INTERACTION BETWEEN CORE I AND CORE II SUBUNITS OF BOVINE bc_1 COMPLEX

By

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NOMENCLATURE

ATP	adenosine triphosphate
NQR	NADH-ubiquinone reductase
SQR	succinate-ubiquinone reductase
QCR	ubiquinol-cytochrome-*c* reductase
CcO	cytochrome-*c* oxidase
kDa	kilodalton
NADH	nicotinamide adenine dehydrogenase
FMN	flavin mononucleotide
FAD	flavin adenine dinucleotide
ADP	adenosine diphosphate
Pi	phosphate
Q	ubiquinone
ISP	iron-sulfur protein
b_L	low potential cytochrome *b*
b_H	high potential cytochrome *b*
MPP	mitochondrial processing petidase
PEP	processing enhancing peptidase
EEDQ	N-(ethyl-carbonyl)-2-ethoxy-1,2- dihydroquinoline
FPLC	Fast protein liquid chromatography

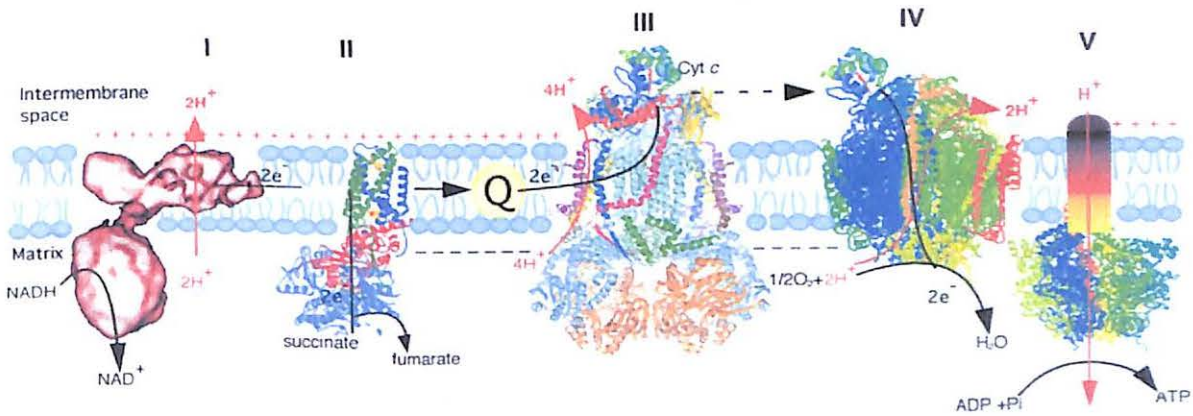
EDTA	ethylenedinitrilo-tetraacetic acid, disodium
pISP	ISP precursor
IPTG	isopropyl-β-D-thiogalactopyranoside
SDS	sodium dodecyl sulfate
PAGE	poly-acrylamide gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
DMSO	dimethyl sulfoxide
PCR	polymerase chain reaction
bp	base pair
cyt.	cytochrome
NTP	nucleoside triphosphate
E.coli	*Escherichia coli*

Chapter I

Introduction

Most of the energy required for eukaryotic organisms is obtained from the process of electron transport coupled to ATP synthesis, known as oxidative phosphorylation. Oxidative phosphorylation takes place in the inner mitochondrial membrane and is carried out by five protein complexes, four of which form the mitochondrial electron transport chain: NADH-ubiquinone reductase (NQR, complex I), succinate-ubiquinone reductase (SQR, complex II), ubiquinol-cytochrome-c reductase (QCR, complex III), and cytochrome c-oxidase (CcO, complex IV) and the fifth complex is the ATP synthase (ATPase, complex V) (Scheme 1). NADH or succinate, which are derived from the citric acid cycle, are oxidized to NAD+ and fumarate by donating their electrons to ubiquinone through complex I or complex II, respectively. The electrons released are transferred to complex III then complex IV and finally to molecular oxygen. The energy released from the oxidation of NADH or succinate by the mitochondrial respiratory chain is conserved as an electrochemical proton gradient, which is then utilized by the ATP synthase complex to synthesize adenosine triphosphate (ATP) (1).

The NADH-ubiquinone reductase complex or Complex I is the largest of these five complexes with a molecular weight of 907 kDa and is composed of 43 subunits. This complex catalyzes the electron transfer from NADH to ubiquinol. One FMN and seven different Fe-S clusters and at least three bound ubiquinone molecules constitute the redox prosthetic groups of this complex (2-6).



Scheme. 1. Electron transport complexes and ATP synthase in mitochondrial respiratory chain

The succinate-ubiquinone reductase or complex II, is composed of five subunits with a molecular weight of 127 KDa. It catalyzes the electron transfer from succinate to ubiquinone, and contains five prosthetic groups: one FAD, three different Fe-S clusters and a *b*-type cytochrome (b_{560}) (7-10).

The ubiquinol cytochrome-c reductase complex or complex III, also known as the bc_1 complex, consists of eleven subunits and has a molecular weight 248 KDa. It catalyzes the antimycin-sensitive electron transfer from ubiquinol to cytochrome c (11). Detailed information about this complex will be presented in the next section.

The cytochrome-c-oxidase (CcO) or complex IV is composed of 13 subunits and has a molecular weight 204 KDa. It catalyzes electron transfer from cytochrome-c to oxygen and has four redox prosthetic groups: two *a*-type cytochromes (a and a3) and two copper centers CuA and CuB (12).

Complex V also known as the ATP synthase is composed of two basic subunits F1 and Fo. Fo is a membrane sector and F1 is a water-soluble catalytic component that binds ADP and Pi and synthesizes ATP (13-15).

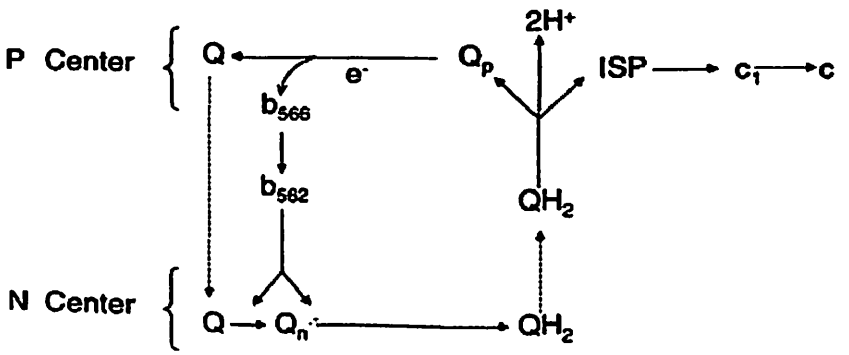
The protonmotive Q-cycle Mechanism

The bc_1 complex acts as a "proton pump" that pumps protons across the membrane using the potential energy generated by the difference in redox potential between ubiquinol and cytochrome c. The most favorable mechanism for electron and proton transfer in the bc_1 complex was first proposed by Peter Mitchell (16) as the "Q cycle mechanism" (Scheme 2) and modified later to explain electron transfer, proton translocation and the 2:1 H^+/e^- ratio in the cytochrome bc1 complex. The two key features of the Q-cycle are:

1- The involvement of two separate binding sites for ubiquinone and ubiquinol: (i) Q_P site where ubiquinol (QH_2) is first oxidized near the P-side (positive side) of the inner mitochondrial membrane and (ii) Q_N site where ubiquinone (Q) is reduced near the N-side (negative side) of the inner membrane.

2- The bifurcated reaction at the Qo site which involves the transfer of one electron to the high potential chain through the 2Fe2S cluster of the iron sulfur protein (ISP) to cytochrome c_1 and then to cytochrome c, and the transfer of the second electron to the low potential chain through heme b_L to heme b_H. From heme b_H, the electron is transferred to ubiquinone to form ubisemiquinone ($Q^{\cdot-}$), which completes half of the Q-cycle with one electron transferred to cytochrome c and two protons released on the P-side of the membrane. The second half of the cycle involves the oxidation of a second QH_2 molecule at the Qo site with one electron transferred to the high potential chain to reduce cytochrome c and the second electron transferred to the low potential chain to reduce ubisemiquinone to QH_2 with the release of another two protons to the P-side of the membrane. In summary, a complete turnover of the cytochrome bc_1 complex oxidizes two molecules of QH_2 at the Qo site and generates one QH_2 molecule at the Qi site with the reduction of two molecules of cytochrome c and the release of 4 protons on the P-side of the membrane:

$$QH2 + 2 \text{ cytochrome } c^{3+} + 2H^{+}_{n} \leftrightarrow Q + 2 \text{ cytochrome } c^{2+} + 4H^{+}_{p}$$



Scheme. 2. Q Cycle mechanism shown in the frame of a functional monomer of the cytochrome bc_1 complex. P center refers to the positive side of the membrane and N center refers to the negative side of the membrane.

A- Ubiquinol-cytochrome c reductase (The cytochrome bc_1 complex or complex III)

The ubiquinol-cytochrome c oxidoreductase is an oligomeric membrane protein complex and one of the fundamental components of the eukaryotic and bacterial respiratory chain (17,18). The complex localizes in the inner mitochondrial membrane of eukaryotic organisms as well as in many aerobic and photosynthetic bacteria (17,18).
In mitochondria, the cytochrome bc_1 complex (Fig. 1) catalyzes electron transfer from ubiquinol to cytochrome c, which is coupled to the translocation of protons across the mitochondrial inner membrane from the matrix space to the intermembrane space and contributes to the electrochemical proton gradient that drives ATP synthesis (18,19). In addition to electron transfer and proton translocation, the cytochrome bc_1 complex can also generate superoxide anion (10,20) and under some specific conditions it can exhibit mitochondrial processing peptidase activity (21).

The bc_1 complex was first purified from bovine heart mitochondria by Hatefi et al in 1962 (19). The purification procedure was modified later to yield this complex with higher purity and activity (20,22,23). The purified mitochondrial cytochrome bc_1 complex contains 11 protein subunits; it consists of 2165 amino acid residues and four prosthetic groups with a total molecular mass of 248 KDa (24-26).

However, *Saccharomyces cerevisiae* (29) and *Neurospora crassa* complexes consist of only 10 subunits.

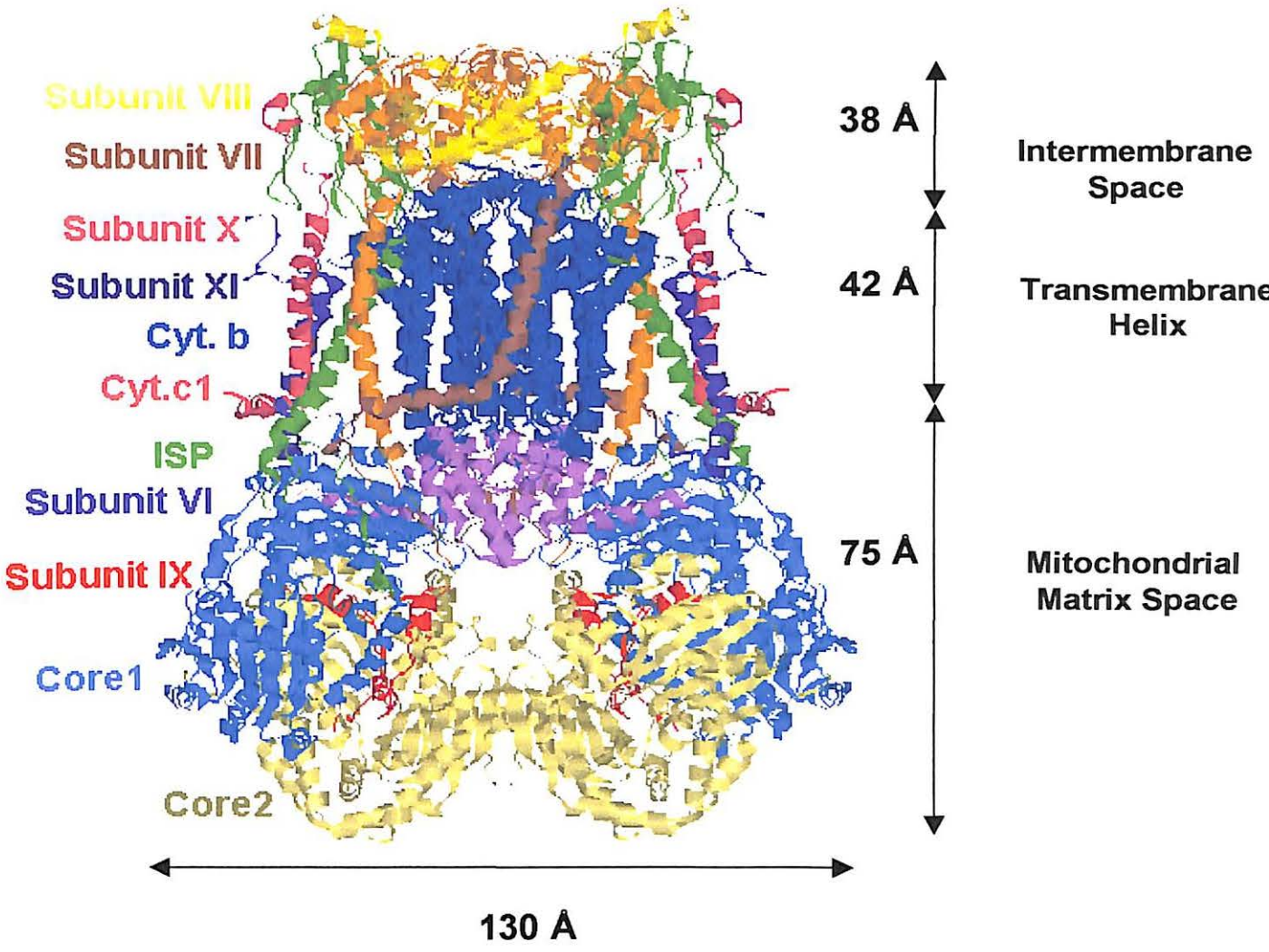


Fig. 1. Ribbon drawing of the dimeric bovine mitochondrial cytochrome bc_1 complex .

1. The redox-containing subunits

Only three of these subunits are catalytic subunits with essential redox prosthetic components and participate in electron transport; the two *b*-type hemes b_L (b_{565}) and b_H (b_{562}) of cytochrome *b*, which is a mitochondrially encoded gene product, in addition to cytochrome c_1 with a *c*-type heme and the iron-sulfur protein (ISP), also known as the Rieske protein, with one high potential iron-sulfur cluster [2Fe-2S] which are encoded by the nuclear genome (26). These three redox-containing subunits are essential subunits of the complex since they are present in all the cytochrome bc_1 complexes and are directly involved in electron and proton transfer.

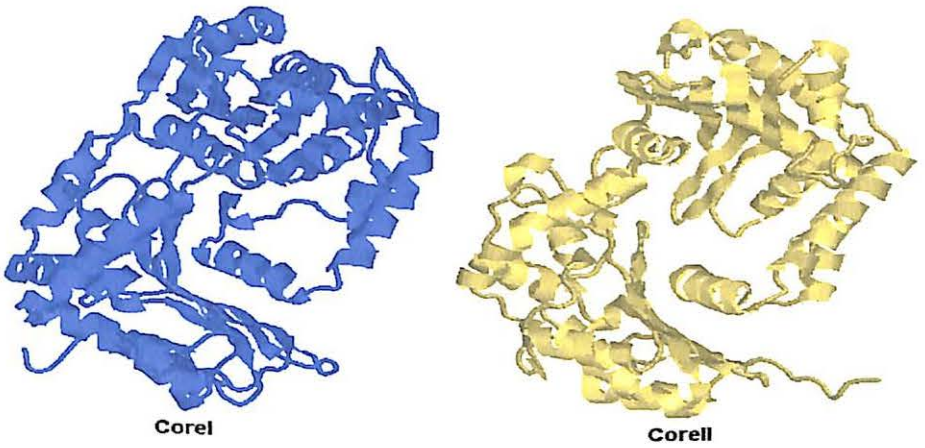
2. Non-redox subunits (supernumerary subunits)

The mitochondrial cytochrome bc_1 complex contains additional subunits known as the non-redox active proteins or the supernumeral subunits. The number of supernumeral subunits in bc_1 complex varies from one organism to another. There are eight supernumerary subunits in bovine bc_1 complex (26), seven in yeast bc_1 complex (27), one in the *Rhodobacter sphaeroides* (28) and none in *Paracoccus denitrificans* (29), Rhodospirillum rubrum (30) and *Rhodobacter capsulatus* (31). Their role in the complex has long been assumed to be structural rather than catalytic (26).

3. Core I and Core II subunits

Over one half of the complex mass, including subunits Core I and Core II (Fig. 2), are on the matrix side of the membrane, while most of the cytochrome *b* subunit is located within the membrane. There are 13 transmembrane helices in each monomer,

(a)



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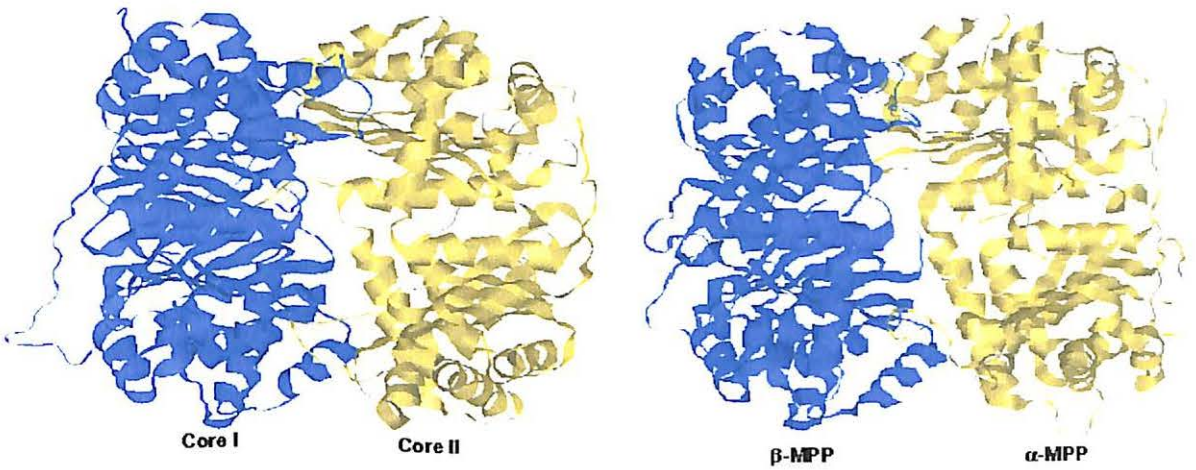
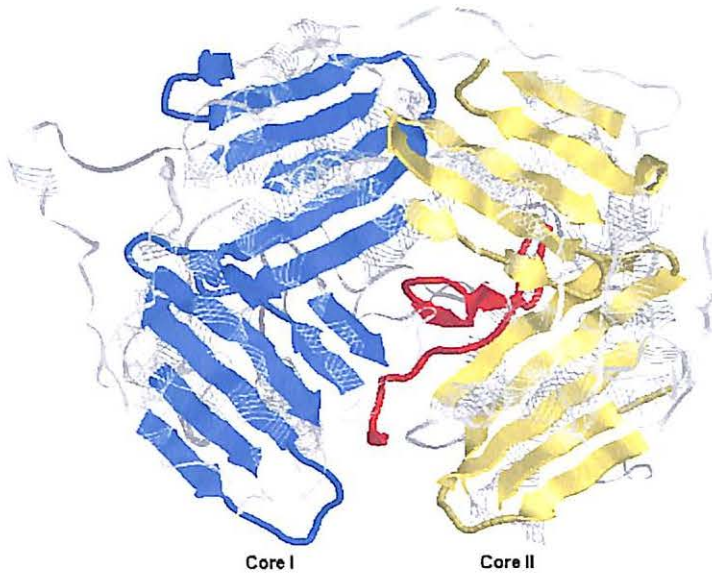


Fig. 2. (a) Ribbon drawing showing the α-helices of Core I (skyblue) and Core II (yellowtint).

(b) Ribbon drawing showing a comparative model between the Core I/Core II subunits of bc_1 complex and the α-MPP/β-MPP subunits of yeast MPP.

eight of them belonging to cytochrome *b* (26). The two large so-called core proteins Core I and Core II are extramembranous subunits that are attached to the transmembrane domains and protrude into the matrix. Both subunits are bowl-shaped and assembled to a hollow ball. Core I and Core II have 21% sequence identity and their three-dimensional structures are remarkably similar (26). Each of these core subunits consists of two structural domains (the N-terminal and the C-terminal domains) of about equal sizes and almost identical folding topology (26). In the assembled bc_1 complex, the NH_2- terminal domain of Core I interacts with the COOH-terminal domain of Core II and the COOH-terminal part of Core I interacts with the NH_2-terminal domain of Core II (26). Both domains are folded into one mixed β sheet of six or five β strands surrounded by three α helices on one side and one α helix from the other domain on the other side (Fig. 3.) (26). Core I and Core II enclose a big cavity and the amino acid residues lining the walls of this cavity are mostly hydrophillic (32,33). The two subunits, Core I and Core II, are synthesized in the cytosol as large precursor proteins of 480 and 453 amino acid residues, respectively, with N-terminal extension peptides or presequences for targeting into the mitochondria (25). The 34 residues of Core I NH_2-terminal and the 14 residues of core II NH2-terminal are proteolytically cleaved during or after the import of the precursors into the mitochondria (25). Removal of the presequences is necessary because they might interfere with the proper folding and assembly of the imported polypeptides or with the function of the protein. The core proteins of the mammalian bc_1 complexes are homologous to the subunits of the general mitochondrial matrix processing peptidase (MPP) that functions in cleaving signal peptides of nuclear encoded proteins after import into the mitochondrion (34).

(a)



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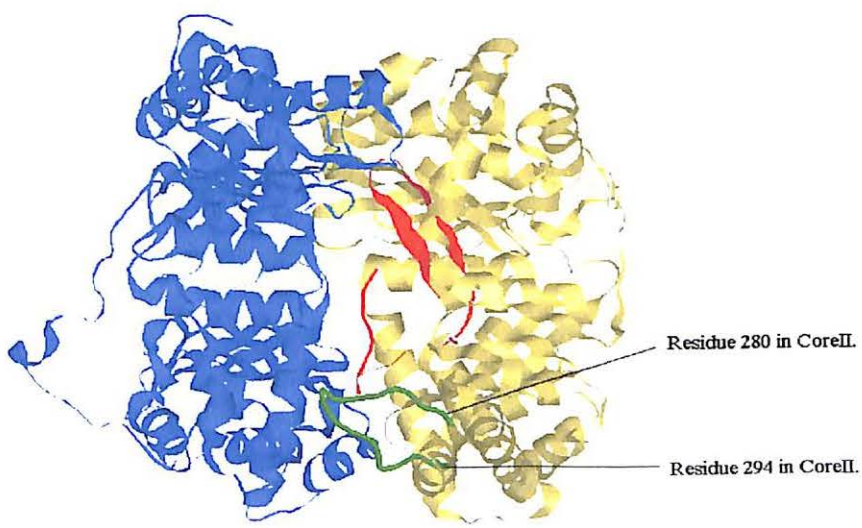

Fig. 3. (a) Ribbon drawing showing the beta sheets of the Core I (skyblue), CoreII (yellowtint) and subunit IX (red).
(b) Ribbon drawing showing the Core I (skyblue), Core II (yellowtint), subunit IX (red), and the so-called glycine rich loop in the Core II subunit (green).

4. Additional Core I and Core II characteristics

A cDNA encoding Core I subunit of bovine bc1 complex was cloned and its amino acid sequence was deduced from nucleotide sequencing (35,36). Besides its function in maintaining electron transport, the bovine Core I subunit, like Core I of *Neurospora crassa* bc_1 complex, has processing peptidase activity. Comparison of the amino acid sequence of Core I with the sequence of the α-subunit of matrix processing peptidase (MPP) from *Neurospora crassa* revealed a high identity of 39% and a similarity of 49% (35). By studying several Core I mutants, it was established that the Core I subunit of the yeast bc_1 complex is required for the assembly of the complex and the conversion of apocytochrome *b* to mature cytochrome *b* (37,38). Similarly, by studying core II-deficient mutants, it was found that the yeast core II is essential in maintaining proper conformation of apocytochrome *b* (39). Treatment of bovine bc_1 complex with N-(ethyl-carbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ), a reagent that chemically modifies buried carboxyl groups resulted in the loss of proton pumping activity in liposome-reconstituted bc_1 complex but with no effect on the passive proton pumping of the proteoliposomes (40). These results suggest that some carboxyl groups in Core II can be involved in proton pumping activity of the complex (40,41).

Subunit VI interacts with cytochrome *b* of one monomer and with Core I and Core II of the other monomer. Core II and cytochrome *b* subunits contribute major dimer interactions across the two-fold symmetry of bovine bc_1 complex. On the other hand, the presence of Core II on the negative side of the membrane, together with subunit VI, may contribute a possible proton pathway to the ubiquinone reduction site (32,33). However, based on the 3-D structure of the bovine bc1 complex, the distance between the Qi site

and core II is not close enough, hence, more evidences are needed to support this suggestion (26,41).

5. Sequence alignment analysis

The Core I protein of the bovine bc1 complex presents 56% sequence identity with the β subunit of rat MPP, 38% sequence identity with the β subunit of yeast MPP and 42% sequence identity with the β subunit of potato MPP, whereas Core II protein of the bovine bc_1 complex is 27% identical to the α subunit of rat MPP, 28% identical to the α subunit of yeast MPP and 30% identical to the α subunit of potato MPP (42,45).

6. Subunit IX (presequence of ISP)

As mentioned before, Core I and Core II subunits enclose a big cavity. This cavity is filled with subunit IX (Fig. 4.). The subunit IX of the bovine heart cytochrome bc_1 complex, with a molecular weight of 8.0 KDa, is the 78 amino acid presequence that is cleaved post-translationally from ISP (subunit V of the bc_1 complex) as it is imported and targeted to the mitochondrial cytochrome bc_1 complex. The Rieske protein in the bovine bc_1 complex is processed in a single proteolytic step and is retained as a subunit (subunit IX) in the complex (27) and it should be noted that a homologous subunit is not present in *Saccharomyces cerevisiae* (46) and *Neurospora crassa* (47) because the processing of the ISP presequence is different and it is removed in two steps and degraded in vivo by another as yet unidentified protease. After undergoing a sequence alignment, 88% of the presequence was found to be identical between the bovine, human and rat species. Removing the presequence of ISP generated the mature ISP protein (27).

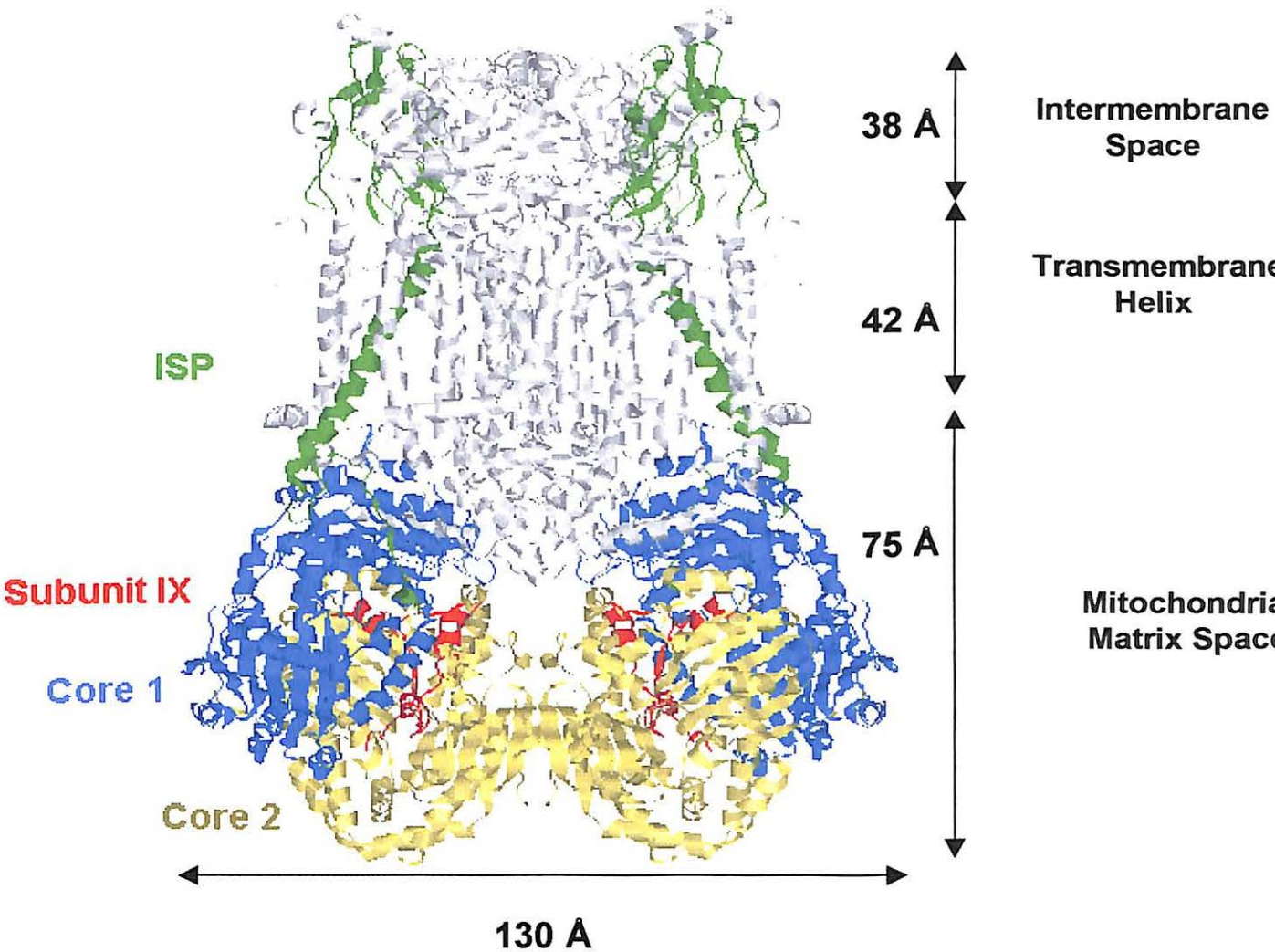

Fig. 4. Ribbon drawing of the dimeric bovine mitochondrial cytochrome bc_1 complex showing the presequence of the iron-sulfur protein retained as a subunit (subunit IX) in the hollow cavity formed by the Core I and Core II subunits.

B- Mitochondrial Processing Peptidase (MPP):

The majority of nuclear encoded mitochondrial proteins are synthesized with amino-terminal presequences that target the preprotein to the intra-mitochondrial location (matrix) where they are cleaved proteolytically by the mitochondrial processing peptidase (MPP) (47).

The mitochondrial processing peptidases also known as the matrix processing peptidases are soluble heterodimeric proteins localized in the mitochondrial matrix and belong to a family of Zn^{2+}-dependent metalloendopeptidases whose members include insulin degrading enzymes from mammals and protease III from bacteria. This family of metalloendoproteases is the pitrilysin family (47,48).

Mammalian MPP consists of α and β subunits. The β-MPP subunit contains the zinc-binding domain $HXXEHX_{76}E$ and the active site of the protease, whereas the α-MPP subunit is thought to bind the leader peptide (44).

The MPPs from *Saccharomyces cerevisiae*, rat liver and *Neurospora crassa* also contain the two α- and β- subunits. In *Saccharomyces cerevisiae* and rat liver both the α- and β-MPP subunits are located in the mitochondrial matrix (48-50), whereas in *Neurospora crassa*, the β-subunit is located in the matrix (51) but the α-subunit is a membrane-associated protein (52).

In plants, and more specifically in potato, spinach and wheat, the MPP subunits are the core subunits of the bc_1 complex and are located in the mitochondrial inner membrane (44,53,54,55).

1. MPP from Saccharomyces cerevisiae

The MPP from *Saccharomyces cerevisiae* contains two polypeptides with apparent molecular weights of 48 and 51 KDa and are believed to be only loosely associated together. Based on Comassie Blue staining, the purified enzyme shows an approximate 1:1 ratio between the two polypeptides (48). The α-MPP was co-expressed with N-terminal 6-His-tagged β-MPP and both subunits assembled into a fully active and soluble enzyme after being co-eluted from a nickel-derivatized affinity resin with a 1:1 stochiometry. Individually purified α- and β-subunits are inactive (56,57) and functionally asymmetric. Cross-linking studies showed that purified α-MPP is responsible for the binding of mitochondrial precursors before their presentation to the catalytic site of MPP in the absence of β-MPP.

2. MPP from rat liver

Mammalian MPP was successfully purified to homogeneity from rat liver mitochondria (49,50). The purified enzyme consists of two subunits; α-MPP (also called P-55) and β-MPP (also called P-52) with apparent molecular masses of 55 and 52 KDa respectively. Unlike in *Saccharomyces cerevisiae*, these two subunits are very tightly associated forming a heterodimer complex (50). The c-DNAs encoding both MPP subunits from rat liver were over-expressed separately in *E.coli* and the recombinant proteins were successfully purified to homogeneity (58). Results showed that both subunits are required for the complex activity, which was reconstituted with a denaturation-renaturation protocol, and the binding of MPP to substrate peptides is found to be 30-fold greater in the dimeric complex than in each subunit monomer (59).

3. MPP from *Neurospora crassa*

In *Neurospora crassa* the α-MPP and β-MPP are also called processing enhancing protein (PEP) (51). The purified enzyme consists of two polypeptides, α-MPP and β-MPP, with a 2.6:1 molar ratio. The molecular masses of the two subunits are 57 KDa and 52 KDa respectively, estimated by SDS-PAGE. These two proteins do not form a complex when separated by FPLC gel filtration (52) but both subunits are required for MPP activity. The purified β-MPP subunit from *Neurospora crassa* alone exhibited very low detectable processing activity. However, the addition of purified α-MPP, which also does not have any processing activity by itself, results in the stimulation of the processing activity, concluding that both of these subunits were required for the full processing activity.

4. MPP from potato tuber

Although the purification of MPPs from plants has not reached homogeneity level, it was reported that in potato and spinach leaf MPP, the MPP activity co-fractionated with the mitochondrial membrane. In both latter species, MPP could not be dissociated from mitochondrial membrane even when treated with high pH (pH,11), high ionic strength (1M KCL), chaotropic reagent (4M urea) or detergent (0.5% triton X-100).

By comparing the processing activity of the membrane and the matrix portion toward a precursor protein, it was found that potato tuber mitochondrial membrane contains at least 100 times more processing activity than the soluble fraction. By immuno-precipitation with antibodies against *Neurospora crassa* MPP, the bc_1 complex was identified as the protein complex associated with MPP activity (44).

5. MPP from spinach leaf

The isolation of MPP (bc_1 complex) from spinach leaf mitochondria was accomplished by its solubilization from mitochondrial membrane with 50mM dodecyl β-maltoside followed by anion-exchange chromatography and gel-filtration chromatography. It was detected that MPP activity was entirely associated with the bc_1 complex (55).

6. Potato bc_1 complex

Potato bc_1 complex consists of 10 protein subunits; three redox containing subunits which are cytochrome *b*, cytochrome c_1 and ISP, and three core subunits with molecular masses of 55, 53 and 51 KDa, in addition to four other supernumerary subunits (44). The purified potato bc_1 complex exhibits MPP activity. The three core subunits were identified by specific antibodies raised against *Neurospora crassa* Core I subunit (60). However the separation of these three core subunits from the complex resulted into the aggregation of the remaining sub-complex and irreversible loss of processing activity (61).

7. Spinach leaf bc_1 complex

Similar to potato bc_1 complex, spinach bc_1 complex consists of 10 protein subunits including three redox subunits, three core subunits with a molecular weight of 61 KDa, 54 KDa and 52 KDa, and four supernumerary subunits. The relationship between spinach Core I protein and β-MPP and between Core II – Core III proteins and α-MPP was

established after the recognition of Core I by specific antibodies raised against yeast β-MPP, and the recognition of Core II - Core III by antibodies against yeast α-MPP (55).

C- Active sites of MPPs and bovine bc_1 complex

MPP belongs to the pitrilysin family of zinc metalloproteases because of the presence of a zinc-binding motif (Fig. 5). MPPs of different species share common amino acid motifs, including the zinc-binding motif. This zinc binding motif HXXEH(X)72-76E is located in the β-MPP subunit of MPP and is actually the inversion of the thermolysin zinc binding motif HEXXHX...(Fig. 6) (62). The histidine residues, together with the glutamic acid residue located 76 residues downstream, are involved in zinc binding and constitute the active site of MPP (62-64). Most of the studies done on the active site(s) of MPP involve the HXXEHX76E motifs of MPPs.

Moreover, it was recently discovered that distal and proximal basic amino acids from cleavage sites are required for an effective processing of the presequence (65,66). The distal basic sites are required as positively charged amino acids, lysine and arginine, and the proximal basic site should be an arginine residue located at the –2 position (P_2). In addition, hydrophobic and hydrophillic residues at P_1 and P_2-P_3, respectively, are also important for effective catalysis (67-69).

The α-MPP regulates the catalytic activity of β-MPP and participates in substrate recognition (70). A so-called glycine rich loop that belongs to the α-MPP subunit was suggested to be involved in substrate binding and/or product release because of it's flexible loop structure (71).

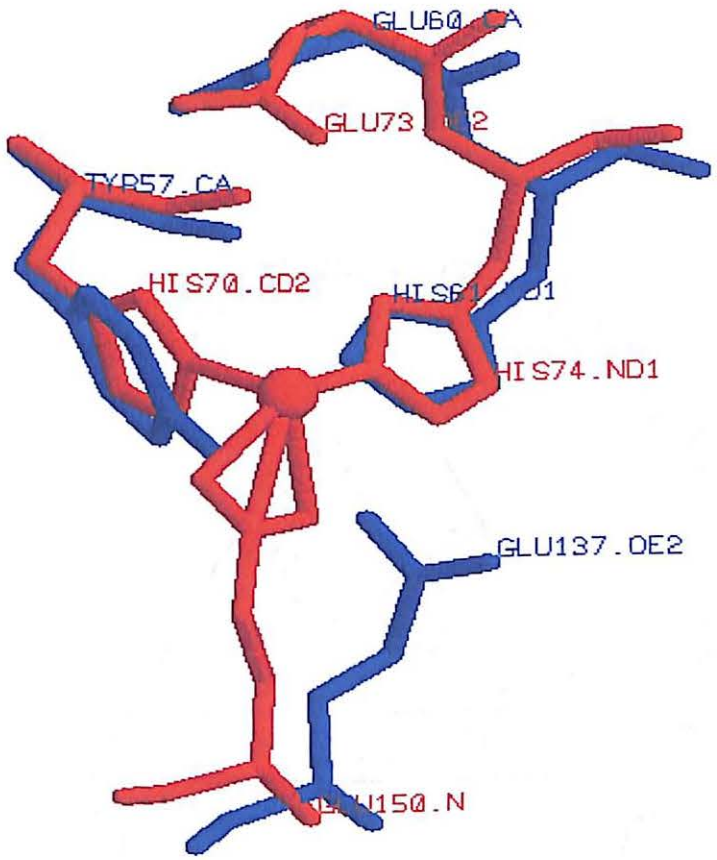

Fig. 5. Drawing model representing residues of the yeast β-MPP zinc binding site (red) superimposed with the residues of the Core I active site of the bovine bc_1 complex (blue).

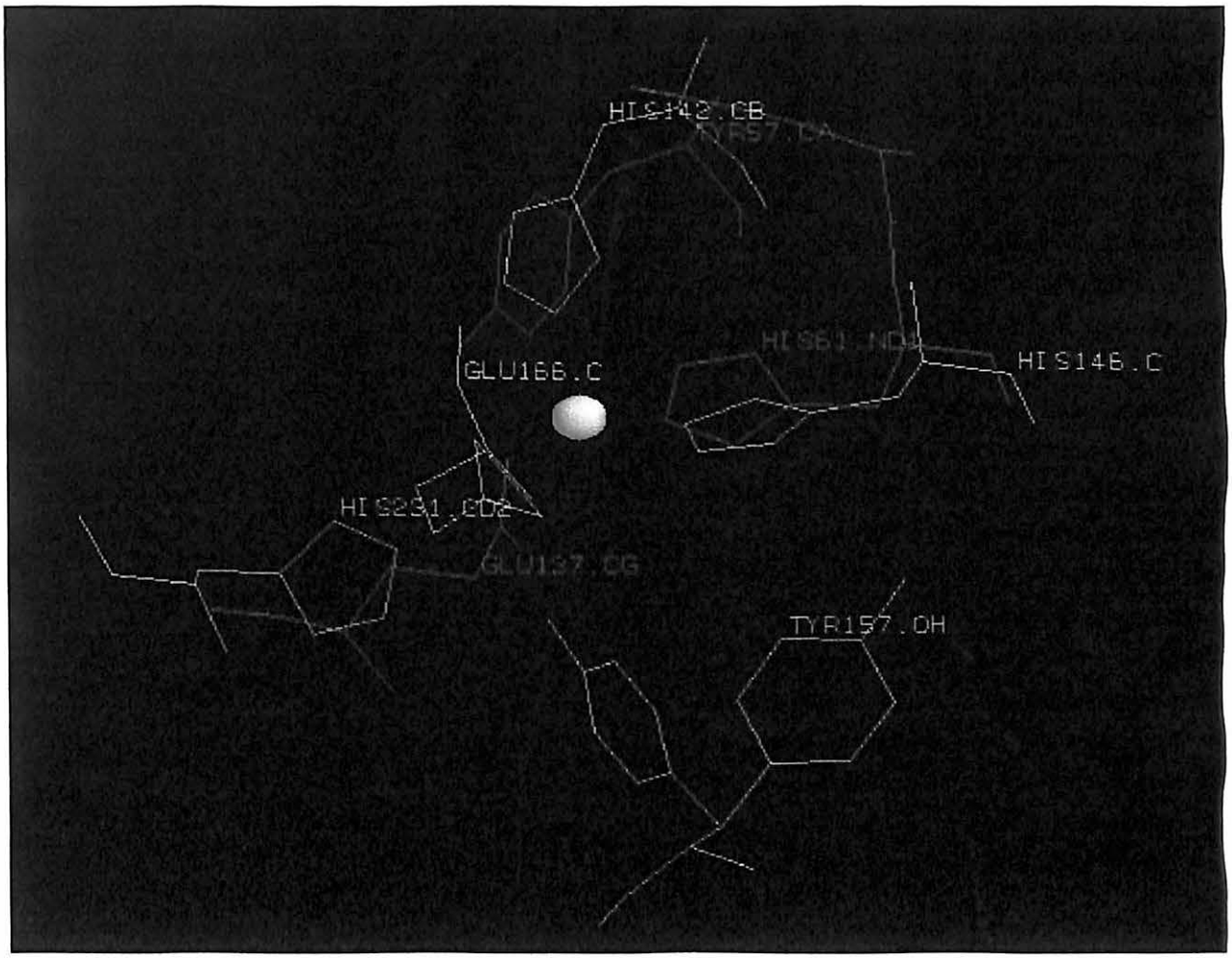

Fig. 6. Drawing model representing residues of the thermolysin zinc binding site (white) superimposed with the residues of the Core I active site of the bovine bc_1 complex (blue).

1. Active site of rat MPP

In the rat β-MPP, the histidine residues and glutamic acid residues of the HXXEHX76E motif were mutated individually, and these mutations decreased the processing activity, which explains the critical role of this motif in the processing activity of MPP (72-74). Some species contain another motif in the α-MPP subunit as the HXXEK motif in rat α-MPP, and mutations along this motif do not have the drastic effects as the ones resulted from mutations in the β-MPP motif (74). More recently, individually mutating Glu191 and Asp195 of rat β-MPP to alanine and quantifying their cleavage activities with that of the wild type using a synthetic substrate obtained evidences that these residues interact with the P2 arginine of precursor protein through ionic and hydrogen bonds, respectively. The E191A and D195A MPP activities were about 50% and 6% of the wild type, respectively, whereas the combined mutation E191A/D195A showed more drastic effect on the activity compared to the wild type. Thus, both Glu^{191} and Asp^{195} seem to be required for the cleavage of extension peptide (75). Hence, β-MPP shows a predominant role in the formation of active site although no direct proof exists for rat β-MPP to be the catalytic site (72-74).

2. Active site of yeast MPP

Unlike in yeast, mutations in the α- and β-subunits indicate that the whole HXXEHX76E region is required for the proper formation of the active site of MPP, and some structural contact was found between the motif and the α-MPP subunit (56).

Substituting the first histidine residue to arginine terminated the zinc binding ability in the mutant β-MPP (76). Besides, deleting a portion of the C-terminal fragment

in α-MPP causes a significant decrease in binding of β-MPP and in processing activity. In addition, the non-conserved central region surrounding lysine-124 of β-MPP was found to be involved in the interaction with α-MPP (56). Recently, the crystal structures of recombinant yeast MPP and a cleavage-deficient mutant of MPP-complexed synthetic presequence peptides was determined and it was found that MPP can bind the short peptide in the extended conformation within a large polar cavity (77). The substrate peptide interacts mainly with β-MPP and its N-terminal site almost reaches the α-MPP.

3. Active site of mitochondrial bc_1 complex

As for the mitochondrial bc_1 complex, a similar zinc-binding motif is found in the Core I subunit. The main variations are that this motif Y57XXE60H61X76E137 has a tyrosine residue at the first position instead of histidine and the distal E137 is close to the five-residue segment in the putative zinc-binding motif of Core I. Since, as mentioned before, histidine residues are required for MPP active site, tyrosine was substituted to histidine. However, the reconstituted activity from Y57H Core I mutant with wild type Core II was the same as the reconstituted activity from wild type Core I and Core II, which suggests that both, tyrosine and histidine, have identical roles in the active site of MPP. Substitution of Y57 with phenylalanine or tryptophan also had no effect on the complex activity, however substitution with threonine eliminated the reconstitutive activity, revealing the functional importance of the aromatic group over the hydroxyl group in position 57 of Core I (78).

D- MPP activaty from bovine heart mitochondrial cytochrome bc_1 complex

Despite the sequence homology between Core I and Core II subunits of the bovine bc_1 complex and the α- and β-subunits of MPP, no MPP activity was detected in the active bovine bc_1 complex.

The bovine heart mitochondrial cytochrome bc_1 complex with electron transfer activity has no mitochondrial processing peptidase activity. The treatment of this complex with increasing concentrations of Triton X-100 at 37°C decreases the electron transfer activity and increases the peptidase activity. Complete inactivation of electron transfer with 1.5mM Triton X-100, a maximum MPP activity is observed. This MPP activity remains constant even with higher concentrations of Triton X-100.

It was established that Triton X-100 disrupts the structural integrity of the bc_1 complex at certain concentrations, uncovered by the loss of its electron transfer activity. Hence, the extent of activity lost indicates the extent of bc_1 structure disruption.

It is suggested that the lack of MPP activity in the mammalian cytochrome bc_1 complex is caused by the binding of an inhibitor polypeptide to the active site of MPP located at the interface of Core I and Core II subunits. This suggestion is based on the three-dimensional structure of the bc_1 complex and the sequence homology between subunits of MPP and the core subunits of beef bc_1 complex.

Hence, Triton X-100 weakens the binding of the inhibitor polypeptide to the active site of MPP in core subunits and activates MPP action. (21)

Similarly to Triton X-100, other non-ionic detergents such as Zwittergent, decanoyl-N-methylglucamide and octyl glucoside also activate MPP in beef cytochrome

bc_1 complex. Even though ionic detergents, such as sodium cholate, deoxycholate and sodium dodecyl sulfate, and other chaotropic reagents such as urea and guanidine HCl inactivate electron transfer, MPP activity is not detected after treating the crystalline bc_1 complex. This is believed to be due to their inhibitory effect on MPP rather than ineffective weakening of the binding of inhibitory polypeptide with the core subunits.

The Triton X-100 activated MPP is pH buffer system, ionic strength, and temperature dependent (21).

1. Optimal conditions for MPP activity

MPP is buffer system and ionic strength dependent. Higher activity is observed in Tris-HCl buffer than in phosphate buffer at pH 8.0. Maximal activity is detected in Tris-HCl buffer at a concentration ranging between 15 and 50mM, and gradual activity decrease is observes at increasing Tris-HCl concentrations. In phosphate buffer, the maximal activity is 80% the maximal activity in Tris-HCl buffer, and similarly it decreases at concentrations higher than 15mM. For both buffer systems the MPP activity decreases with a salt concentration higher than 100 mM.

The optimal pH for MPP activity is between 6.5 and 8.5. At pH values lower than 6.5 a decrease in activity is observes but more drastic decrease is observed at pH values higher than 8.5 (21).

Temperature wise, the maximal activity is observed at 37°C and drops drastically at 42°C because of some partial denaturation, unlike MPP in plant bc_1 complex, which is active at 50°C (79).

The optimal conditions at which the maximal MPP activity is observed is after 2 hours of incubating the bc_1 complex with 15mM Tris-HCl buffer pH 8.0 containing 1.5mM Triton X-100 at 37°C. Prolonged incubation under the same conditions decreases slightly MPP activity.

MPP activity requires the presence of metal ions but cannot be stimulated by the addition of divalent cations. The addition of metal ion chelators such as EDTA inhibits it and increasing concentrations of chelators gradually lowers it. But this chelator-inhibited activity is restored in different extents by the addition of divalent cations like Fe2+, Zn2+, Co2+, Mn2+, and Mg2+ indicating how essential they are and required for MPP activity (21).

MPP is a substrate specific enzyme and this specificity is governed by the length of the NH2-terminal end of the mature subunit rather than the length of the COOH-terminal end of the presequence. This conclusion was reached after synthesizing several polypeptides composed of various lengths of COOH-terminal presequences and NH2-terminal sequences of mature subunits and tested as substrates to examine where specifically MPP cleaves the peptide (21).

E- Reconstitution of MPP activity from Core I and Core II subunits of bovine bc_1 complex

To study the structure-function of MPP/bc_1 complex, reconstituted active Core I and Core II subunits of bovine bc_1 complex are required.

The reconstituted active subunits were obtained by gene expression where recombinant Core I and Core II were generated. The pET expression system was used for this purpose since it can introduce a 6-His-tag upstream of the N-terminus of the expressed protein, which allows a one-step purification of recombinant protein with Ni-NTA gel.

Hence, recombinant Core I and Core II were generated for reconstitution studies, and since pISP is the natural substrate for MPP/bc_1 which cleavage product is subunit IX, pISP was generated for studies of MPP/ bc_1 inhibition by subunit IX (78)

1. Expression conditions

Several factors such as IPTG concentration, induction growth time, medium and temperature are believed to affect the expression of recombinant His-tagged Core I, Core II, and pISP.

The yield increases as the IPTG concentration and induction growth time are increased, reaching a maximum at 0.5mM of IPTG and 3 hours of post-induction growth. Running a western blot using antibodies against the respective proteins revealed that a post-induction growth longer than 3 hours results in a lower yield and increased degradative products.

The expression levels for core I, Core II and pISP in *E.coli* are very high using LB medium at 37°C and represent almost 30% of the total cellular protein. However, 95% of the expressed protein was found in the inclusion precipitate and when they were solubilized in 8M urea, dialyzed and passed through a Ni-NTA column, the proteins were recovered in very small amounts and inactive (78).

This problem was overcame by adding betaine and sorbitol to the LB medium to a final concentration of 2.5mM and 0.44M respectively, and with the IPTG induction done at 27°C for 3 hours (80,81). This solubilization increased the yield of the soluble expressed protein in *E.coli*, and about 6mg of purified recombinant Core I, 5mg of Core II and 6mg of pISP were obtained from 500ml of their respective cell cultures.

2. Reconstitution of MPP from purified recombinant Core I and Core II

Like α- and β- subunits of MPP, individually purified recombinant Core I and Core II show no MPP activity. However, the activity is reconstituted by mixing the purified proteins together. The reconstituted MPP activity increases as the molar ratio of Core I to Core II increases and maximum activity is detected when this molar ratio is 1. These results indicate that recombinant Core I and Core II are active.

The MPP activity detected in Triton X-100 treated complex is associated with Core I and Core II and the structural integrity of the bc_1 complex is not required for MPP/bc1 activity.

Similar to Triton X-100-activated MPP in the bc_1 complex, the processing activity of the reconstituted MPP is inhibited by metal ion chelators. Likewise, the chelator-inhibited activity can be partially restored by the addition of divalent cations and the most effective one is Zn^{2+}, which leads to the conclusion that MPP/bc_1 is a Zn-metallopeptidase. But the role of Zn^{2+} in MPP/bc_1 and yeast MPP is different, although, yeast MPP has been also shown to be a zinc metallopeptidase (76). MPP/bc_1 activity is not totally dependent on Zn^{2+} presence whereas in yeast, Zn^{2+} is required for MPP activity.

The reconstituted MPP/bc1 substrate specificity, like Triton X-100 activated MPP in bovine bc_1 complex, depends more on the length of the amino acid sequence of the mature protein portion rather than on the presequence portion (21).

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Chapter II

Interaction between Core I and Core II Subunits of Bovine bc_1 Complex

In this chapter, a detailed scheme about plasmid construction, overexpression and purification of Core $I_{C\text{-}term\ His\text{-}tag}$/Core II_{Untag} complex and mutants generation will be discussed. To study the interaction between Core I and Core II, five mutations were designed in Core I subunit where the residues that are in close proximity to Core II were mutated to alanine. The expression level of Core I and the binding affinity of Core I to Core II in each of the mutants was analyzed and compared to wild type.

A- Study of Core I/Core II interaction

Core I and Core II proteins, which similarly to α-MPP and β–MPP are not active unless found in a complex form, form a heterodimer complex in the bovine bc_1 complex with two contact regions between them revealed in the crystal structure of the bovine complex (1). The first contact region is located between the C-terminal part of Core I and the N-terminal part of Core II and includes residues S291 and H289 of Core I (Fig. 7), and the second contact region is located between the N-terminal part of Core I and the C-terminal part of Core II and includes residues E80 and K77 of Core I (Fig.8). These residues will be mutated singly and in combination to determine the essentiality of each in the binding affinity of Core I to Core II.

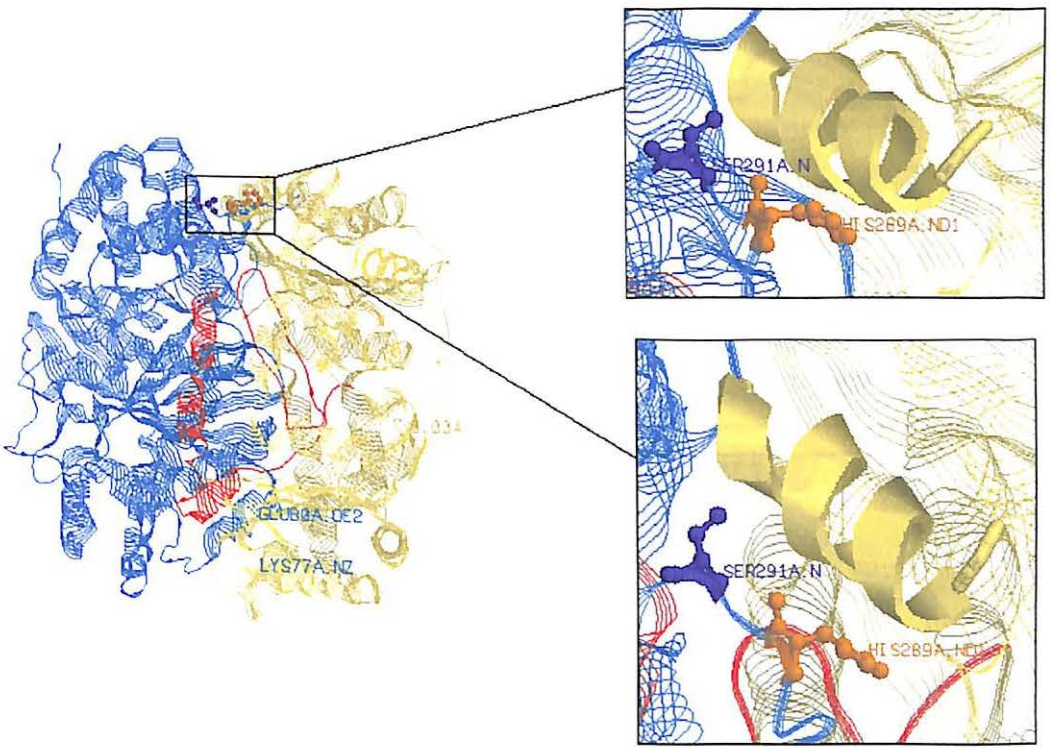


Fig. 7 . Drawing model representing the close distance between the residues H289, and S291 of the Core I subunits and one of Core II alpha helices.

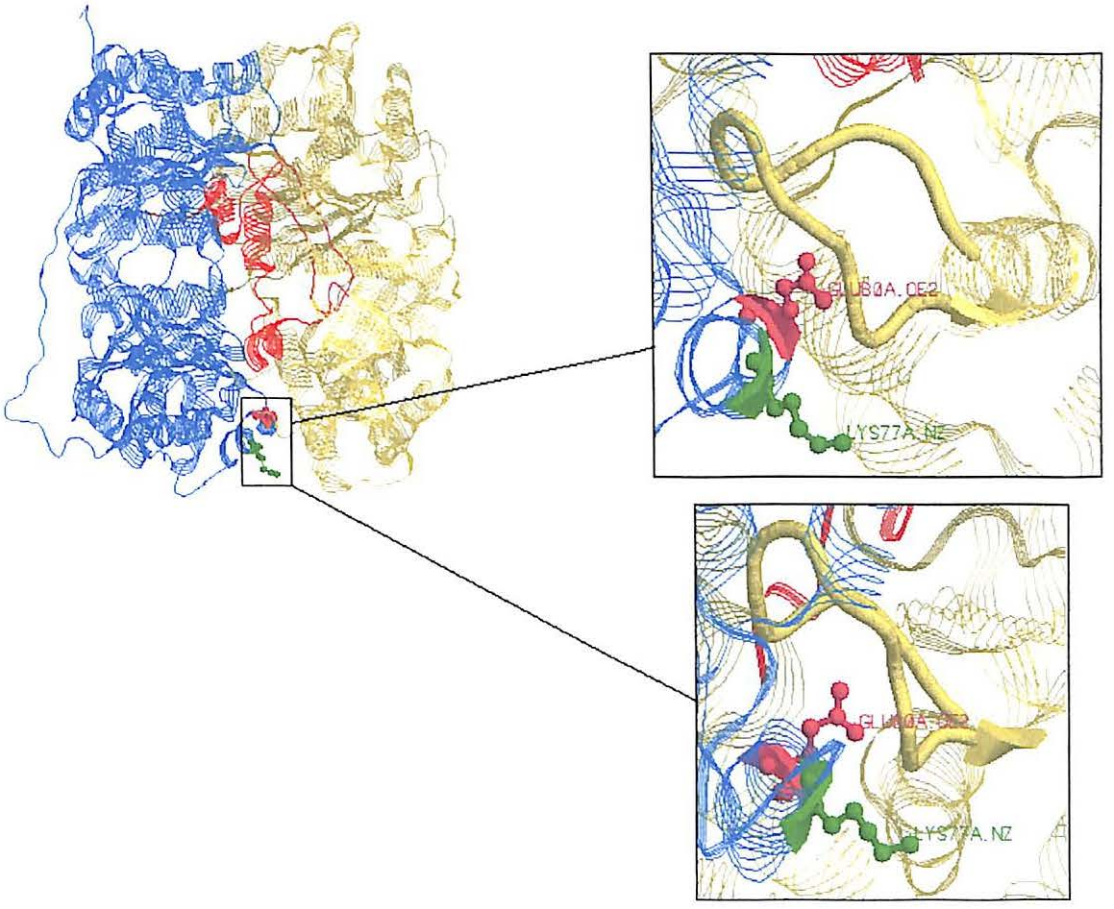


Fig. 8. Drawing model representing the close distance between the residues K77 and E80 of the Core I subunits, and the so-called glycine rich loop of the Core II subunit.

Core I/Core II complex formation will be studied using site directed mutagenesis followed by Ni-NTA column separation, SDS-PAGE and Western Blot analysis. Mutant Core I will be expressed in a His-tagged vector whereas wild type core II will be expressed in an untagged vector. Hence, untagged core II will interact with tagged Core I and the complex can be purified by Ni-NTA column. Expressing the mutant Core I as a his-tag protein eliminated the possibility that that the mutation actually resulted in decreased expression level of the protein rather than decreased the binding affinity. For instance, if a mutation in Core I decreases the expression level of untagged Core I as compared to wild type, and wild type Core II is expressed as a His-tagged protein, then less Core I will be available to interact with Core II and upon purification of the complex, mutant His-tagged Core I/ wild type untagged Core II ratio will be less than wild type His-tagged Core I/ wild type untagged Core II ratio even though the mutation does not actually affect the binding affinity of Core I to Core II. For this reason, expressing the mutant Core I as the His-tagged protein and wild type Core II as the untagged protein followed by purification by Ni-NTA column will remove all the excess wild type Core II and the only reason for a decreased mutant His-tag Core I/wild type untagged Core II ratio can be that the mutation in Core I affects the binding affinity of Core II to Core I.

B- Materials

LB broth and LB agar were purchased from Invitrogen and Life Technologies Inc. respectively. The TA Cloning kit was from Invitrogen. *Taq* DNA polymerase, T4 DNA ligase and restriction endonucleases were obtained from either Promega or Life Technologies,Inc. Kanamycin and ampicillin were from Fisher Scientific. Betaine,

sorbitol, pheny-methyl-sulfonyl fluoride, dimethyl sulfoxide, and imidazole were obtained from Sigma. Centriprep YM-30 was from Amicon. Nitrocellulose membranes were from Osmonics. Oligonucleotides were synthesized by the DNA/Protein Core Facility at Oklahoma State University or were obtained from Sigma Genosys. Antibodies against bovine bc_1 complex were generated in rabbits and purified in our laboratory. Antibodies against His-Tag were obtained from Bio-rad. Isopropyl β-D-thiogalactopyranoside was from Bio Vectra. Plasmid "Mini-prep" kit, "Gel extraction" kit and Ni-NTA gel were purchased from Qiagen. Other chemicals were obtained commercially in the highest purity available.

E.coli INVα'F (F'*end*A, *rec*A, *hsd*R17 (r_k-m_k-), *sup*E44, *thi*-1, *gyr*96, *rel*A1, ϕ80*lac*ZΔm15, Δ(*lac*ZYA-*arg*F)U169λ-), *E.coli* BL21c$^+$ (DE3) (F' *ompT hsdS*(r_B- m_B-) *dcm*$^+$ Tet^r *gal* λ(DE3) *endA* Hte [*argU ileY leuW* Cam^r] were used as hosts for pCR2.1 (invitrogen) and for pET 22b$^+$ or pET 30a$^+$ (Novagen) plasmids.

C- Experimental Procedures

1. DNA Manipulation

Mini-preparation of plasmid was performed according to Qiagen user's manual. DNA sequencing and oligonucleotide synthesis were performed by the Recombinant DNA/Protein Resource Core Facility at OSU.

2. Isolation of cDNAs of Core I and Core II

The cDNAs for Core I and Core II were isolated from bovine heart cDNA library in λZAP (Stratagene) by PCR amplification. Sense- and antisense- primers were

synthesized based on the 5'-end and 3'-end of the cDNA sequences of mature Core I and Core II with restriction enzyme sites at the 5'-end of the oligonucleotides (2).

3. Construction of Core I and Core II expression vectors

The *Nde*I-*Hind*III Core I fragment and the *Nde*I-*Hind*III Core II fragment were ligated to an enzyme-cut pET $22b^+$ and pET $30a^+$ vector, respectively. Both vectors contain a gene encoding a 6His-tag located upstream of the multiple cloning site to allow one step purification of Core I or Core II with Ni-NTA affinity column. The resulting plasmids, pET$22b^+$/Core I and pET$30a^+$/Core II were transformed into BL21c^+(DE3) competent cells by chemical transformation. The core proteins expressed by the *E. coli* transformants were identified by western blot using antibodies against bovine bc_1 complex.

4. Generation of pET/Core $I_{C\text{-terminal His-tag}}$ and pET/Core $II_{C\text{-terminal His-tag}}$

The DNA fragments encoding mature Core I and Core II were amplified by polymerase chain reaction (PCR) using in each case two specific synthetic primers containing the full restriction site of the specified endonucleases (NdeI or HindIII) at their 5' ends, using the pET/Core $I_{N\text{-terminal His-tag}}$ and pET/Core $II_{N\text{-terminal His-tag}}$ (2) as template, respectively.

The following primers were used for Core I amplification:

sense primer	5'-CATATG (NdeI) ATACCGCCACCGCCACCTACGCC-3'
antisense primer	5'-AAGCTT (HindIII) GAAGCGCAGCCA-3'

The following primers were used for Core II amplification:

sense primer	5' -CATATGGTCCCTCAAAGTTGCT- 3' (NdeI)
antisense primer	5' -AAGCTTCAACTCATCAATGAA- 3' (HindIII)

The PCR mixture contained 1x Mg free PCR buffer, 75pmoles $MgCl_2$, 125ng of each of the sense primer and anti-sense primer, 50ng of the respective template, 20nmoles dNTPs and 2.5 units of *Taq* polymerase.

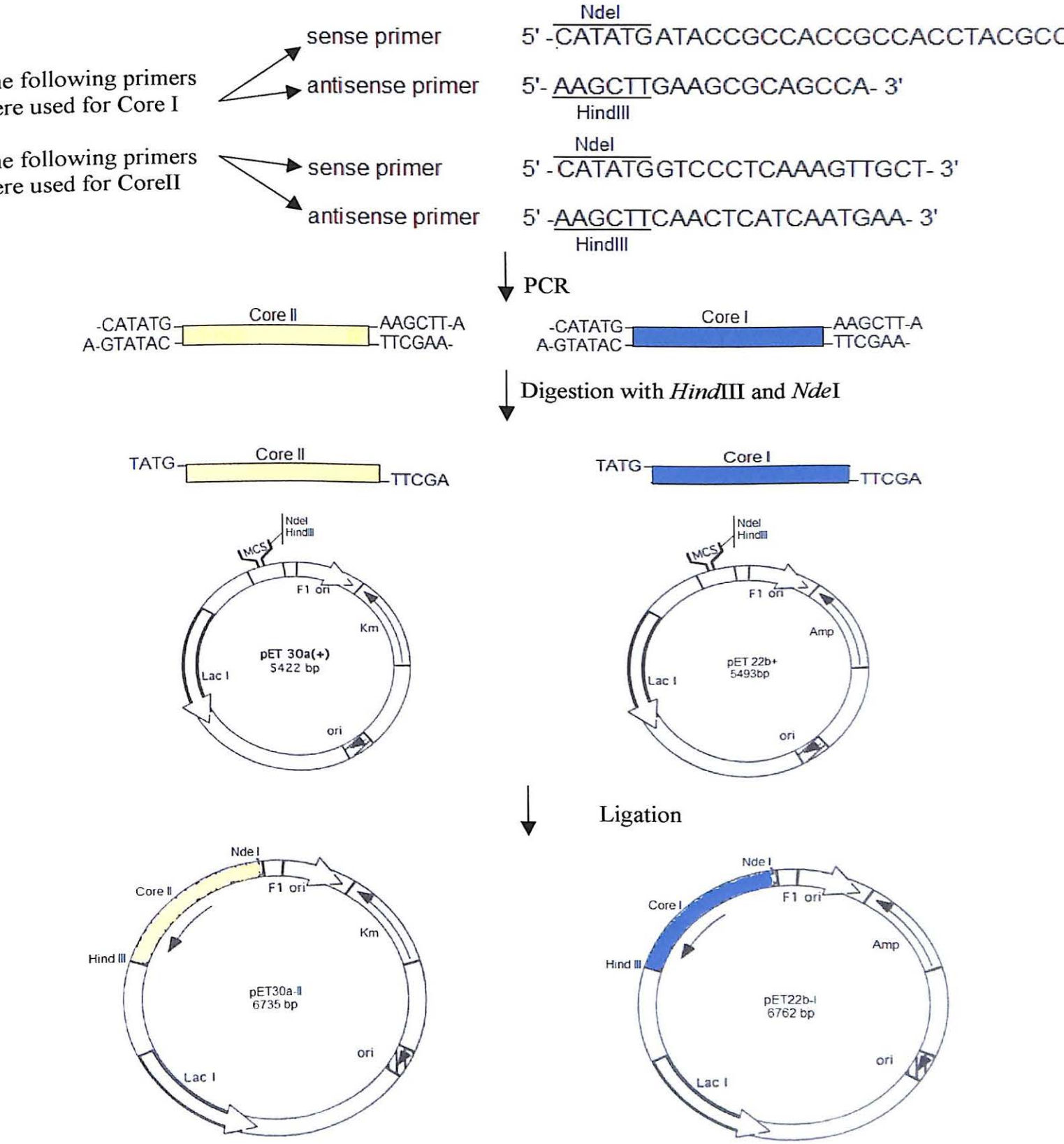
The PCR reaction was performed as follows: initial denaturation at 94°C for 1 minute, denaturation at 94°C for 45 seconds, hybridizing at 55°C for 30 seconds and extension at 72°C for 1.5 minutes. The reaction was carried through 16 cycles with a final extension step at 75°C for 10 minutes. Four µl of PCR mixture was electrophoresed on a 1% agarose gel, stained with ethidium bromide, to confirm the size of the PCR product.

The generated NdeI-HindIII fragments encoding Core I or Core II were digested with the proper enzymes (NdeI and HindIII) and ligated to their respective vectors (Scheme. 3):

- Core I fragment was ligated to NdeI-HindIII digested $pET22b^+$ to generate the plasmid pET/Core $I_{C\text{-terminal His-tag}}$
- Core II fragment was ligated to NdeI-HindIII digested $pET30a^+$ to generate the plasmid pET/Core $II_{C\text{-terminal His-tag}}$

Ligated plasmids were transformed to $BL21c^+(DE3)$. pET/Core $I_{C\text{-terminal His-tag}}/Bl21c^+(DE3)$ cells were selected on ampicillin plates and pET/Core $II_{C\text{-terminal His-}}$

$_{tag}$/BL21c^{+}(DE3) cells were selected on kanamycin plates. The sequences of the inserts were confirmed by DNA sequencing.



Scheme. 3. Generation of Core $I_{C\text{-term His-tag}}/pET22b^{+}$ and Core $II_{C\text{-term His-tag}}/pET30a^{+}$

5. Generation of Core $II_{untag}/pET30a^+$

The $(His)_6$ tag was deleted from the pET/Core $II_{C\text{-terminal His-tag}}$ plasmid by the QuickChange® site directed mutagenesis method using the QuickChange® site directed mutagenesis kit from Stratagene in addition to two specific synthetic primers from Sigma, to generate the pET/Core II_{untag}.

The following primers were used:

sense primer	5' –CGGCCGCACTCGAGTGAGATCCGGCTG- 3'
anti-sense primer	3' –GCCGGCGTGAGCTCACTCTAGGCCGAC- 5'

The QuickChange® site directed mutagenesis reaction contained 1x cloned *pfu* buffer, 50ng of the template pET/Core $II_{C\text{-terminal His-tag}}$, 125ng of each of the sense and antisense primers, 25nmolesdNTP, and 2.5 units of *pfu* polymerase. The QuickChange® site directed mutagenesis reaction was performed as follows: initial denaturation at 95°C for 30 seconds, denaturation at 95°C for 30 seconds, hybridization at 55°C for 1 minute and extension at 68°C for 12 minutes. The reaction was carried through 18 cycles. The product of this reaction was first digested with *Dpn*I to digest the parental strands and then transformed to XL1-Blue. The plasmid was purified from XL1-Blue and sequenced to confirm the mutation and then transformed to $BL21c^+(DE3)$. pET/Core $II_{untag}/BL21c^+(DE3)$ cells were selected on kanamycin plates.

6. Co-transformation of pET/Core $I_{C\text{-terminal His-tag}}$ x pET/Core II_{untag}

Competent cells were prepared from pET/Core $II_{untag}/BL21c^+(DE3)$.

pET/Core $I_{C\text{-terminal His-tag}}$ was then transformed into pET/Core II_{untag}/BL21c^{+}(DE3), and cells were selected on (ampicillin+kanamycin) plates to generate:

[pET/Core $I_{C\text{-terminal His-tag}}$ x pET/Core II_{untag}]/BL21c^{+}(DE3)

7. Generation of pET/ Core $I_{C\text{-terminal His-tag}}$ mutants

pET/Core $I_{C\text{-terminal His-tag}}$ were generated by QuickChange® site directed mutagenesis reaction, following the reaction mentioned above except that the template used is pET/ Core $I_{C\text{-terminal His-tag}}$ and the following primers were used for each mutant:

E80A mutant:

sense primer

5' –GCCTTGGAGAAGGAGGTGGCGAGCATGGGGGCCCATC- 3'

anti-sense primer

3' –CGGAACCTCTTCCTCCACCGCTCGTACCCCCGGGTAG- 5'

S291A mutant

sense primer

5' –GGTGGCGGAGCGCACCTGGCCAGCCCACTGGCTTCCATTG- 3'

anti-sense primer

3' –CCACCGCCTCGCGTGGACCGGTCGGGTGACCGAAGGTAAC- 5'

K77A

sense primer

5' –CCTGGCAATGCCTTGGAGGCGGAGGTGGAGAGCATGGGGG- 3'

anti-sense primer

5' –CCCCCATGCTCTCCACCTCCGCCTCCAAGGCATTGCCAGG- 3'

H289A

sense primer

5' –CGGTGGCGGAGCGGCCCTGTCCAGCCCAC- 3'

anti-sense primer

5' –GTGGGCTGGACAGGGCCGCTCCGCCACCG- 3'

The QuickChange® site directed mutagenesis reaction was performed as mentioned above. The product of this reaction was first digested with *Dpn*I to digest the parental strands and then transformed to XL1-Blue. The purified plasmid's sequence was confirmed by DNA sequencing and transformed to BL21c^{+}(DE3). pET/Core $I_{C\text{-terminal His-tag}}$/BL21c^{+}(DE3) mutant cells were selected on ampicillin plates.

8. Generation of the double mutant pET/(E80A/S291A)-Core$I_{C\text{-terminal His-tag}}$

The generation of the double mutant pET/(E80A/S291A)-Core $I_{C\text{-terminal His-tag}}$ was performed by QuickChange® site directed mutagenesis reaction as mentioned above except that pET/S291A-Core $I_{C\text{-terminal His-tag}}$ was used as template and the E80A mutant sense and anti-sense primers listed above were used.

9. Co-transformation

pET/Core $I_{C\text{-terminal His-tag}}$ mutants were transformed to already prepared pET/Core II_{untag}/BL21c^{+}(DE3) competent cells, and cells were selected on (ampicillin+kanamycin) plates to generate the following [pET/Core$I_{C\text{-terminal His-tag}}$x pET/Core$II_{untag}$]/BL21c^{+}(DE3) mutants:

- [pET/ K77A-Core $I_{C\text{-terminal His-tag}}$ x pET/Core II_{untag}]/BL21c^{+}(DE3) or K77A

- [pET/ E80A-Core $I_{C\text{-terminal His-tag}}$ x pET/Core II_{untag}]/BL21c^{+}(DE3) or E80A

- [pET/ H289A-Core $I_{C\text{-terminal His-tag}}$ x pET/Core II_{untag}]/BL21c^{+}(DE3) or H289A

- [pET/ S291A-Core $I_{C\text{-terminal His-tag}}$ x pET/Core II_{untag}]/BL21c^{+}(DE3) or S291A

- [pET/(E80A/S291A)-Core $I_{C\text{-terminal His-tag}}$ x pET/Core II_{untag}/BL21c^{+}(DE3) or E80A/S291A

10. Co-Expression of recombinant Core $I_{C\text{-terminal His-tag}}$ /Core II_{untag} complex in *E.coli*

A fresh colony is inoculated in 25ml LB broth in a 125ml flask containing 125μg/ml ampicillin and 50μg/ml kanamycin and grown overnight at 37°C. The whole inoculate is then transferred to a 2 liters flask containing 500ml LB broth containing 2.5mM betaine and 0.44M sorbitol, in addition to ampicillin and kanamycin to the corresponding final concentration. Cells were grown at 37°C until $O.D_{600}$ reached 0.7. The medium is then cooled down to the required induction temperature (23°C) and induced for 3 hours with 1.0mM IPTG (100mM IPTG stock solution was used). Cells are harvested by centrifuging the whole medium at 8000xg for 25 minutes. About 2.0 g of cell paste per 500ml culture were obtained and stored at -20°C until used.

11. Core I/Core II complex Purification

Around 2.0 gram of cell paste, obtained from 500ml of cell culture, were suspended in 8.0ml of 50mM sodium/phosphate (Na/K) buffer, pH 8.0, containing 300mM NaCl (PBS). Phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 1.0mM from a stock solution of 500mM in dimethyl sulfoxide (DMSO).

Some DNase, RNase and magnesium chloride $MgCl_2$ were added to the suspended cells in very small amounts. The cells were broken by sonication using Fisher ultrasonics cell disruptor two times for 1.5 minutes with a 2 minute-interval. Triton X-100 was added to the broken cell suspension to a final concentration of 1% (w/v). The suspension was then stirred for 45mins at 0°C and centrifuged at 19K for 25 minutes.

The supernatant was recovered and applied twice in a very slow flow rate to 10ml of Ni-NTA agarose slurry gel, which was equilibrated with the sonication buffer (PBS). The column was first washed with the sonication buffer until the UV absorption at 280 nm of the effluent dropped reached below 0.01. The column was then washed with 20-column volume of 50mM Na/K phosphate buffer pH 6.0, containing 300mM NaCl and 10% glycerol, followed by another 20-column volume wash with sonication buffer containing 20 mM imidazol. The last washing step was done with 15-column volume of sonication buffer containing 35mM imidazol. The recombinant core proteins were then co-eluted from the column with 15ml of sonication buffer containing 100mM imidazol and 0.01%LM. The eluted solution was concentrated to a final volume of 600µl by centriprep YM-30 and frozen at –80°C until use.

12. SDS-PAGE and Western Blot

Proteins were treated with 1% β-mercaptoethanol and 1% SDS for 2 hours at 37°C. Different amount of the digested samples were applied to the wells of a 10% SDS-PAGE gel.

Analytical 10% SDS-PAGE was performed in a Bio-Rad Mini-Protein dual slab cells.

The proteins on the SDS-PAGE gels were electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked with 20mM Tris-Cl buffer pH 7.5, 500mM NaCl (TBS), containing 3% gelatin for one hour at room temperature and washed with TBS for 5 minutes and with TBS containing 0.05% Tween-20 (TTBS) for 5 minutes. The membrane was then incubated with anti-mitochondrial bc_1 antibodies (15μg/ml) in TTBS containing 1% gelatine at room temperature overnight. After incubating with the primary antibodies, the blots were washed twice with TTBS for 5 minutes and then incubated with the protein A-HRP conjugate (1:2500 in TTBS containing 1% gelatin) for one hour. After washing the membrane with TTBS and TBS for 5 minutes respectively, the proteins were detected by showing HRP color development with hydrogen peroxide as substrate. Prestained protein molecular weight standard was used to estimate the size of proteins on the gel and the membrane.

The prestained protein molecular weight standard contained phosphorylase B 113.000Da, bovine serum albumin 92,000Da, ovalbumin 52.300Da, carbonic anhydrase 35.300Da, Soybean Trypsin inhibitor 28.700Da, Lysozyme 21.300Da.

13. Densitometric analysis

The densitometric study was done with a GS-700 imaging densitometer using a captured picture of the SDS-PAGE.

D- Results and Discussion

The Core I/Core II complex interaction can be studied in two ways: *in vivo* or *in vitro*. In *in vitro* studies, each of Core I and Core II is expressed separately in *E-coli* followed by the protein purification. Afterward, Core I and Core II are mixed together and the complex is purified. In this case, if Ni-NTA column is to be used for the complex purification, only one of the two proteins can be tagged with histidine and purified by Ni-NTA. If both proteins are tagged, the complex can be purified by gel filtration. In *in vivo* studies, both proteins are co-expressed in the same cell with one of the two proteins tagged followed by purification of the complex by Ni-NTA column. The latter method was used in this study to analyze the effect of several mutations on the interaction of Core I with Core II. For this purpose, an expression vector for each of Core I and Core II proteins was constructed and both were expressed in the same E-coli cell. As explained in the previous chapter, the his-tag should be on the mutated subunit to eliminate the error of the effect on the expression of the protein. In this study, the residues mutated for the interaction study are located on Core I, hence, Core I protein is his-tagged whereas Core II is not and the complex is purified by Ni-NTA column. The conditions used to co-express and purify the recombinant Core $I_{His\text{-}tag}$/Core II complex from a single cell are stated in the Materials and Methods section.

Theoretically, Core I and Core II amounts in crude cell extract should be similar on SDS-PAGE stained with Coomassie Blue. However, Core I protein seems to be predominant, which is due to a much better expression of Core I compared to Core II. Densitometric analysis of Core I to Core II ratio in crude cell extract is around 4.0, which means that Core I is expressed 4 times better than Core II.

In the soluble fraction of the cell extract, Core I and Core II amounts are almost the same with Core I slightly more than Core II.

Around 10% of Core I proteins of the crude cell extract were expressed as soluble proteins and 90% were expressed in the inclusion bodies. Whereas 35% of Core II proteins of crude cell extract were expressed as soluble protein and 65% as inclusion bodies. As mentioned before Core I protein is four times more expressed than core II in the crude cell extract, which explains the slight abundance of the soluble fraction of Core I compared to Core II.

Structural integrity of these two proteins is studied by mutating several residues in Core I in the two contact regions where the distance between Core I and Core II is minimal. After constructing the Core I mutants co-expressed with Core II in *E. coli* and purifying the complex with Ni-NTA affinity column, a comparative study was done to examine the effect of these mutations first on the protein expression and second on their structural integrity. The comparison was done in the eluted sampled between each of the mutant complexes and the wild type complex. The wild type complex is Core $I_{C\text{-}term\ His\text{-}tag}$/Core II untag. The mutated residues in Core I are K77, E80 and S291. Each of these residues was mutated to alanine individually to generate the Core I mutants K77A-Core $I_{C\text{-}term\ His\text{-}tag}$, E80A-Core $I_{C\text{-}term\ His\text{-}tag}$ and S291A-Core $I_{C\text{-}term\ His\text{-}tag}$, respectively. In addition, a double mutant was constructed, (E80A/S291A)Core $I_{C\text{-}term\ His\text{-}tag}$, where both residues E80 and S291 were mutated to alanine.

As compared to wild type, only around 50% of Core I is expressed in E80A-Core$I_{C\text{-}term\ His\text{-}tag}$/Core II_{untag} whereas the expression level of Core I in each of the mutants, K77A-Core$I_{C\text{-}term\ His\text{-}tag}$/Core II_{untag} and S291A-Core$I_{C\text{-}term\ His\text{-}tag}$/Core II_{untag} is around 90%

of wild type (Fig.9). These results suggest that mutating the residue E80 to alanine significantly decrease the expression level of Core I while mutating either of residues K77 and S291 to alanine has a minimal effect on the expression. In the double mutant (E80A/S291A)-Core $I_{C\text{-}term\ His\text{-}tag}$/Core II_{untag}, the level of Core I expression is equal to the level of Core I expression in E80A-Core $I_{C\text{-}term\ His\text{-}tag}$/Core II_{untag}, which is 50% of wild type Core I (Fig. 9). The decrease in Core I expression level can be explained by the degradation of the expressed protein by the cell as being a toxic foreign protein.

To study the structural integrity between Core I and Core II, the ratio of Core I to Core II was calculated with Core I set as control (100%) and the amount of Core II was compared to its respective Core I. The calculation was done based on the densitometric integration. The ratio of Core I to Core II reflects the structural binding affinity of Core I to Core II. The closer the ratio is to 1.0, the higher the affinity binding is between the subunits. In the wild type complex, Core II amount is 90% that of Core I amount in the eluted sample (Core I to Core II ratio is 1.1) (fig. 10.). The amount of Core I is slightly higher than Core II since the His-tag is located on Core I. In both, K77A-Core $I_{C\text{-}term\ His\text{-}tag}$/Core II_{untag} and S291A-Core $I_{C\text{-}term\ His\text{-}tag}$/Core II untag, the amount of Core II is 90% the amount of Core I (Core I to Core II ratio is 1.1) and hence comparable to wild type, which implies that individually mutating each of the residues K77 and S291 to alanine do not affect the binding of Core I to Core II. However, the amount of Core II is 80% the amount of Core I (Core I to Core II ratio is around 1.25) in the eluted sample of the double mutant (E80A/S291A)-Core $I_{C\text{-}term\ His\text{-}tag}$/Core II_{untag} (Fig. 10). This effect implies that mutating residue E80 to alanine affects the binding of Core I to Core II. Based on these results, it is likely that the charged residue glutamic acid at position 80 in Core I is

favored for better affinity binding between Core I and Core II whereas at position 77 or 291, either lysine (K) or serine (S), respectively, or alanine (A) allow preeminent interaction in the complex.

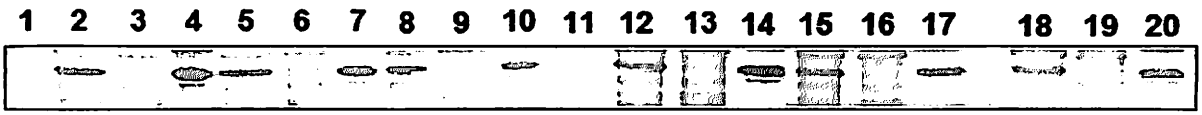


Fig. 9. SDS-PAGE of recombinant complex Core I $_{\text{C-term His-Tag}}$/ Core II $_{\text{untag}}$. Insoluble cell extract were suspended in suspension buffer in the same volume as that of total cell extract. The volume of soluble extract was adjusted to the same as that of total cell extract. Four µl were used on SDS-PAGE for total cell extract, soluble and insoluble cell extract. Lane 1: molecular standard (ovalbumin 49.3 kDa); lane 2: total lysate of the wild type Core I $_{\text{C-term His-tag}}$/ Core II $_{\text{untag}}$; lane 3: soluble fraction of the wild type Core I $_{\text{C-term His-tag}}$/ Core II $_{\text{untag}}$; lane 4: insoluble fraction of the wild type Core I $_{\text{C-term His-tag}}$ / Core II $_{\text{untag}}$; lane 5: total lysate of the mutant Core I S291A $_{\text{C-term His-tag}}$ / Core II $_{\text{untag}}$; lane 6: soluble fraction of the mutant Core I S291A$_{\text{C-term His-Tag}}$/ Core II $_{\text{untag}}$; lane 7: insoluble fraction of the mutant Core I S291A$_{\text{C-term His-Tag}}$/ Core II $_{\text{untag}}$; lane 8: total lysate of the mutant Core I E80A $_{\text{C-term His-Tag}}$/ Core II $_{\text{untag}}$; lane 9: soluble fraction of the mutant Core I E80A$_{\text{C-term His-Tag}}$/ Core II $_{\text{untag}}$; lane 10: insoluble fraction of the mutant Core I E80A$_{\text{C-term His-Tag}}$/ Core II $_{\text{untag}}$; lane 11: molecular standard (ovalbumin 49.3 kDa); lane 12: total lysate of the mutant Core I K77A $_{\text{C-(His)6-Tag}}$/ Core II $_{\text{untag}}$; lane 13: soluble fraction of the mutant Core I K77A$_{\text{C-(His)6-Tag}}$/ Core II $_{\text{untag}}$; lane 14: insoluble fraction of the mutant Core I K77A$_{\text{C-term His-Tag}}$/ Core II $_{\text{untag}}$; lane 15: total lysate of the double mutant Core I [E80A/S291A] $_{\text{C-term His-Tag}}$/ Core II $_{\text{untag}}$; lane 16: soluble fraction of the double mutant Core I [E80A/S291A]$_{\text{C-term His-Tag}}$/ Core II $_{\text{untag}}$; lane 17: insoluble fraction of the double mutant Core I [E80A/S291A]$_{\text{C-term His-Tag}}$/ Core II $_{\text{untag}}$; lane 18: total lysate of the mutant Core I H289A $_{\text{C-term HisTag}}$/ Core II $_{\text{untag}}$; lane 19: soluble fraction of the mutant Core I H289A$_{\text{C-term His-Tag}}$/ Core II $_{\text{untag}}$; lane 20: insoluble fraction of the mutant Core I H289A$_{\text{C-term His-Tag}}$/ Core II $_{\text{untag}}$.

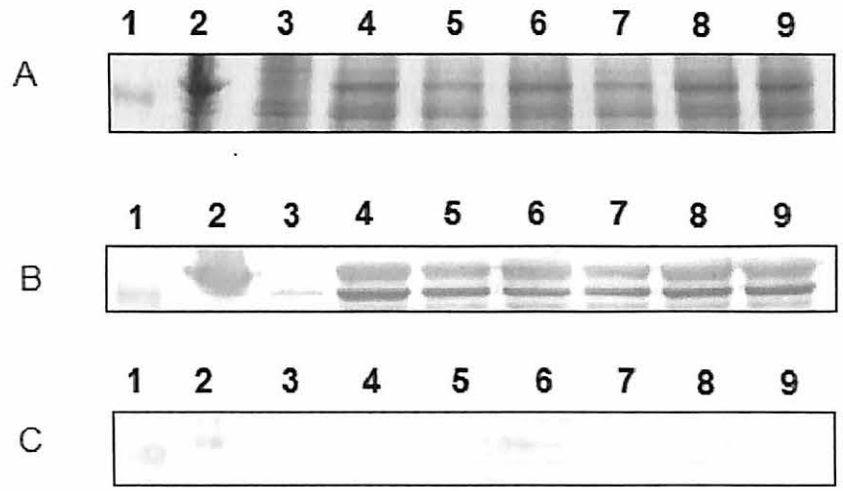


Fig. 10. (A) SDS-PAGE of recombinant complex Core I $_{\text{C-term His-Tag}}$/ Core II $_{\text{untag}}$. (B) Western-blot of the samples in (A) using mitochondrial bc_1 complex antibody as first antibody. (C) Western-blot of the samples in (A) using His-tag antibody as first antibody. Lane 1: molecular standard (ovalbumin 49.3 kDa); lane 2: total lysate of the wild type Core I $_{\text{C-term His-tag}}$; lane 3: total lysate of the wild type Core II $_{\text{untag}}$; lane 4: eluted sample of the wild type Core I $_{\text{C-term His-tag}}$/ Core II $_{\text{untag}}$; lane 5:eluted sample of the mutant Core I E80A $_{\text{C-term His-Tag}}$/ Core II $_{\text{untag}}$; lane 6: eluted sample of the mutant Core I S291A $_{\text{C-term His-Tag}}$/ Core II $_{\text{untag}}$; lane 7: eluted sample of the double mutant Core I [E80A/S291A]$_{\text{C-term His-Tag}}$/ Core II $_{\text{untag}}$; lane 8: eluted sample of the mutant Core I K77A $_{\text{C-term His-Tag}}$/ Core II $_{\text{untag}}$; lane 9: eluted sample of the mutant Core I H289A $_{\text{C-term His-Tag}}$/ Core II $_{\text{untag}}$.

Strain	Core I Expression
W.T	100%
K77A	90%
E80A	50%
S291A	90%
H289A	90%
E80A/S291A	50%

Table. 1. Comparative table for Core I expression in Wild type and mutant Core I. Wild Type Core I expressed in the crude extract was set as control (100%). Each of the expressed mutants Core I in the respective crude extract was compared to wild type.

Strain	Binding affinity of Core II to Core I
W.T	90%
K77A	90%
E80A	80%
S291A	90%
H289A	90%
E80A/S291A	80%

Table 2: Comparative table for the binding affinity between Core I and Core II. In the eluted sample of wild type and each of the mutants, Core I and Core II were quantified and compared to each other by setting Core I as control (100%) and comparing Core II to it.

E- Conclusion

As compared to wild type, K77A, H289A and S291A mutants do not seem to have any significant effect on the expression level of Core I and the binding affinity of Core II to Core I. Therefore, mutating the polar residue at position 77, 289 or 291 to a nonpolar residue do not affect the interaction between Core I and Core II, which suggest that these residues are not involved in any electrostatic interaction with other residues in Core II. On the other hand, E80A mutation in Core I affects the expression level of Core I and the binding affinity of Core II to Core I. Hence, mutating the charged residue glutamic acid at position 80 to a nonpolar residue may be disrupting the structural integrity between Core I and Core II to a certain extent. The side chain of glutamic acid at position 80 is pointed towards the so-called glycine rich loop in Core II and is 3.6Å distant from Aspargine 290 in the loop (Fig. 11), which may predict hydrogen-bonding between the two residues. Therefore, changing the polarity of the residue at position 80 of Core I may disrupt the hydrogen-bonding interaction and affect the affinity binding between Core I and Core II.

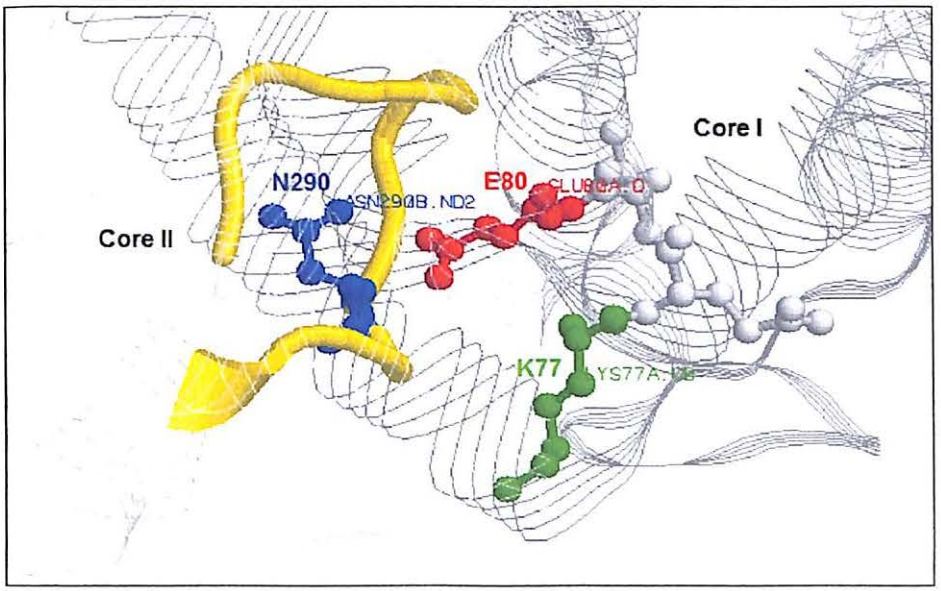


Fig. 11. Drawing model representing the close distance between residues E80 of Core I subunit and residue N290 of the so-called glycine rich loop of Core II subunit.

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