2.12 ± 0.06	20 ± 3
MDH + (bc_1 + Zinc ion)	1.84 ± 0.14	4 ± 8

[a], Stigmatelin-, antimycin A- or zinc ion ($ZnCl_2$)-treated bc_1 complex was incubated with MDH and assayed for MDH activity.

[b] Enhancement of MDH activity at the reversed reaction. N=3, means ± SE.

[c & d] The bc_1 dilute buffer or inhibitors themselves was added as negative controls.

2.4.7 Cytochrome bc_1 complex changes the enzyme kinetics of MDH.

It was interesting to see whether the kinetics of MDH under this unidirectional enhancement changed or not. The K_m for NADH and the V_{max} for the reverse direction of MDH were determined by Lineweaver-Burk plots. The K_m for NADH decreased from 22.2±0.5 μM to 15.4 ± 0.5 μM and the V_{max} increased from 1.6± 0.2 mmol/min/mg to 2.5±0.2 mmol/min/mg when bc_1 complex was added to MDH (Table 2-3 and Fig. 2-7). Also the K_m of OAA decreased from 10.7±0.7 μM to 8.9±0.9 μM and the V_{max} increased from 2.2±0.1 mmol /min/mg to 3.5±0.1 mmol /min/mg (Table 2-3 and Fig. 2-8). A similar kinetics analysis was also carried out in the forward direction, but no significant difference was detected. Therefore, the binding of bc_1 complex might induce some conformational change of MDH, and thus lower its K_m for NADH and OAA, and meanwhile increase the V_{max}, which enhances the activity of the enzyme.

Table 2-3. Kinetics analysis of the MDH in the present of bc_1 complex.

Enzyme	NADH		OAA	
	K_m (μM)	V_{max} (mmol/min/mg)	K_m (μM)	V_{max} (mmol/min/mg)
MDH	22.2±0.5	1.6±0.2	10.7±0.7	2.2±0.1
MDH + bc_1	15.4±0.5	2.5±0.2	8.9±0.9	3.5±0.1

0.1mM, 0.05mM, 0.025mM, 0.016mM, 0.0125mM of NADH and 0.2 mM OAA were used in the assay for analyzing the K_m of NADH. 0.1mM, 0.025mM, 0.0125mM, 0.00625mM of OAA and 0.2 mM NADH were used in the assay for analyzing the K_m of OAA. MDH only or together with bc_1 complex was used in both of the assays and V_{max} was also calculated via this Lineweaver–Burk plot.

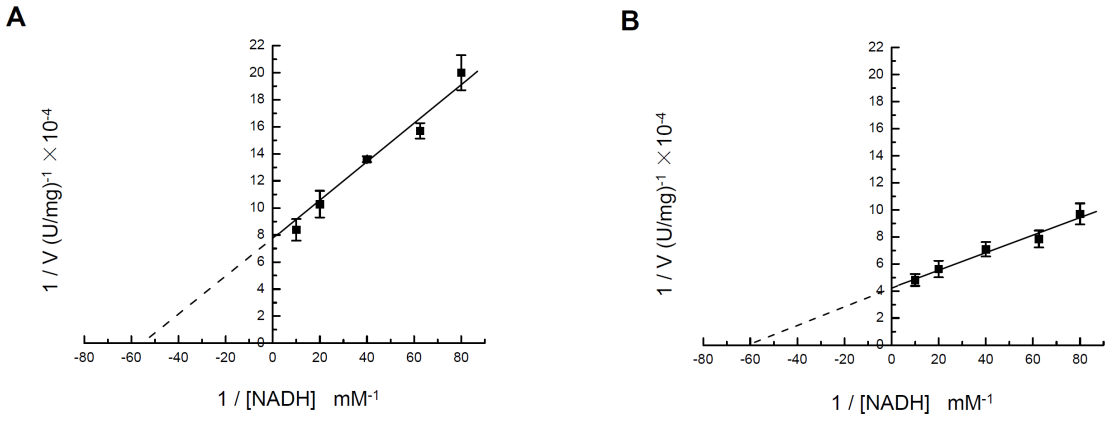

Fig. 2-7. Lineweaver Burk plots for the MDH reverse directional activity by the change of NADH. 0.1mM, 0.05mM, 0.025mM, 0.016mM, 0.0125mM of NADH and 0.2 mM OAA were used in the assay for analyzing the K_m of NADH. All values (presented in Table III of main text) are averages from three independent experiments.

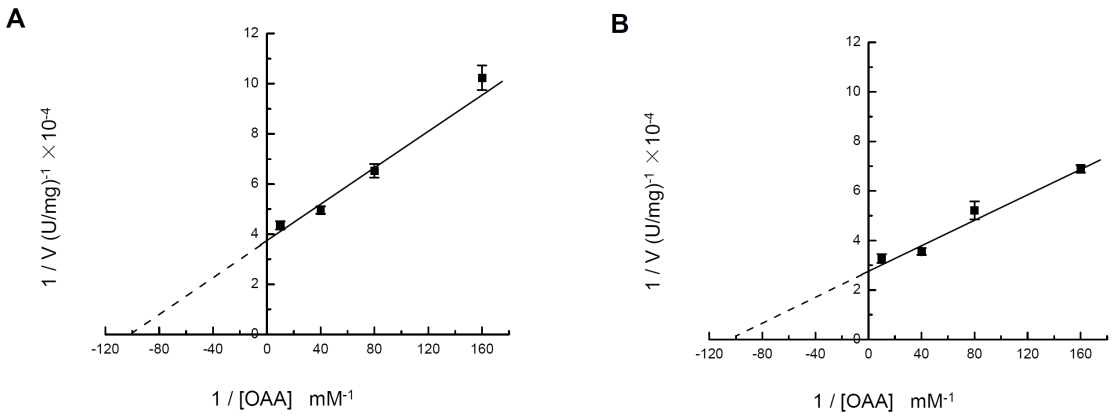

Fig.2-8. Lineweaver Burk plots for the MDH reverse directional activity by the change of OAA. 0.1mM, 0.025mM, 0.0125mM, 0.00625mM of OAA and 0.2 mM NADH were used in the assay for analyzing the K_m of OAA. All values (presented in Table III of main text) are averages from three independent experiments.

2.4.8 The crosstalk may regulate the mitochondrial bioenergetics.

The cell regulates the ATP generation in mitochondria depending on the energy requirements of different situations. When the cell needs less ATP, mitochondria will slow down the citric acid cycle and thus slow down the ATP generation via the decrease of electron transport and ATP synthase activity. Based on the present work, we hypothesize that when the cell needs to slow down the ATP generation, it triggers the crosstalk between MDH and the bc_1 complex (See Fig. 2-9). Once the bc_1 complex binds to MDH, it will enhance the reversed activity of MDH and thus decrease the OAA concentration in the matrix. Since OAA is the crucial member of the citric acid cycle, a decrease in its concentration will result in lowering the concentrations of NADH and $FADH_2$, which are substrates of Complex I and II in the electron transport chain, respectively. The decrease in the electron transfer activity resulting from the limited input of substrates will yield a negative regulation of ATP generation. At this moment we offer no good explanation on why the interaction between the bc_1 complex and MDH enhances the bc_1 activity, but to speculate that the regulation of cellular energy metabolism may depend on the interplay between the electron transport chain and citric acid cycle activities.

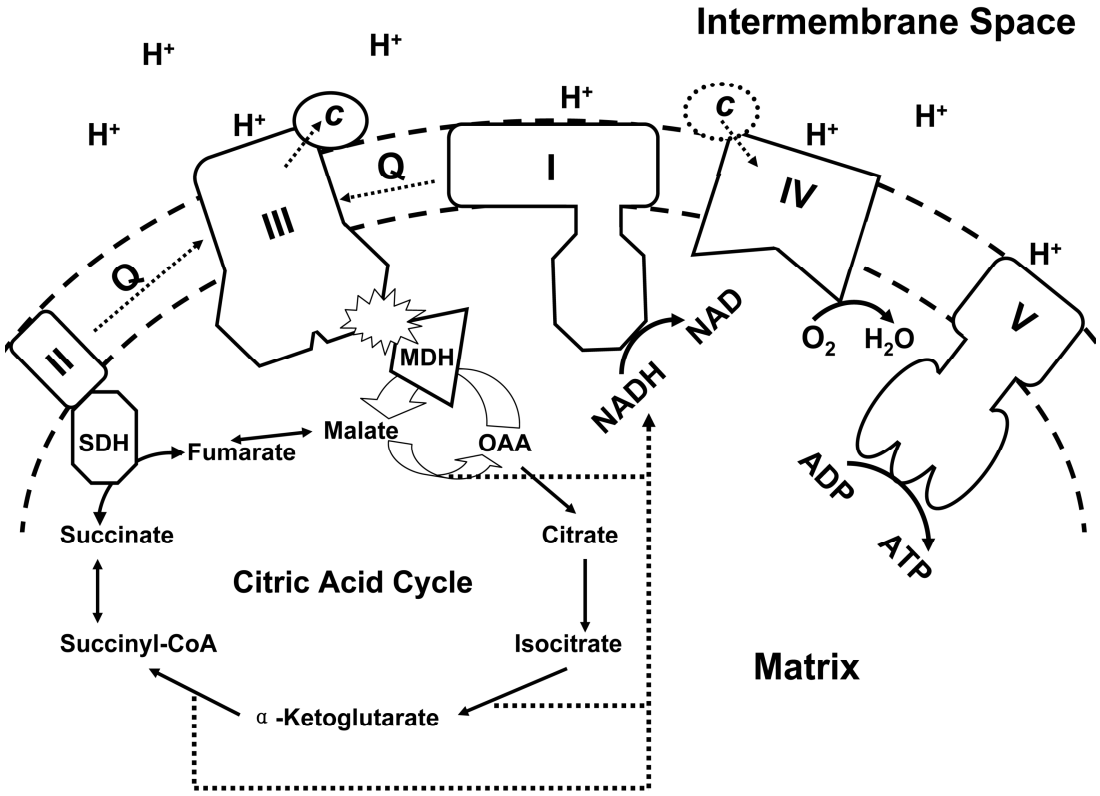

Fig. 2-9. The schematic illustration for the crosstalk between MDH and bc_1 Complex. Solid arrows show the reactions in the matrix. Dashed arrow in the membrane stands for the electron transport. Dashed arrow in the matrix stands for the NADH generation form the citric acid cycle. Dashed circled cyt *c* shows its translocation from Complex III to Complex IV.

The crosstalk could also be involved in the gluconeogenesis or in helping the correction of mitochondrial dysfunction during dypoxia/ reoxygenation(30). In the gluconeogenesis, the substrate OAA can not get out of mitochondria directly by itself. It has to be converted into malate first via the reversal of the citric acid cycle catalyzed by MDH. Once the malate is transported into the cytosol, it is converted to OAA and yields NADH by cytosolic MDH.

What is still yet to be resolved is the molecular mechanism associated with this crosstalk between the bc_1 Complex and MDH. Since it apparently plays an important role in controlling ATP generation, it is likely that some factors or signals are responsible for the regulation of the enhancing or breaking up the interaction. Work of determining the molecular mechanism of this crosstalk is undergoing in our laboratory.

2.4.9 Discussion about our new precipitation pull-down technique

Most of the proteins need to bind with other proteins and affect each other's biological functions. Therefore, the methods of studying protein-protein interaction are very important to most of the biological research field. Here we will discuss some popular assays and compare them with our new precipitation pull-down technique.

Coimmunoprecipitation (Co-IP), a traditional method for detection of protein-protein interactions, has been employed in many experiments. In the Co-IP experiment, after antibody is added to the cell lysates, the antigen is precipitated with bound proteins which can be analyzed. Purified protein or synthetic peptide coupled to the carrier can be used as antigen for antibody production. Alternatively, an epitope tag, the antibodies of

which are commercially available, can be fused to the protein; or a protein tag which is able to bind to the beads can also be used in the Co-IP experiment. (*19*)

There are several advantages for coimmunoprecipitation using crude lysates. First, a built-in specificity control exists in the experiment due to the presence of all the competing proteins in the crude lystate. Second, the proteins exist in the same proportional concentrations as in the cell. Third, the protein complexes are in their natural state, which might not be easy to assemble in vitro. Fourth, the proteins have natural posttranslational modifications which might be required for the protein interaction. There are also some apparent disadvantages. First, the coimmunoprecipitating proteins might not have direct interactions with the antigen. Second, monoclonal antibodies are needed in this experiment. If the proteins you study do not have these monoclonal antibodies, it would be a difficult way to get the antibodies first. Third, Co-IP is not very sensitive, when compared with some other methods, like protein chromatography.

It may be a good idea to use anti-bc_1 antibody to pull down proteins from the matrix extraction. However, this method still has other disadvantages for the possible interacting proteins. First, the antibody could just bind to the interaction sites where the possible proteins need to bind or in other words, the antibody can compete with other proteins to bind with bc_1 complex, thus we may obtain some false negative results. Second, even if the antibody does not bind to the exact binding sites, its huge molecular weight can still cause big problems for other proteins to interact with bc_1 complex, especially when such polyclonal antibodies generated from rabbit are used, which may bind and cover most parts of the surface of bc_1 complex.

Two-Hybrid System (*20-22*) uses transcriptional activity to assay protein-protein interaction. It is based on the nature of some site-specific transcriptional activators, consisting of a transcriptional activation domain and a DNA-binding domain (*23-25*). The DNA binding domain is used to bind specific genes, and the activation domain activate transcription by collecting other necessary proteins of the transcriptional machinery. The two domains do not need to be linked covalently and can come together by protein-protein interaction. Two hybrids are constructed for the application of the method. The two domains are fused to proteins X and Y individually. If the two proteins have interaction with each other, they will be brought together to initiate the transcription of the reporter gene. If the genes of the candidate proteins are available, we can use yeast two-hybrid to detect their interaction (*26, 27*).

There are some limitations for yeast two-hybrid screens. First, it can only study soluble proteins and is not feasible to study integral membrane proteins' interaction. Second, the fusion proteins must be able to enter the nucleus because they need to work as a whole transcription activator. Third, by constructing fusion proteins, the site essential for protein-protein interaction might be occluded. Fourth, if the post-translational modification necessary for the interaction does not happen in yeast cells, the protein-protein interaction can not be detected.

For Yeast two-hybrid method, it is good to be used to study interaction between small proteins, at least soluble proteins, because its mechanism is based on the fusion proteins which can still work as a whole transcriptional factor within the nucleus. However for our bc_1 complex, which is a huge membrane protein with MW around 480k, it is impossible to use such yeast two hybrid method.

Pull down assays-"Pull-down" is a technique which employed some other affinity system other than functional antibody and it is similar to immunoprecipitation (IP). Here, glutathione agarose beads or metal chelate agarose beads are used to capture GST-tagged protein or His-tagged protein. The fusion-tagged protein functions as the "bait" to acquire its binding partner ("prey"). In a typical pull-down assay, the bait protein is added to a cell lysate; after extensive washing, the "preys" are selectively eluted which can be analyzed in-gel or by Western blot. His-Tag or GST-Tag pull down assay can be used in many protein-protein interaction studies. However, our bc_1 complex is purified from bovine mitochondria and has no tag on it. Therefore, we can not use this normal pull down assay.

Precipitation pull-down technique- Cytochrome bc_1 complex is a membrane protein and it can only be soluble when there is detergent in the solution. If it lacks the detergent, bc_1 complex will aggregate together and become precipitate in the solution. When bc_1 complex become precipitate, they use their trans-membrane parts to bind each other and then aggregate together. In this case, the matrix parts are exposed outside to face the solution just like the detergent's hydrophilic part. However, we actually just want to study the interaction between this matrix part and other soluble proteins in the matrix. Therefore, the precipitate itself does not affect the interaction between the bc_1 matrix part and other proteins. This method is good for those membrane proteins which contain a large portion other than its trans-membrane part and it has a potential to bind with other soluble proteins.

However this method may also contain some insufficient points like all the other interaction assays. Non-specific binding is a common issue for all these assays. For our

assay, when the membrane protein forms precipitate, some soluble proteins may also be non-specifically pulled down by this big precipitating complex. Thus, further confirmation need to be done to make sure that this binding is specific or non-specific. We have at least two approaches to check if the interaction is specific or not. First, the soluble proteins and bc_1 complex recovered in the precipitates by the dialysis/ precipitation method can be re-solubilized and their activities can be determined. And this can also show us whether the interacting proteins can affect each others' activity. Therefore, we can know if this interaction is functional or just non specific binding. Second, for non specific binding, it is unsaturable. The possibility of non-specific binding can be excluded if bc_1 can only pull down certain amount of interacting proteins even with excessive amount of matrix extraction solution in our pull down assay.

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CHAPTER III

ACONITASE SCAVENGES SUPEROXIDE GENERATED FROM THE CYTOCHROME BC1 COMPLEX

Qiyu Wang, Ting Su, Mingquan Zhang, Linda Yu and Chang-An Yu

Manuscript

3.1 Abstract

Another matrix protein, pulled down by the bc_1 complex using the dialysis pull-down method, was identified by MALDI-TOF Mass spectrometry as mitochondrial aconitase. Using the crosslinking technique, subunits II (core II) of the bc_1 complex was identified as the interacting site for aconitase. When purified aconitase was incubated with the detergent dispersed bc_1 complex, a slight increase in aconitase activity, but no change in bc_1 activity was observed. Upon the binding of aconitase, the superoxide generating activity of bc_1 complex is greatly decreased. However, when aconitase was added to the bacterial bc_1 complex, little effect on its superoxide generating activity was observed. The lack of core subunits I and II in the bacterial bc_1 complex may be the reason for this difference. The interaction between bc_1 complex and aconitase can be enhanced by calcium, which is considered as an active factor for mitochondrial bioenergetics process. These results suggest that the interaction between mitochondrial bc_1 complex and aconitase might play an important role in superoxide scavenging when mitochondria is in a high energy producing state, which is crucial for mitochondria and the whole organism.

3.2 Introduction

With the dialysis pull-down technique (*1*), we found that soluble proteins from the mitochondrial matrix interacting with detergent dispersed bc_1 complex can co-precipitate with bc_1 complex upon removal of detergent by dialysis followed by centrifugation. Several matrix proteins were pulled down in the precipitates. One of the proteins was identified as MDH (*1*). Another protein pulled-down was identified as aconitase, by

MALDI-TOF Mass spectrometry. Aconitase (EC 4.2.1.3) catalyzes the reversible conversion between citrate and isocitrate via cis-aconitate as the intermediate. Although the catalytic function of aconitase involved non-redox- active process, it contains a covalently bound [4Fe-4S] iron-sulfur cluster. The iron sulfur cluster is required for both the catalytic and the superoxide scavenging activities (*2*).

Herein we report the identification of aconitase as one of the matrix enzymes that interacts with bc_1 complex, the interaction between aconiatse and the bc_1 complex, and the effect of bc_1 complex on the superoxide scavenging activity of the aconitase.

3.3 Experimental procedures

Chemicals-- Cytochrome *c* (horse, type III), sodium cholate, sodium citrate, isocitrate dehydrogenase, $NADP^+$, and porcine aconitase were purchased from Sigma. Cross linker, Sulfo-SBED (Sulfosuccinimidyl-2- [6-(biotinamido)-2-(p-azidobenzamido) hexa- noamido] ethyl-1,3′- dithiopropionate) was purchased from Pierce. 2,3-Dimethoxy-5-methyl-6-(10-bromodecyl)-1,4-benzoquinol ($Q_0C_{10}BrH_2$) was prepared as previously reported (*3*).

Cytochrome bc_1 complex preparation and activity assay— Preparation of bovine heart mitochondrial cytochrome bc_1 complex, the concentration determination of cytochromes *b* and c_1, and the activity assay for mitochondrial bc_1 complex were the same as in chapter II.

The his_6-tagged, wild type *R. sphaeroides* cytochrome bc_1 complex was prepared as previously reported (*4*). A condition similar to that used for the mithochondrial complex was used for the assay of the bacterial complex, except that the bacterial complex was diluted with 50 mM Tris-HCl, pH 8.0, containing 200 mM NaCl and 0.01% DM to a final concentration of cytochrome *b* of 1 μM.

Superoxide detection –Superoxide production by the bc_1 complex in the presence and absence of aconitase was detected by measuring the chemiluminescence of MCLA-$O_2^{\cdot-}$ adduct (*5*). An Applied Photophysics stopped-flow reaction analyzer SX.18MV-R (Leatherhead, UK) was used for the assay by leaving the excitation light off and registering light emission (*6*). Reactions were carried out at 23 °C by mixing 1:1 of solutions A and B. Solution A contains 100 mM Na^+/K^+ phosphate buffer, pH 7.4, 1 mM EDTA, 1 mM NaN_3, 0.1% bovine serum albumin, 0.01% DM, and 5.0 μM cytochrome bc_1 with or without aconitase, Solution B contains 125 μM $Q_0C_{10}BrH_2$ and 4 μM MCLA in the same buffer. The produced fluorescence was consecutively monitored for 2s once the reaction started. One volt equals the chemiluminescence (maximum peak height of light intensity) produced by 0.5 unit of xanthine oxidase which uses 100 μM hypoxanthine as a substrate in the Applied Photophysics stopped-flow reaction analyzer SX.18MV-R.

Aconitase Activity assay-- The activity of aconitase was determined as described in (*7*). Briefly, aconitase alone or together with bc_1 complex were added in the reaction buffer consisting of 50 mM Tris-HCl (pH7.4), 5 mM cis-aconitate, 0.6mM $MnCl_2$, 0.2mM $NADP^+$, and 1 unit isocitrate dehydrogenase. The reaction was followed by the

reduction of $NADP^+$ (the increment of the absorption at 340nm) in a Shimadzu UV-2401PC spectrophotometer at 25 °C, using an extinction coefficient of 6.2 $mM^{-1}cm^{-1}$.

Biotin-labled Aconitase Pull down-- 150ul aconitase solution (33 mg/ml) in phosphate buffer, was incubated with 3 mg NSH-biotin-linker, and the sample was incubated on ice for 2 hour. Then it was dialysised against 1L matrix protein buffer (*1*) to remove the extra soluble biotin-linker. Centrifuge at 100,000 ×g for 40 min to remove any denatured protein. Final protein was stored in -80℃ for later use. Twenty µL of Biotin- aconitase and 7 µL bc_1 complex (150 µM cyt b) were incubated in 500 µL MP buffer for 1 hr on ice. Then the mixture was dialyzed against 1L MP buffer for 3 hour to remove the detergent. After dialysis, the mixture was centrifuged down at 100,000 ×g for 40 min. And make sure that the supernatant was completely removed. Re-desolve the precipitate with phosphate buffer containing 1% KDOC for the following experiments.

Mass Spectroscopy, Cross linkingand and *Western blotting* are the same methods described in chapter II.

3.4 Results and discussion

3.4.1 Identification of mitochondrial aconitase as an interacting protein with the bc_1 complex.

Aliquots of a given amount of the detergent dispersed bc_1 complex were incubated with various amounts of the matrix extract at 4 ˚C for 2 hours. The mixtures were then dialyzed against the matrix protein buffer for 4 hours with 3 changes of buffer to remove the detergent, and then centrifuged at 100,000 ×g for 1 hour to recover the precipitates of the bc_1 complex. Fig. 3-1A shows the SDS-PAGE of the recovered precipitates of bc_1 complex under the UV light. In addition to uv bands shown by the cytochrome bc_1 complex, a protein band was observed and its intensity increased as the matrix proteins used in the incubation increased. This protein band was cut out from the gel, subjected to in-gel digestion with trypsin, and analyzed by MALDI-TOF mass spectrometry. This protein was identified as bovine mitochondria aconitase based on PMF (Peptide Mass Fingerprinting) analysis (Fig. 3-1B).

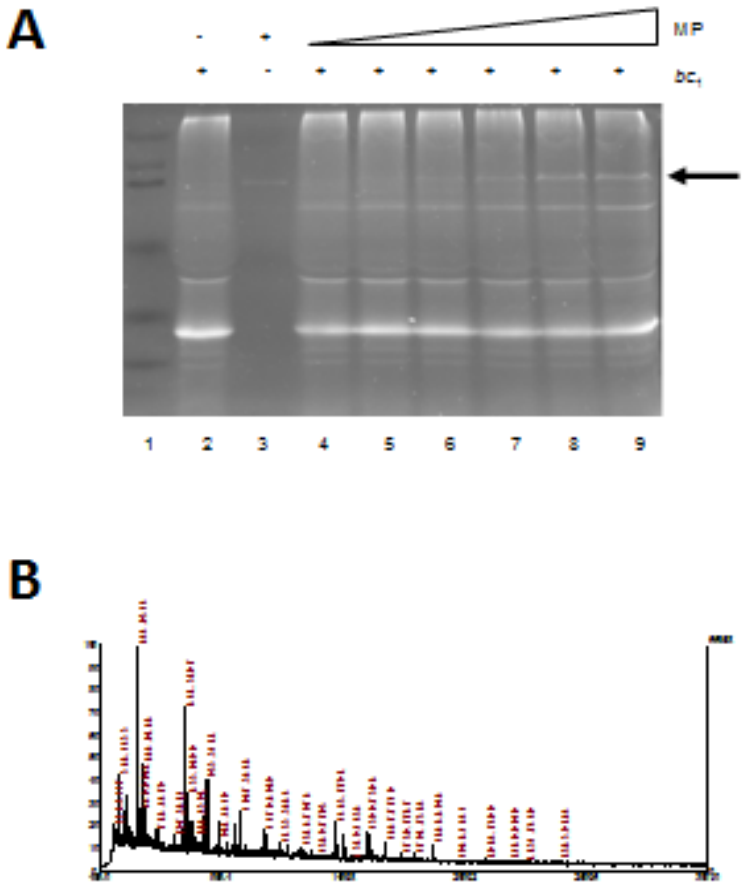

Fig. 3-1 Identification of Aconitase as an interacting protein with the bc_1 complex. ***A.*** SDS PAGE analysis of bc_1 pulled-down proteins from matrix extract under the UV light. Lane 1, standard proteins; lane 2~3, bc_1 complex and MP only as controls to show the precipitates after centrifugation; lane 4~9, bc_1 complex together with the pulled-down proteins from matrix extract under the UV light. Solid arrow shows the main matrix protein being pulled down by the bc_1 complex. ***B.*** MS spectra of protein sliced from SDS-PAGE gel. Spectrum data were collected from a MALDI-TOF Mass spectrometer. It consisted of a series of multiple charged ions from each fragment on a mass-to-charge (m/z) ratio scale. 100% intensity represents the highest peak.

To further confirm the interaction between aconitase and bc_1 complex, purified bovine aconitase is need for the interaction study. However, expression of bovine aconitase in BL21 system did not work due to its huge molecular weight (80k). And there is no commercial bovine aconitase available. But the porcine aconitase shares 98% sequence identity with the bovine aconitase and is available from Sigma-Aldrich. Thus, purified porcine aconitase was used in the pull-down experiment. However, this commercial aconitase is only about 60% purity and the co-precipitation was very weak. Later, we changed it to another commercial aconitase from Japan. We detected the pulled-down aconitase by bc_1 complex through western blotting. Since the anti-aconitase antibody did not work well in the western blotting, a biotin-labeled aconitase was used instead (Fig. 3-2, upper).

The effect of ionic strength and calcium iron on the amount of aconitase pulled down by bc_1 complex was studied. Fig, 3-2 shows the differences of the amount of aconitase pulled-down by bc_1 complex under various conditions. When the ionic strength increased, especially when calcium ion is present, a tighter interaction between aconitase and bc_1 complex was observed indicating that the interaction between aconitase and bc_1 is hydrophobic. And calcium can enhance the binding between them.

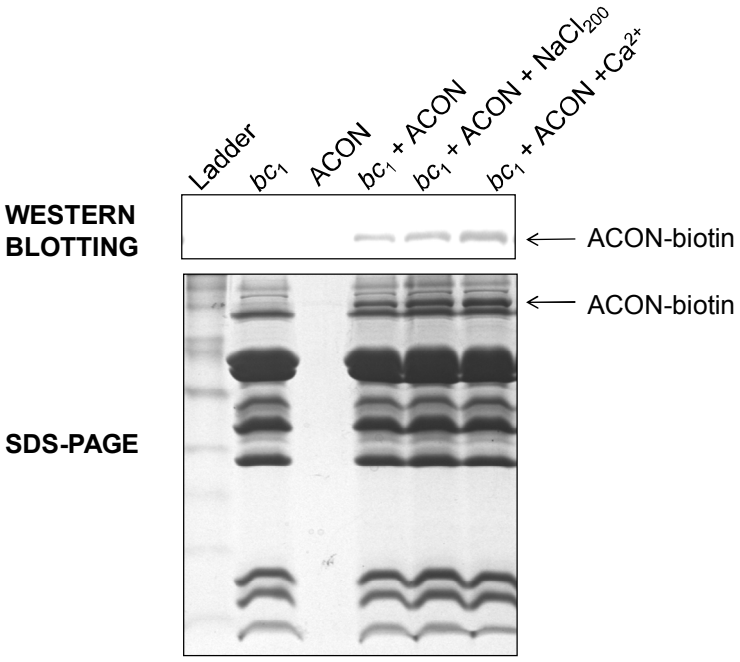

Fig.3-2 SDS-PAGE and Western blotting of biotin-labeled aconitase pulled down by bc_1 complex in high salt and calcium environment. With the method described in the material and method part, we incubated and dialyzed bc_1 complex together with biotin-labeled aconitase with vary solutions (normal MP solution, MP solution containing 200 mM NaCl or MP solution containing 10 mM Calcium). Bottom figure shows the SDS-PAGE result of the precipitates obtained from the pull-down assay. The figure on the top shows the corresponding western blotting result of biotin-labeled aconitase pulled-down by bc_1 complex. The lanes of bc_1 complex and acon show the control by themselves.

The effect of redox state of bc_1 complex on the interaction between aconitase and bc_1 complex was studied as Fig. 3-3. Since bc_1 complex has different redox states, it is of interest to see whether the redox state affects the interaction or not. The purified bc_1 complex from bovine heart mitochondria is about 10% reduced. To obtain the fully reduced form, bc_1 complex was treated with succinate and complex II on ice over night. Instead, cyt *c* and cyt *c* oxidase were used for oxidizing bc_1 complex, However, no matter the fully reduced or the fully oxidized bc_1 complex was used for the assay, the amount of aconitase pulled down was comparable to that pulled down by the untreated bc_1 complex. The results indicate that the interaction does not depend on the redox state of the cytochrome bc_1 complex.

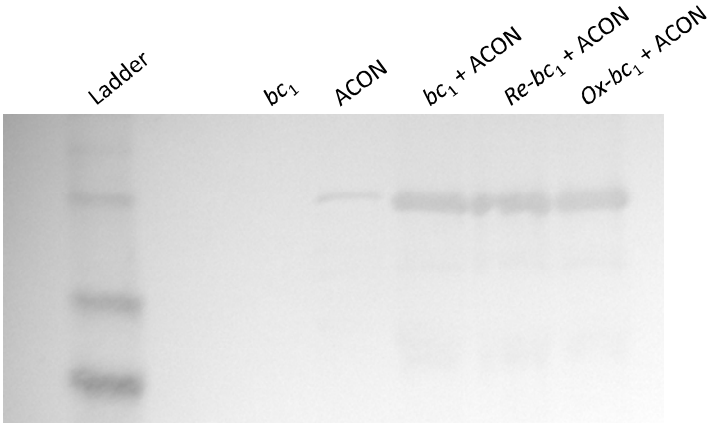

Fig. 3-3 Western blotting of biotin-labeled aconitase pulled down by reduced and oxidized bc_1 complex. bc_1 stands for 10% reduced form of purified bc_1 complex; Re- bc_1 stands for bc_1 complex reduced by succinate and complex II; Ox- bc_1 stands for bc_1 complex oxidized by cytochrome *c* and complex IV. Lanes marked as bc_1 and ACON are the two controls of those two proteins themselves.

3.4.2 Identification of the subunits in bc_1 complex that interact with aconitase.

To identify the subunit(s) in the bc_1 complex that interacts with aconitase, a biotin labeled transfer cross-linker, Sulfo-SBED from Pierce, was used (*1*). Fig. 3-4A shows the SDS-PAGE and the Western blotting analysis of Sulfo-SBED labeled aconitase illuminated by UV in the presence or absence of the bc_1 complex. The UV activated samples were treated with β-mercaptoethanol before subjected to SDS-PAGE. The horseradish peroxide conjugated streptavidin was used to probe proteins having a transferred biotin tag. Three protein bands, with apparent molecular masses of 70-, 54- and 51KD were shown to contain the biotin tag in the western blotting. The 70KD band is aconitase and 51KD band is core II of the bc_1 complex. The 54 KD band is from the aconitase preparation albeit its similar molecular mass to the core I (53KD) of the bc_1 complex. The identification of subunit II in the bc_1 complex as the interaction partner for aconitase is in line with the 3-D structure of the bc_1 complex (shown as Fig.3-4B). Subunits I and II account for a major portion of the matrix part of the bc_1 complex. Since the mitochondrial aconitase is an enzyme of the citric acid cycle and locates in the matrix, interaction of aconitase with the bc_1 complex through subunits II in the matrix region is expected.

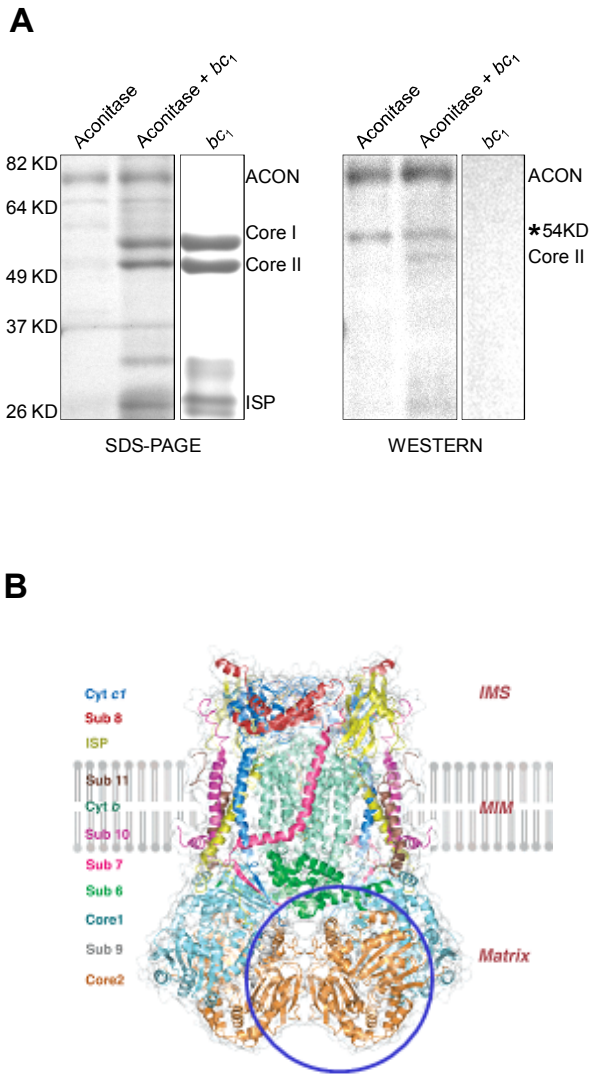

Fig. 3-4 SDS-PAGE and Western blotting of sulfo-SBED cross linked Aconitase and bc_1. ***A.*** Ten µg of aconitase was incubated with sulfo-SBED for 120 minutes on ice in the dark followed with desalting. Labeled aconitase was then incubated with 30 µg bc_1 complex for 60 minutes and the mixture was exposed to ultraviolet light (365 nm) at a distance of 5 cm for 15 minutes. The photolyzed samples were treated with ß-ME and subjected to SDS-PAGE and Western blotting. Aconitase with sulfo-SBED was the control. The extra band in the western blot is the subunit of bc_1 complex which got the transferred biotin from Aconitase. The blots were probed with streptavidin-horseradish peroxidase conjugate. ***B.*** The interaction partner for aconitase was shown in the 3-D structure of the bc_1 complex.

3.4.3 Effect on the activities of the cytochrome bc_1 complex and aconitase upon interaction.

In the previous chapter, we reported that bc_1 complex and Malate dehydrogenase have crosstalk, which means they can affect each other's activity (*1*). It is interesting to see whether such crosstalk between bc_1 complex and aconitase exists. Fully reduced bc_1 complex was used for the assay to prevent the reduction of cyt c_1 and mimic the physiologic condition in which aconitase is not accessible to the intermembrane space part of bc_1 complex. Fig. 3-5 shows that in the presence of fully reduced bc_1 complex, the activity of aconitase was increased about 20%. However, no effect was found on the electron transfer activity of bc_1 complex by interacting with aconitase. To make sure there is no effect on the very early time of the reaction, stop-flow was used to check the presteady state reduction of cytochrome *b* and cytochrome c_1 (shown as Fig.3-6). The rates of the reduction of cytochorme *b* and c_1 are similar regardless aconitase is present or not. These results indicate that binding of aconitase to bc_1 complex has no effect on its electron transfer acitivity.

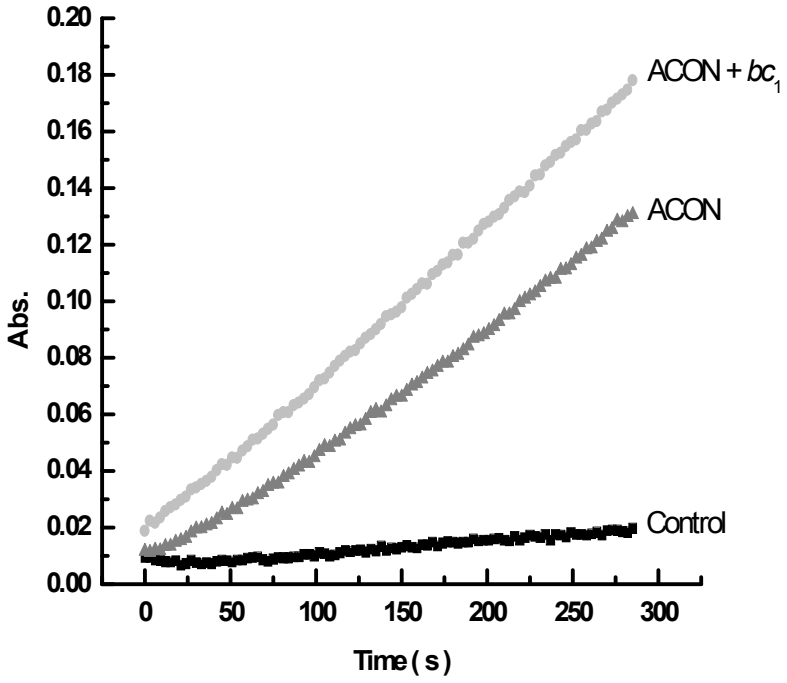

Fig. 3-5 Effect of bc_1 complex on the activity of aconitase. According to the method described in the material and method part, aconitase only or aconitase together with bc_1 complex were added separately in the aconitase activity assay mixtures. Data was obtained from the reduction of NADP at 340nm. The sample of substrates with bc_1 complex in the absence of aconitase was used as control.

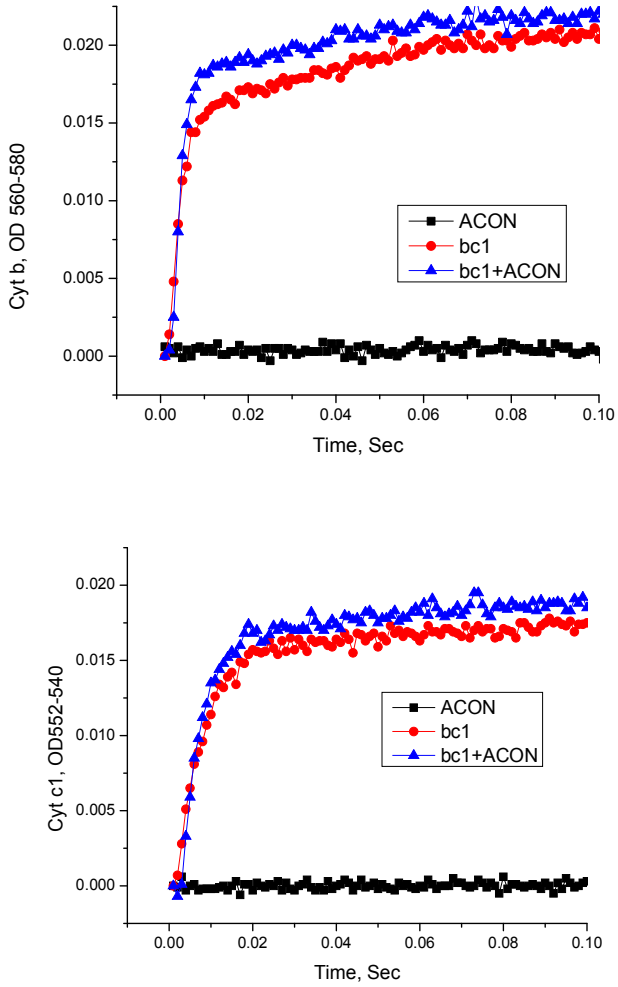

Fig.3-6 Time courses of cytochrome *b* and c_1's reduction by $Q_0C_{10}BrH_2$. *A.* Cytochrome *b*'s reduction by $Q_0C_{10}BrH_2$ when aconitase was present or not. ***B.*** Cytochrome c_1's reduction by $Q_0C_{10}BrH_2$ when aconitase was present or not. Aconitase itself was used as control in both figures.

3.4.4 Aconitase scavenges superoxide generated by bc_1 complex.

The cytochrome bc_1 complex is one of the two major sites that generate superoxide during the process of electron transfer in the mitochondrial electron transport chain (*8*). Aconitase is known to have superoxide scavenge activity (*9*). It is interesting to see whether aconitase in complex with bc_1 complex can prevent or decrease the superoxide generation from the latter or it can scavenge superoxide after release. Fig.3-7A shows the effect of aconitase on generation of superoxide by cytochrome bc_1 complex. The amount of superoxide generated by a given amount of bc_1 complex decreased as the amount of aconitase used increased. However, when the same assay was carried out with bacterial bc_1 complex and aconitase, only very little effect on the superoxide generation was observed (Fig. 3-7B). Fig. 3-7C shows the calculated relationship between the protein ratio and superoxide generation. In contrast to bacterial bc_1 complex, the interaction between mitochondrial bc_1 complex and aconitase is clearly specific. This is also indirectly confirmed by the observation that aconitase interacts with bc_1 complex through its subunit II, which is lacking in the bacterial bc_1 complex.

The titration of bc_1 complex with aconitase showed the maximal effect of aconitase on the generation of superoxide by bc_1 complex reached around the ratio 2.4 between the two enzymes. This indicates that two moles of aconitase might bind per mole of bc_1 complex assuming some inactive aconitase is present in the commercial preparation. Since each bc_1 complex contains two monomers and aconitase works only as a monomer, each subunit II of bc_1 complex may interact with one aconitase.

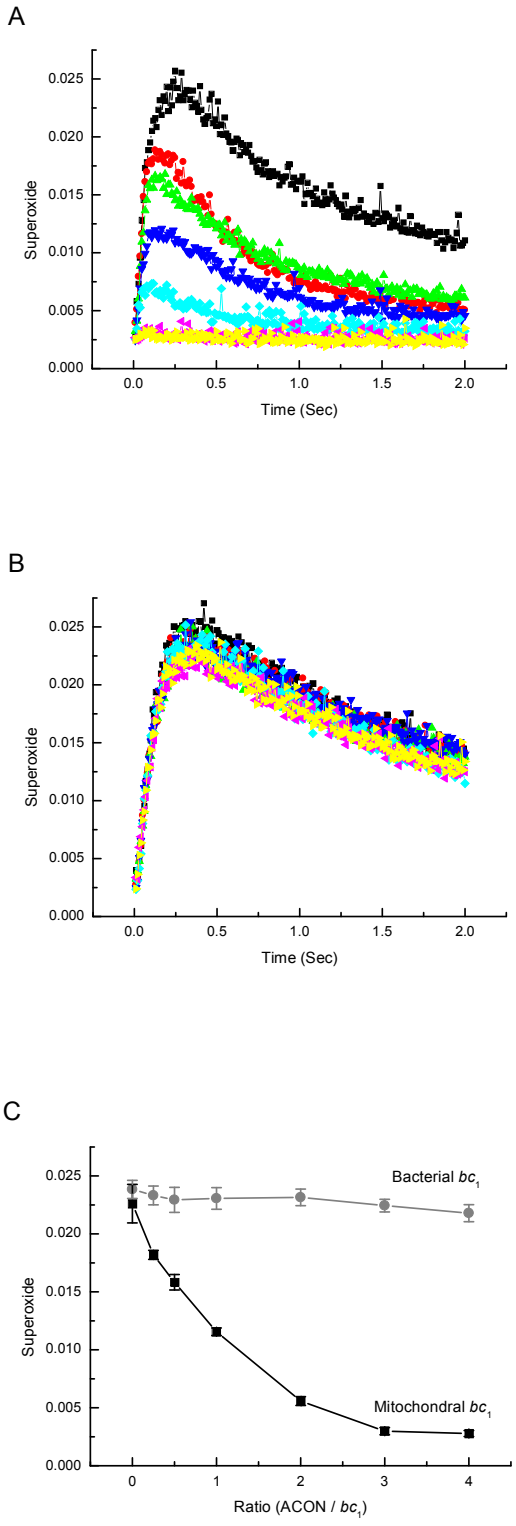

Fig. 3-7 Effect of aconitase on the superoxide generation by the mitochondrial (A and ■ in C) or bacterial (B and ● in C) bc_1 complex. Various amounts of aconitase were

incubated with a given amount of mitochondrial bc_1 complex on ice for 10 minutes. The mixtures were then subjected to the assay for superoxide generation in Stop-Flow. The same ratio between aconitase and bacterial bc_1 complex was repeated in the same assay. The relationship between the ratio of these two proteins and the superoxide generation was shown together in C.

3.4.5 The interaction may protect the mitochondria from the damage of superoxide.

In most organisms, there are a number of toxicities mediated by "oxidative stress." In the oxidative stress, the reactive oxygen species (ROS) may interact with cellular constituents such as DNA/RNA, proteins, carbohydrates, and unsaturated lipids, and causes an irreversible damage to these components and thus induces aging and apoptosis (*10, 11*). Cumulative oxidative damage has been involved in many disease states, e.g. the cancer, inflammation, ischemia-reperfusion injury and so on. (*12-16*). The ROS, which include reactive radicals (hydroxyl or superoxide) and reactive non-radical species (singlet oxygen, peroxynitrite, and peroxide), can inactivate the iron-sulfur clusters of complexes I and III in the mitochondria electron transport chain leading to damage of the mitochondrial respiration (*17*). Mitochondria have long been believed to be the primary site of intracellular ROS generation (*18, 19*). About 1-2% of oxygen consumption doesn't participate in oxidative phosphorylation, and this part is involved in the formation of the superoxide anion (*20*). Complex I and complex III are considered as the main sites for the superoxide production (*21*). In complex I, the electron donors for superoxide production are the flavin radical and the ubiquinone radical (*22*). In bc_1 complex, the ubisemiquinone radical at the QP site (*23*) and the reduced heme b_{566} (or b_L) (*24*) were considered to be responsible for electron leakage which induce superoxide production

during bc_1 complex catalysis. More recently a mechanism which shows direct participation of molecular oxygen in the bifurcated oxidation of ubiquinol was suggested for the superoxide generation in bc_1 complex (*25*).

In mitochondria, the superoxide dismutases (SOD) could catalyze superoxide dismutation to form oxygen and hydrogen peroxide. Recently, aconitase is found to have superoxide scavenging activity and the iron-sulfur cluster is essential for such scavenging activity (*26, 27*). In the present work, we found that aconitase could interact with bc_1 complex and the interaction could be involved in the superoxide scavenging process in mitochondria. The effect of aconitase on superoxide depletion activity could be due to interaction between bc_1 complex and aconitase or simply due to the scavenging activity of aconitase. If the effect on superoxide depletion is based on their interaction, a simple stoichiometric relation between bc_1 and aconitase should be observed. If the effect is resulted from intrinsic scavenging activity of aconitase and has nothing to do with its interaction with bc_1 complex, then the effect would be aconitase concentration dependent. In contrast to bacterial bc_1 complex, mitochondrial bc_1 complex could bind aconitase through its subunit II and thus enhance superoxide scavenging activity. The stoichiometric relation between bc_1 and aconitase could be 2, which indicates that each mitochondrial bc_1 complex can bind with two aconitase. And once both of the two subunit II in the bc_1 complex bind with aconitase, the fastest speed for aconitase to scavenge superoxide can be reached. However, aconitase itself can also react with nonspecific superoxide other than from bc_1 complex. Xanthine oxidase, an superoxide generator, is considered as a good control here to compare with mitochondrial bc_1 complex. However, for some unknown reason, this xanthine oxidase assay was not very

stable. So we have to use other superoxide generator. Since bacterial bc_1 complex cannot bind with aconitase, we used it as a nonspecific superoxide generator to confirm the specific interaction between mitochondrial bc_1 complex and aconitase. The superoxide scavenging activity is much higher when it binds with mitochondrial bc_1 complex (Fig. 3-8). Therefore, aconitase could scavenge the superoxide more effectively with the help of bc_1 complex. And this interaction may play an important role to protect the mitochondria from the damage of superoxide.

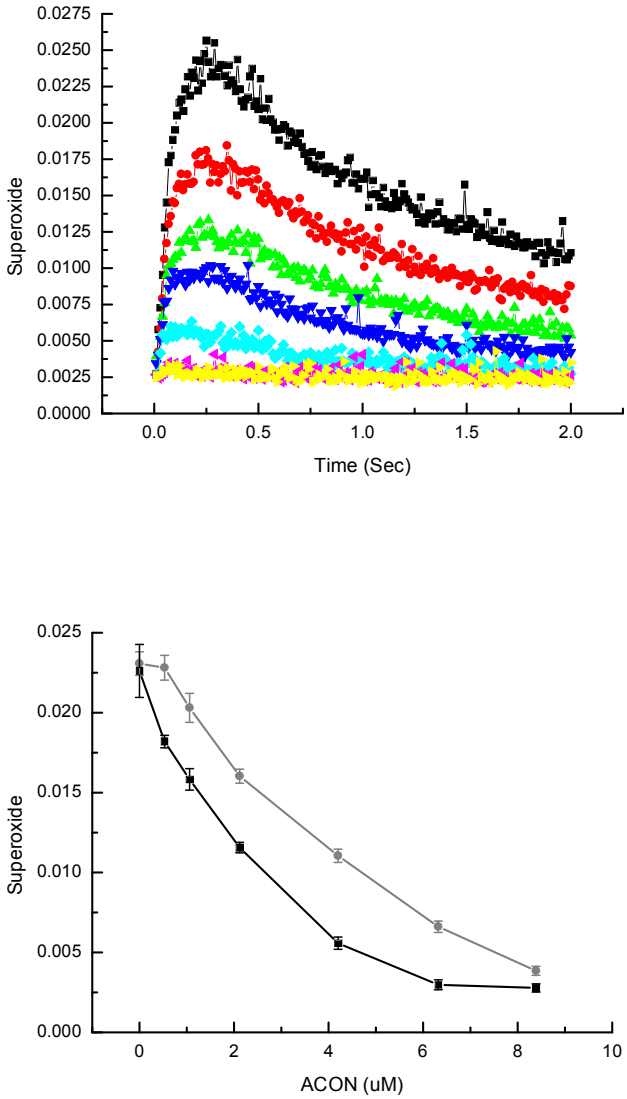

Fig. 3-8 Comparison of superoxide generation from mitochondrial and bacterial bc_1 complex. ***A*** Same amount of aconitase according to figure-3-8A was used in the bacterial superoxide generation assay. ***B*** Comparison of the superoxide generation in Figure 3-8A and Figure 3-9A. Gray line stands for bacterial bc_1's superoxide generation and black line stands for mitochondrial bc_1's superoxide generation.

At this moment we offer no good explanation on why the interaction between the bc_1 complex and aconitase enhances its activity. It is likely that bc_1 complex can stabilize aconitase by protecting the iron-sulfur cluster from destruction by superoxide. And it is also likely that bc_1 complex can help aconitase to recycle from inactive state to active state by offering electron.

When there is calcium present in the pull-down assay, more aconitase was pulled down by bc_1 complex. Therefore, calcium may be considered as an important factor for enhancing this interaction. This is also meaningful for the cell to regulate the energy metabolism (Fig. 3-9). Calcium is known as an activator for mitochondrial energy metabolism. Once the system need to be active, every member involved in the mitochondrial bioenergetics process should also increases their activity, and meanwhile, some harmful side product like superoxide should also be controlled by this system. Here we speculate how mitochondria regulate this complicated bioenergetics process through the collaboration between the electron transport chain and citric acid cycle.

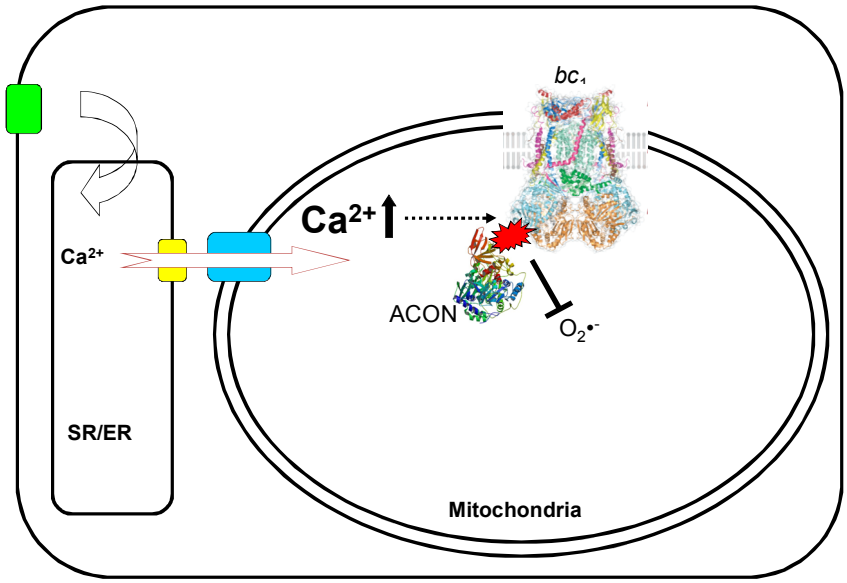

Fig. 3-9 The schematic illustration for the aconitase and bc_1 complex in the cell.

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CHAPTER IV

A NOVEL SHORT MALATE DEHYDROGENASE IN BOVINE MITOCHONDRIA

Qiyu Wang, Ting Su, Christopher Hoover, Linda Yu, and Chang-An Yu

Manuscript

4.1 Abstract

In the database of Genbank, there are two kinds of mitochondrial malate dehydrogenase in bovine (*Bos taurus*). One is a short protein with 278 amino acid residues (NP_001013605.1; AAX46375.1; DAA15016.1), which is the NCBI reference sequence for MDH. The other is a longer protein with 338 amino acid residues (AAI09598.1; Q32LG3.1). In most cases, the mitochondrial malate dehydrogenase reported is actually the longer form. With RT-PCR, we confirmed that both of them exist in bovine heart mitochondria. We expressed and purified both of the malate dehydrogenase in *E.coli*. We found that in contrast to the long MDH, the short form of protein does not have the activity. In our previous paper we reported that the long form of MDH may bind with bc_1 and this interaction can down-regulate the bioenergetics in mitochondria. The analysis of this short MDH indicates that the database may put the sequence of the nonfunctional one as the NCBI reference sequence for the correct enzyme in the citric acid cycle. However this short MDH may help the normal long MDH together to regulate the bioenergetic process of mitochondria.

4.2 Introduction

Mitochondrial malate dehydrogenase (EC 1.1.1.37) catalyzes the conversion of malate into oxaloacetate, which is the final reaction of the citric acid cycle. The reaction is NAD^+-dependent and oxidizes malate's secondary hydroxyl group to a corresponding ketone (*1*). The MDH also catalyzes the reversible reaction from oxaloacetate to malate. The crystal structure of mitochondrial malate dehydrogenase from porcine heart has

already obtained. And in this MDH crystal structure, it contains four identical subunits in the asymmetric unit of a monoclinic cell.(*2*) Besides the TCA cycle, MDH also participates in the malate-aspartate shuttle, translocating electrons (NADH) produced during glycolysis across the mitochondrial inner membrane. Malate dehydrogenase has two forms in the shuttle system: mitchondrial malate dehydrogenase and cytosolic dehydrogenase. In the cytosol, malate dehydogenase catalize the reaction of oxaloacetate and NADH to produce malate and NAD^+. Then malate is imported into mitochondrial matrix by the Malate-alpha-Ketoglutarate antiporter, and meanwhile alpha-ketoglutarate is exported from the matrix into the cytosol. Once transported into matrix, malate will be converted to oxaloacetate by mitochrondrial malate dehydrogenase. And NAD^+ is also reduced to NADH (*3*).

Malate dehydrogenase is also involved in gluconeogenesis, in which glucose is synthesized from smaller molecules. Mitochondrial malate dehydrogenase reduces the oxaloacetate to malate, which is transported through the inner mitochondrial membrane. Then malate is oxidized by the cytosolic malate dehydrogenase to form oxaloacetate in the cytosol, which is converted to phosphoenol pyruvate by phosphoenol pyruvate carboxy kinase (PEPCK) finally. (*4*)

Recently, we found that MDH can bind with bc_1 complex and affect each other's activity. This novel crosstalk between citric acid cycle enzymes and electron transfer chain complexes might play a regulatory role in mitochondrial bioenergetics (*5*). The negative regulation cannot be always functional and there should be some negative regulator to work together with it. In the present work, we found there is a short form of

MDH from mitochondria. It has the NCBI reference sequence but it does not have the MDH activity. It may bind with bc_1 complex and break the interaction between the normal MDH and bc_1 complex, and functions as an up-regulator for energy process. Both the up and down regulations together influence the TCA cycle and thus regulate ATP generation according to the need of organisms.

4.3 Experimental procedures

Chemicals- Cytochrome *c* (horse, type III), sodium cholate, deoxycholate, and bovine malate dehydrogenase were purchased from Sigma. 2,3-Dimethoxy-5-methyl- 6-(10-bromodecyl)-1,4-benzoquinol ($Q_0C_{10}BrH_2$) was prepared as previously reported (*6*).

Cloning, expression, and purification of proteins- The recombinant proteins corresponding to the long-MDH, Short-MDH, C-Long, C-Short and their mutant versions were depicted in Fig. 4-2. The cDNAs of bovine cell were purchased from Invitrogen. All His-tag fusion constructs were cloned into the expression vector pET28a (a pET28a variant with the first his-tag coding sequence deleted) between the *Xba* I and *Xho* I sites. All MDH proteins were expressed in *Escherichia coli* strain BL21 (DE3). *E. coli* cultures were shaken at 200 rpm and 37 °C until the OD_{600} reached 0.5–0.7. Isopropyl β-D-thiogalactopyranoside (IPTG) was then added to the culture at a final concentration of 1 mM, and the protein expression was induced for an additional 4 h at 30 °C. All His-tagged fusion proteins were purified with His•Bind® Purification Kits (Novagen).

Cytochrome bc_1 complex preparation and activity assay, and *MDH activity assay* are the same as described in chapter II.

4.4 Results and discussion

4.4.1 Comparing the amino acid sequence of long and short MDH

In the database of Genbank, the NCBI reference sequence for bovine MDH is NP_001013605.1 (or AAX46375.1; DAA15016.1) which codes for 278 amino acids residues MDH (including the signal peptide). However in the most publications, MDH has 338 amino acids (including the signal peptide), which has the sequence of AAI09598.1(or Q32LG3.1). The amino acid sequence alignment shows that the N-terminal parts (1-195) of these two MDH are exactly the same (shown as Fig. 4-1). The C-termini (196-278 and 196-338) are totally different, which means the short one may not be spliced from the longer one. The longer one (from bovine mitochondria) has 95% identity with the human mitochondrial malate dehydrogenase, and even 100% identity with the porcine MDH. Therefore, it is interesting to know whether this short bovine mitochondrial MDH can work like other MDH.

278 aa (NP_001013605.1; AAX46375.1; DAA15016.1) [*Bos taurus*]

```
  1 MLSALARPAG AALRRSFSTS AQNNAKVAVL GASGGIGQPL SLLLKNSPLV SRLTLYDIAH
 61 TPGVAADLSH IETRATVKGY LGPEQLPDCL KGCDVVVIPA GVPRKPGMTR DDLFNTNATI
121 VATLTAACAQ HCPEAMICII SNPVNSTIPI TAEVFKKHGV YNPNKIFGVT TLDIVRANAF
181 VAELKDLDPA RVNVPRHRRP RWENHHPLDL PVHPQGGISP GPADHPHRSD PGSWHRGGQG
241 EGRSGLCHPV HGIRWSPLCL LPRGRDEWKG RSRRMFLR
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338 aa (AAI09598.1; Q32LG3.1) [*Bos taurus*]

```
  1 MLSALARPAG AALRRSFSTS AQNNAKVAVL GASGGIGQPL SLLLKNSPLV SRLTLYDIAH
 61 TPGVAADLSH IETRATVKGY LGPEQLPDCL KGCDVVVIPA GVPRKPGMTR DDLFNTNATI
121 VATLTAACAQ HCPEAMICII SNPVNSTIPI TAEVFKKHGV YNPNKIFGVT TLDIVRANAF
181 VAELKDLDPA RVNVPVIGGH AGKTIIPLIS QCTPKVEFPQ DQLTTLTGRI QEAGTEVVKA
241 KAGAGSATLS MAYAGARFVF SLVDAMNGKE GVVECSFVKS QETDCPYFST PLLLGKKGIE
301 KNLGIGKVSP FEEKMIAEAI PELKASIKKG EEFVKNMK
```

Fig. 4-1 Amino acid sequence analysis for Long and Short MDH from bovine mitochondria. The mitochondrial location signal peptides are shown in blue; the same parts in the N-termini are shown in black; the different parts in the C-termini are shown in red.

4.4.2 RT-PCR confirmed the existence of short MDH

First, we needed to confirm that this short form MDH exist in bovine mitochondria. Since all the sequences of these MDH in the Genbank are obtained through the cDNAs, it is easy to design RT-PCR assay with the whole cDNAs from bovine cell as the template. Because there is another cytosolic MDH which also share high identity with mitochondrial MDH, the primers with the codes for mitochondrial location peptide are designed. Both the short and long MDH bands were found (Shown as Fig. 4-2) in the DNA agarose gel. These bands were extracted from the gel and ligated into T-easy vector for sequencing. Both of them were correct with the given sequences from the database. This confirmed that the short form of MDH does exist in the cell.

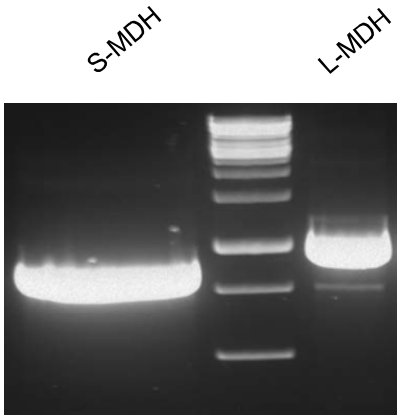


Fig. 4-2. RT-PCR result of the Long and Short MDH from bovine cell.

4.4.3 Expression and Purification of the long and short form MDH

To obtain the long and short form MDH, a His-tag was added on each sequence through another PCR with his-tag primer (shown as Fig. 4-3). Then the proteins of long and short MDH were expressed in *E.coli* BL21 with modified vector pET-28a. Purified long and short MDH were shown in SDS-PAGE and western blotting as compared to the one obtained from Sigma. Since the purified long MDH contains the signal peptide and His-tag, its molecular weight is bigger than the one purchased from sigma, which was purified from the bovine tissues. Surprisingly, we found that the polyclonal antibody for MDH can only bind with purified long MDH and the sigma one (shown in Fig. 4-4). This antibody may be against the C-terminus of MDH and cannot recognize the N-terminus. Therefore, we cannot use it to check the protein level of the short MDH in comparison with the long one in the cell.

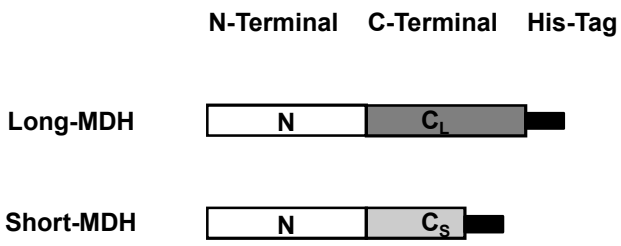


Fig. 4-3. Schematic representation of the proteins used in this study. N stands for N-terminus; C_L and C_S stand for the Long and short C-terminus. The dark gray thin bar stands for the 6His-tag.

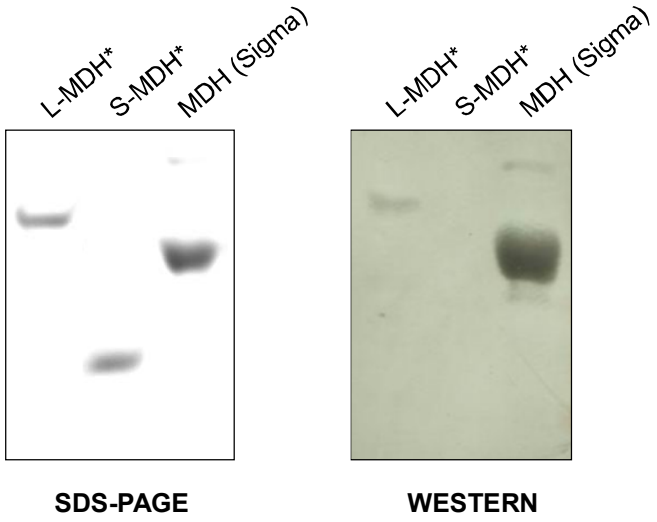


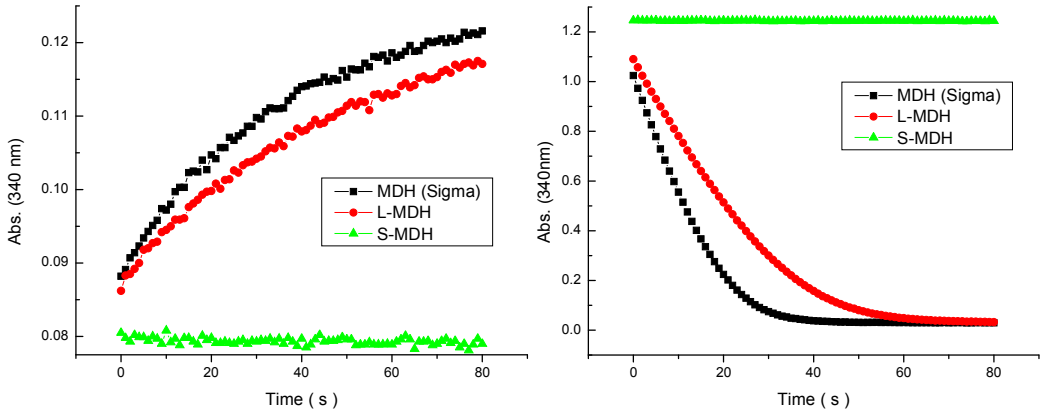
Fig. 4-4 SDS PAGE and western blotting results of the Long and Short MDH. *Both long and short MDH contain the mitochondrial signal peptide and His-tag. Anti-bovine mitochondrial MDH antibody was used.

4.4.4 Short MDH does not have the normal enzyme activity of long MDH

Once the protein of the short form MDH is obtained, it is important to know whether the protein is enzymatic active. Since long-MDH catalyzes both forward and reverse reaction in the citric acid cycle, both activities of short-MDH were assayed by the same activity assay method. The purified Long -MDH was also checked. The Long-MDH had the similar enzyme activity as the one purchased from sigma. However, Short-MDH did not have the normal MDH enzyme activity (Shown as Fig. 4-5). Since the short-MDH exists in the cell but do not have activity, it may have some important function other than the in the citric acid cycle.

4.4.5 Short MDH may compete with Long MDH on the binding site of bc_1 complex

Previous report indicated that long MDH can interact with bc_1 complex and enhance the activity of bc_1 complex. It is interesting to see whether or not the short-MDH has the same function. The bc_1 activity enhancement assay was carried out with the purified short-MDH. The results show that short-MDH can enhance the bc_1 activity much more as compared to the long MDH (Fig. 4-6). This result suggests that short-MDH can interact with bc_1 complex. The interaction or binding between the bc_1 complex and short-MDH may be stronger than that of long-MDH.



malate + $NAD^+ \leftrightarrow$ oxaloacetate + NADH + H^+

Fig. 4-5 MDH activity assay in the forward and reverse directions. The activity of Long MDH (L-MDH) is in red; the activity of Short MDH (S-MDH) is in blue; the activity of the MDH purchased from Sigma is in black.

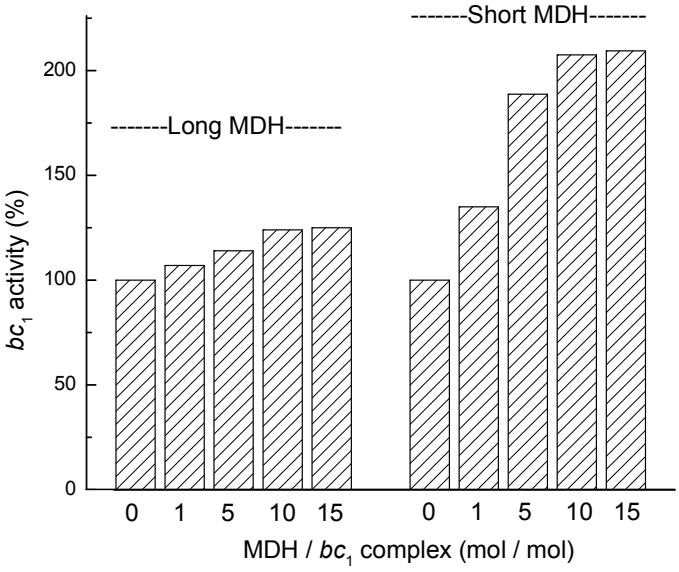


Fig. 4-6. Long and short MDH's effect on the activity of bc_1 complex.

However, the pull-down assay (*5*) using matrix proteins has failed to recover the short-MDH in the precipitate. Although the short-MDH is detected in matrix proteins fraction, the concentration may be too low for the pull down assay. It is also possible that the conditions of the pull-down assay preferred by the Long-MDH may not suitable for the short-MDH to bind with bc_1 complex. To verify the binding between short-MDH and bc_1 complex, recombinant short-MDH was used in the pull down assay (Fig. 4-7). In the SDS-PAGE, it shows clearly that short-MDH was pulled down by bc_1 complex, when comparing bc_1 only with bc_1 plus short-MDH. The effect of ionic strength on the amount of short MDH pulled down by bc_1 complex was also studied. Fig, 4-7 shows the difference of the amount of short MDH pulled-down by bc_1 complex under 200 mM NaCl. When the ionic strength increased, no short-MDH was observed indicating that the interaction between short MDH and bc_1 is hydrophilic.

MDH was considered as a dimer in the cell. The crystal structure shows that the two monomers form a dimer through their N-termini. And the active site is mainly formed from amino acid residues from the C-terminus part. The short MDH contains the same N-terminus but different C-terminus and it does not have the enzyme activity. But the short MDH binds with bc_1 complex stronger than the long MDH. If it can compete with Long-MDH to bind with bc_1 complex, it will break the crosstalk between bc_1 complex and MDH (the long MDH) and thus have a special role in regulating the bioenergetic process in the mitochondria.

It is still not clear what factor can switch between Long-MDH and Short-MDH on binding with bc_1 complex. This part of work is currently undergoing in our lab.

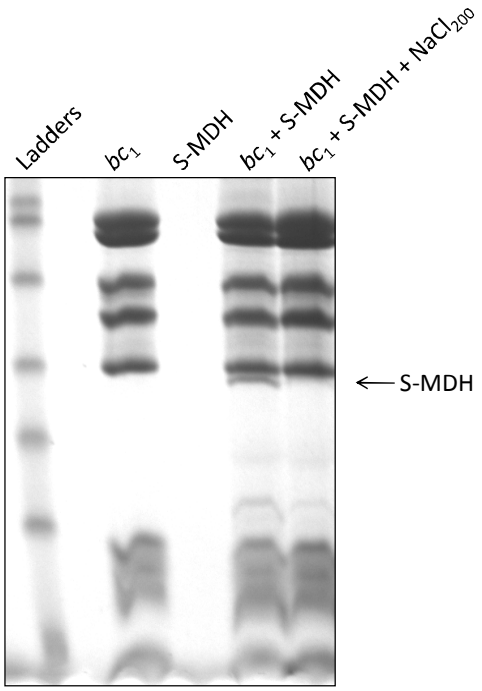


Fig. 4-7. SDS-PAGE result of the short MDH pulled down by bc_1 complex in low and high salt solutions. $NaCl_{200}$ stands for the addition of 200 mM NaCl to the pull down assay.

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CHAPTER V

CO-CRYSTALLIZATION OF CYTOCHROME BC1 COMPLEX WITH MITOCHONDRIAL MALATE DEHYDROGENASE OR ACONITASE

Qiyu Wang, Mingquan Zhang, Linda Yu and Chang-An Yu

5.1 Abstract

To study the co-complex of mitochondrial bc_1 complex and malate dehydrogenase (or aconitase), we developed two approaches to obtain the co-crystals. The first one is to use bc_1 to pull down MDH or ACON when bc_1 becomes crystals; the second one is to use the soluble parts (core I and core II) of bc_1 complex to get the co-crystal together with MDH or Aconitase. With these approaches, we got some crystals and further study is still undergoing.

5.2 Introduction

In chapter II and III, I reported that bovine mitochondrial bc_1 complex could bind with mitochondrial malate dehydrogenase and aconitase. The interaction between them may play an important role in regulating the bioenergetic process in mitochondria. However, the mechanism of the interaction is still unclear. To further understand the interaction between those proteins, we need to know the structural information for those co-complexes. We have already known all the structures for these three proteins. The crystal structures of bovine mitochondrial bc_1 complex was obtained in 1997(*1*), and the crystal structures of mitochondrial malate dehydrogenase and aconitase were obtained in 1994 (*2, 3*).(Fig. 5-1) However, it is still impossible for us to simulate the co-complex through computer so far. Because all of them are too big for calculating and we don't know the orientation.

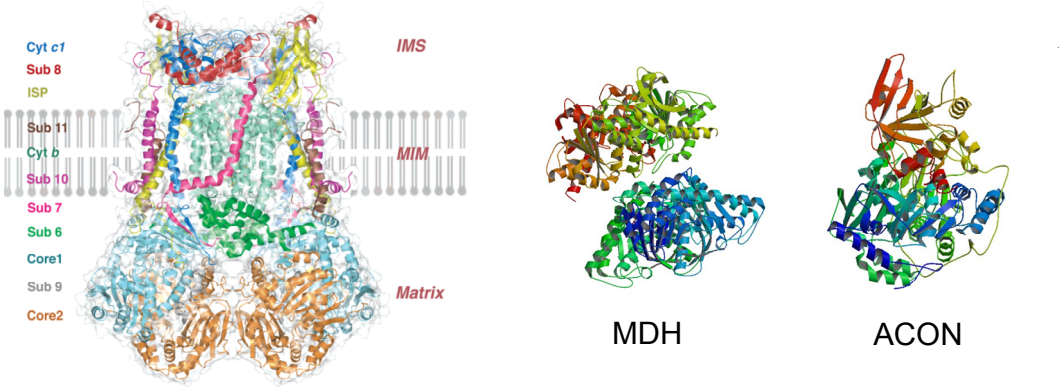


Fig. 5-1 3-D structures of the mitochondrial bc_1 complex, malate dehydrogenase and aconites.

Therefore we still have to get the co-crystals first to obtain the structure of co-complex. But we don't need very high resolution at this point, since we have already known their structures individually. With this aim, we have two approaches. The first one is aimed at getting the co-crystals by using pull-down method. As we mentioned in the previous chapters, bc_1 can pull down the MDH and Aconitase. And we also have a developed method to obtain the crystal of bc_1 complex. Therefore, we will try to use bc_1 to pull down MDH or ACON when bc_1 forms crystals. If it works, we may see both bc_1 complex and MDH (or aconitase) in the co-crystals. The second one is aimed at getting co-crystals of core I or core II with MDH (or aconitase). As we reported in chapter II and III, core I and core II (also known as subunit I and II) of bc_1 complex have the binding sites for MDH, and core II has the binding site for aconitase. Core I and core II are soluble and stable in the solution although we separate them from other subunits. We will express and purified the core I and core II from *E.coli* and try to get the co-crystals with MDH or Aconitase.

With these approaches, we got some crystals and used SDS-PAGE to check whether the crystals contain those two proteins or not beside the bc_1 complex. And the further study is still being done.

5.3 Experimental procedures

Chemicals- Sodium cholate, deoxycholate, and bovine malate dehydrogenase were purchased from Sigma. 2,3-Dimethoxy-5-methyl- 6-(10-bromodecyl)-1,4-benzoquinol ($Q_0C_{10}BrH_2$) was prepared as previously reported (*4*).

Cloning, expression, and purification of proteins- The cDNAs of bovine cell was purchased from Invitrogen. Both core I- and core II-His-tag fusion constructs were cloned into the expression vector pET28a (a pET28a variant with the first his-tag coding sequence deleted) between the *Xba* I and *Xho* I sites. These two proteins were expressed in *Escherichia coli* strain BL21 (DE3). *E. coli* cultures were shaken at 200 rpm and 37 °C until the OD_{600} reached 0.5–0.7. Isopropyl β-D-thiogalactopyranoside (IPTG) was then added to the culture at a final concentration of 1 mM, and the protein expression was induced for an additional 4 h at 30 °C. All His-tagged fusion proteins were purified with His•Bind® Purification Kits (Novagen).

Cytochrome bc_1 complex preparation- Cyt bc_1 complex from the bovine heart mitochondria was prepared as described in chapter II. For crystallization, an additional 15-step ammonium acetate fractionation was carried out to remove impurities from the bc_1 complex. Then bc_1 complex was recovered from the precipitates formed between 18.5% and 33.5% ammonium acetate saturation. The final bc_1 complex was dissolved in 50 mM Tris–HCl buffer（pH 7.8, containing 0.66 M） sucrose to a protein concentration

of 26 mg/ml and frozen at −80 °C until use. The concentrations of cyt *b* and c_1 were determined spectroscopically, using millimolar extinction coefficients of 28.5 cm^{-1} mM^{-1} (*5*) and 17.5 cm^{-1} mM^{-1} (*6*) for cyt *b* and c_1, respectively.

Crystallization- Purified bc_1 complexes were adjusted to a final concentration of 20 mg/ml in the crystalliztion buffer (50 mM Mops at pH 7.2, 20 mM ammonium acetate, 20% (w/v) glycerol, and 0.16% (w/v) sucrose monocaprate). One ul of MDH (19.7 mg/mL) or Aconitase (21.2 mg/mL) was added into the co-crystallization solution. This crystallization solution with or without MDH (or Aconitase) was set up for crystallization as described in previous publications (*1, 7*). Crystals appeared in two days by using the hanging drop method, and one to two weeks in the sitting drops. Both bc_1 crystals and co-crystals had a rectangular shape.

5.4 Results and discussion

5.4.1 The first approach to get the co-crystals

Our lab has developed a stable method for crystallizing the mitochondrial bc_1 complex. And we have already obtained the purified proteins of mitochondrial bc_1 complex, malate dehydrogenase and aconitase (Fig. 5-2). Therefore, in the first approach, we used the same method but put the proteins together to obtain some crystals. First, we dialyzed MDH and aconitase against the crystallization buffer to remove their original buffer. Then, we concentrated those two proteins and got them in high concentration (MDH 19.7 mg/mL, aconitase 21.2 mg/mL). In this way, very few volume of MDH or aconitase would be added into the total mixture. Therefore, the conditions for crystallizing bc_1 were only changed very little.

With this method, we got some crystals in the hanging drop assay first (Fig. 5-3). And actually, when I added MDH into bc_1 complex, I got the crystal faster than the bc_1 itself, and the crystals were also bigger. Then to make sure whether the co-crystals contain those two proteins or not, I used the SDS-PAGE to check the co-crystals. In the first time, I dried the crystals on the filter paper. In Fig. 5-4, it looks like the co-crystals contain MDH or Aconitase as compared to the crystals of bc_1 only. In the second time, I washed the crystals several times in the similar solution for crystallization first before they were subjected to the SDS-PAGE. In Fig.5-5, the MDH band in the co-crystal was much weaker than Fig. 5-4, but all the bands were very weak. Therefore, it is still not able to confirm the co-crystals yet. We need get the X-ray data to confirm the co-crystals later.

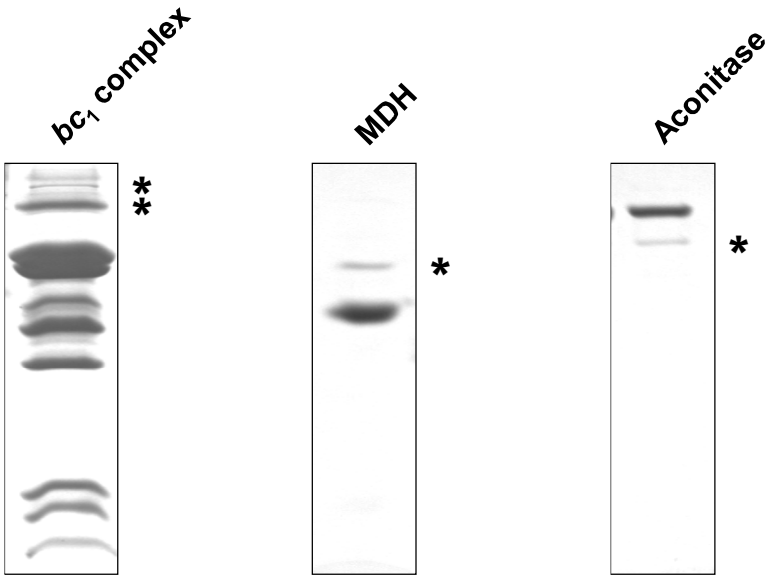
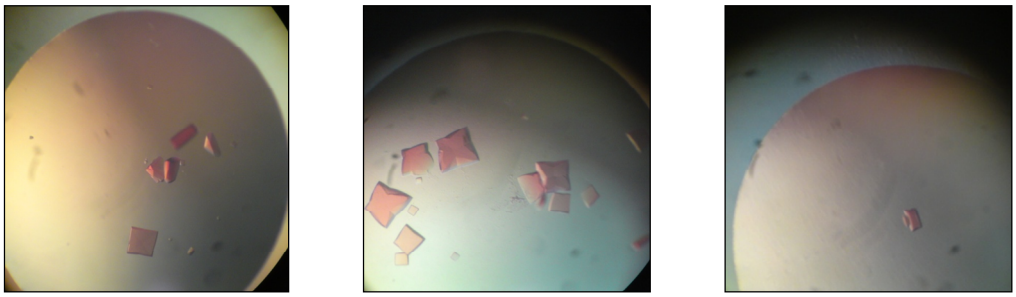


Fig. 5-2 SDS-PAGE of the purified mitochondrial bc_1 complex, malate dehydrogenase and aconites . * stands for the contamination in the purified proteins.



bc_1 bc_1+MDH bc_1+ACON

Fig.5-3 Crystals of mitochondrial bc_1 complex in the presence of malate dehydrogenase and aconitase.

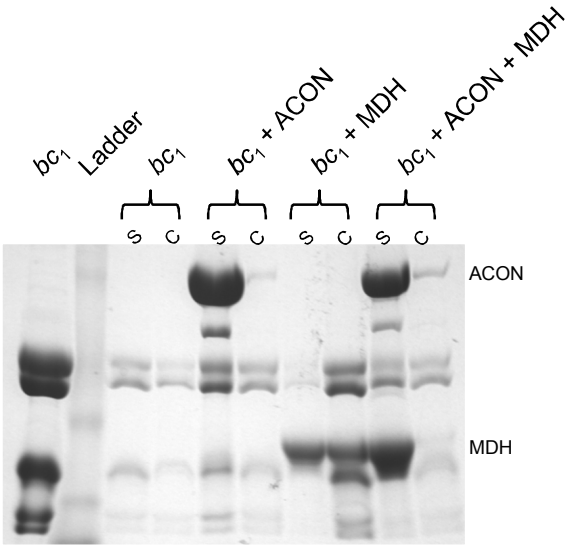


Fig. 5-4 SDS-PAGE of the co-crystals for the mitochondrial bc_1 complex together with malate dehydrogenase and aconitase after being dried on the filter paper. 10% SDS-PAGE was used here. S, solution; C, crystal. Crystals were dried on the filter paper.

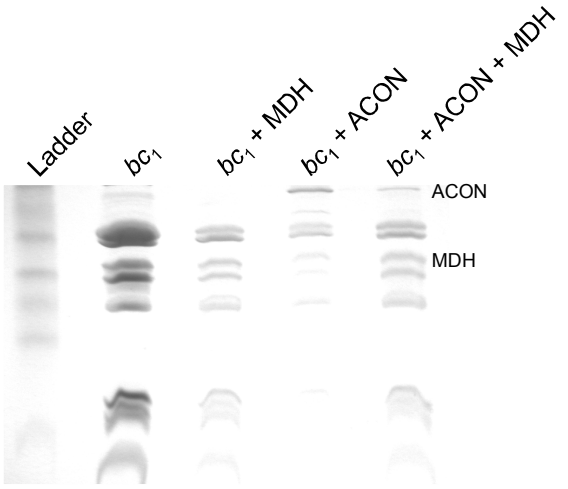


Fig. 5-5 SDS-PAGE of the co-crystals for the mitochondrial bc_1 complex together with malate dehydrogenase and aconitase after being washed in the solution. 15% SDS-PAGE was used here. ACON, aconitase.

5.4.2 The second approach to get the co-crystals

For the second approach, we need to use the matrix part core I and core II to get the co-crystals of bc_1 complex with MDH or aconitase. Since we have already obtained the purified MDH and aconitase (Fig.5-2), we need to purify the core I and core II of bc_1 complex. Core I and II could be soluble by themselves and their MW are around 45 kD, which are not too big for expression in the *E.coli.* system. Therefore, we used *E.coli.* BL-21 to express those two proteins. I just finished the clone part of the second approach, and in the SDS-PAGE (Fig. 5-6), we can see the induced expression of core I and core II clearly. However, I have not purified them yet and I will continue this part of work later.

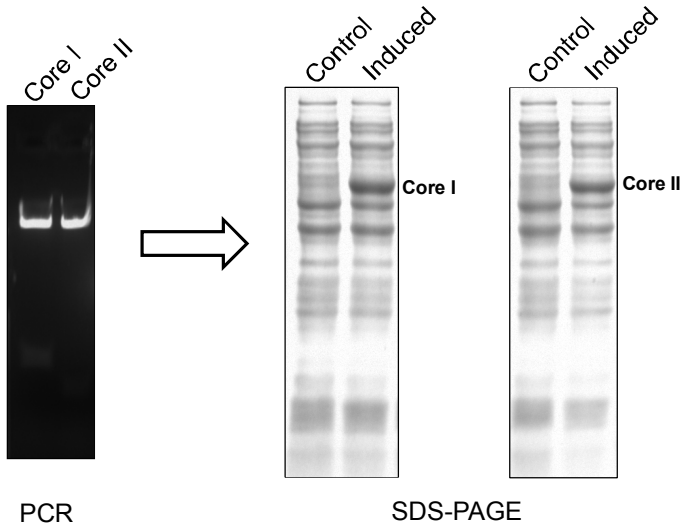


Fig. 5-6 PCR and SDS-PAGE results for mitochondrial Core I and II. PCR shows the DNA segments for the Core I and II obtained from RT-PCR of bovine heart cell cDNA. SDS-PAGE shows the proteins expressed in *E.coli.* BL 21.

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APPPENDICES

ADP	adenosine diphosphate
ATP	adenosine triphosphate
β-ME	β-mercaptoethanol
cyt	cytochrome
DNA	deoxyribonucleic acid
E. coli	Escherichia coli
EPR	electron paramagnetic resonance
ISP	Iron sulfur protein
kDa	kilodaltons
kb	kilo base pairs
PAGE	polyacrylmide gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
Pi	phosphate
Q	ubiquinone

$Q_0C_{10}Br$	2,3-dimethoxy-5-methyl-6-(10-bromo)-decyl-1,4-benzoquinone
Rs.	*Rhodobacter sphaeroides*
MDH	Malate dehydrogenase
SDH	Succinate dehydrogenase
SDS	sodium dodecylsulfate
SMP	submitochondrial particle
UHDBT	5-*n*-undecyl-6-hydroxyl-4,7-dioxobenzothiazole
UV	ultra viole

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Contributed Presentations

Poster, "Crosstalk between Mitochondrial Malate Dehydrogenase and Cytochrome bc_1 Complex", Biophysical Society 53rd Annual Meeting, Boston, MA (2009)

Poster, "Interaction between the Mitochondrial Cytochrome bc_1 Complex and The matrix Enzymes", Biophysical Society 55rd Annual Meeting, Baltimore, MD (2011)

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Title of Study: CROSSTALK BETWEEN CYTOCHROME BC1 COMPLEX AND MITOCHONDRIAL MATRIX ENZYMES

Pages in Study: 132 Candidate for the Degree of Doctor of Philosophy

Major Field: Biochemistry and Molecular Biology

Scope and Method of Study:

Protein pull-down assay, western blotting, protein activity assay, superoxide assay, Surface Plasmon Resonance, Mass Spectroscopy, Cross linking.

Findings and Conclusions:

The interactions between the mitochondrial cytochrome bc_1 complex and matrix soluble proteins were studied by a precipitation pull-down technique. One of the matrix proteins pulled down by bc_1 complex was identified as mitochondrial malate dehydrogenase (MDH) by MALDI-TOF Mass spectrometry and confirmed by Western blotting with anti-MDH antibody. Using cross linking technique, subunits I, II (core I & II) and V of the bc_1 complex were identified as the interacting sites for MDH. Incubating purified MDH with the detergent dispersed bc_1 complex results in the increase of the activities of both the bc_1 complex and MDH. The effect of the bc_1 complex on the activities of MDH is unidirectional (oxalacetate → malate). These results suggest that the novel crosstalk between citric acid cycle enzymes and electron transfer chain complexes might play a regulatory role in mitochondrial bioenergetics. We also confirmed the existence of a short form of MDH. We found that it does not have the normal enzyme activity but may help the normal long MDH together to regulate the bioenergetics process.

Another matrix protein pulled down by bc_1 complex was identified as mitochondrial aconitase. Subunit II of the bc_1 complex was identified as the interacting site for aconitase. Unlike MDH, no effect on the activity of the bc_1 complex was observed when purified aconitase was interacted with the detergent dispersed bc_1 complex. And the activity of aconitase was only enhanced slightly by cytochrome bc_1 complex. Upon the binding of aconitase, the superoxide generating activity of bc_1 complex is greatly decreased. However, when aconitase was added to the bacterial bc_1 complex, little effect on its superoxide generating activity was observed. The lack of core subunits I and II in the bacterial bc_1 complex may be the reason for this difference. The interaction between bc_1 complex and aconitase can be enhanced by calcium, which is considered as an active factor for the bioenergetic process of mitochondria. These results suggest that the binding between mitochondrial bc_1 complex and aconitase might play an important role in superoxide scavenging when mitochondria is in a high energy producing state, which is crucial for mitochondria and the whole organism.

To study the co-complex of mitochondrial bc_1 complex and malate dehydrogenase (or aconitase), we developed two approaches to obtain the co-crystals. With these approaches, I got some crystals and am conducting further studies.

ADVISER'S APPROVAL: Chang-An Yu