STRUCTURE-FUNCTION STUDIES OF BOVINE HEART MITOCHONDRIAL SUCCINATE-Q REDUCTASE

By

SUDHA K. SHENOY

Bachelor of Science
University of Madras
Madras, India
1982

Master of Science
University of Madras
Madras, India
1986

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
December, 1998

STRUCTURE-FUNCTION STUDIES OF BOVINE HEART MITOCHONDRIAL SUCCINATE-Q REDUCTASE

Thesis Approved:

Thesis Adviser

Dean of the Graduate College

ACKNOWLEDGMENTS

I wish to express my sincere appreciation to my major advisor, Dr. Linda Yu for her intelligent supervision, constructive guidance, inspiration and friendship. I am also equally grateful to Dr. Chang-an Yu for his encouragement and guidance during my study. I want to express my thanks to them and the Department of Biochemistry and Molecular Biology for providing financial support and research opportunity.

My sincere appreciation also extends to my committee members, Dr. Ulrich Melcher, Dr. Olin Spivey, and Dr. Robert Burnap for their advice and guidance.

I am greatly indebted to my husband, Murali for his love, patience, endurance through out my study. I wish to offer this thesis to him and my two children Shivany and Siddharth. Finally I want to thank my parents and parents in law for their encouragement and blessings.

TABLE OF CONTENTS

LIST OF FIGURES

Figure Page

LIST OF TABLES

LIST OF SCHEMES

LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
Azido-Q	3-azido-2-methyl-5-methoxy-6-geranyl-1,4-benzoquinone
3-azido-Q_0C_{10}	3-azido-2-methoxy-5-methyl-6-decyl-1,4-benzoquinone
5-azido- Q_0C_{10}	5-azido-2,3-dimethoxy-6-decyl-1,4-benzoquinone
BCIP	5-bromo-4-chloro-3-indolylphosphate-toluidine salt
CAPS	3-[cyclohexylamino]-1-propanesulfonic acid
CcO	cytochrome *c* oxidase
DCPIP	dichlorophenolindophenol
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EPR	electron paramagnetic resonance
FAD	flavin adenine dinucleotide
$F^{\cdot}$	Flavin radical
$FH^{\cdot}$	protonated form of flavin radical
FP	flavoprotein
FPLC	fast protein (peptide) liquid chromatography
FRD	quinol-fumarate reductase
GST	glutathione S-transferase
[^{3}H]azido-Q	3-azido-2-methyl-5-methoxy-6-(3,7-dimethyl-octyl)-1,4 benzoquinone
[^{3}H]-3-azido- Q_0C_{10}	3-azido-2-methoxy[^{3}H]-5-methyl-6-decyl-1,4-benzoquinone
[^{3}H]-5-azido- Q_0C_{10}	5-azido-2,3-dimethoxy[^{3}H]-6-decyl-1,4-benzoquinone

HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IP	Iron-sulfur protein
β-ME	β-mercaptoethanol
IPTG	isopropyl-β-D-thiogalactopyranoside
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced form of nicotinamide adenine dinucleotide
NBT	p-nitroblue tetrazolium chloride
NQR	NADH-ubiquinone reductase
PAGE	polyacrylamide gel electrophoresis
PBS	20 mM sodium/sodium phosphate buffer, pH 7.3, containing 150 mM NaCl
PMSF	phenylmethylsulfonylfluoride
PVDF	polyvinylidene difluoride
Q	ubiquinone
$Q^{\cdot}$	radical of ubiquinone
$QH^{\cdot}$	protonated form of ubiquinone radical
QH_2	ubiquinol
Q_0C_{10}	2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone
QCR	quinol-cytochrome *c* reductase
QFR	quinolfumarate reductase
QPc	ubiquinone-binding proteins in QCR
QPs	ubiquinone-binding proteins in SQR
SCR	succinate-cytochrome *c* reductase
SDH	succinate dehydrogenase
SDS	sodium dodecylsulfate

SOC	growth medium containing 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 20 mM glucose, 2.5 mM KCl, and 10 mM $MgCl_2$ / $MgSO_4$
SQR	succinate-ubiquinone (Q) reductase
TBS	20 mM Tris-HCl buffer, pH 7.4, 500mM NaCl
TTBS	20 mM Tris-HCl buffer, pH 7.4, 500mM NaCl, 0.05% tween-20
TTFA	2-Thenoyltrifluoroacetone
UV	ultra-violet
X-gal	5-bromo-4-chloro-3-indolyl-β-galactopyranoside

INTRODUCTION

Bovine heart mitochondrial succinate-ubiquinone (Q) reductase (SQR), also known as Complex II, catalyzes oxidation of succinate to fumarate in the citric acid cycle and transfers electrons to ubiquinone in the mitochondrial respiratory chain. The other components of the respiratory chain are NADH-ubiquinone reductase (NQR, Complex I), Ubiquinol-cytochrome *c* reductase (QCR, Complex III) and cytochrome *c* oxidase (CcO, Complex IV) (Figure 1). Succinate and NADH which are produced in the citric acid cycle, are oxidized to fumarate and NAD^+ by donating electrons to ubiquinone via SQR and NQR, respectively. The electrons are then transferred to cytochrome *c* by QCR. Cytochrome *c* is oxidized when it donates electrons to molecular oxygen by CcO. Electrons transferred through NQR, QCR, and CcO, are accompanied with the translocation of protons across the inner mitochondrial membrane to generate a proton gradient for ATP synthesis (1, 2). Although the electron transfer catalyzed by SQR does not generate proton gradient for ATP synthesis, study of SQR is nevertheless important because of its unique position in cellular metabolism in bridging the TCA cycle and the electron transport chain.

Bovine SQR was first isolated from bovine heart mitochondria by Ziegler and Doeg in 1962 (3). However, this isolation procedure was not ideal; the yield was poor and purification complicated by the use of organic solvent fractionation. A simpler and more reliable method to prepare active and pure enzyme complex was introduced by our group in 1982 (4). This method involves the treatment of SCR with Triton X-100 followed by calcium phosphate column chromatography at different pH's.

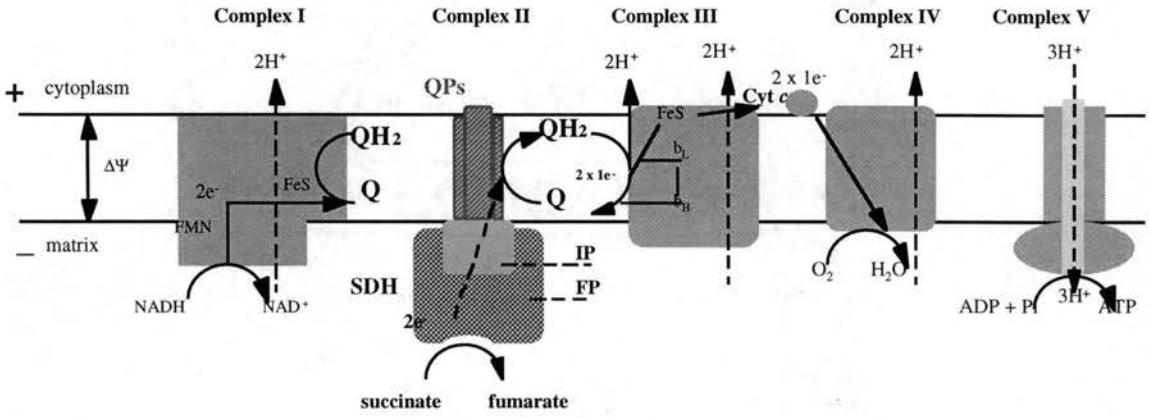


Figure 1. Electron and Proton Transfer Paths in the Mitochondrial Respiratory Chain.

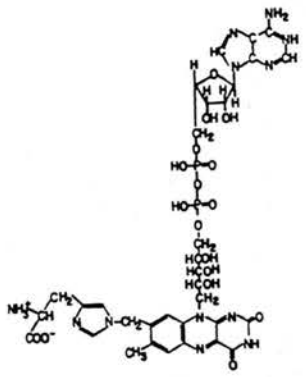
(Modified from references 9 and 53)

Purified SQR has five subunits with apparent molecular weights of 70, 28, 14, 11, and 9 kDa as estimated by high resolution SDS-PAGE. The active complex is associated with six prosthetic groups, namely, a covalently linked FAD, three Fe-S clusters, cytochrome *b* heme, and ubiquinone (Figure 2).

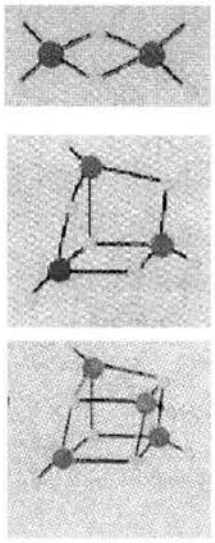
Mitochondrial SQR can be resolved into two reconstitutively active fractions: a soluble SDH and a membrane anchoring fraction. The soluble SDH which has been purified and characterized by different groups of researchers (5 - 7) is made up of the larger two polypeptides, namely 70 kDa flavoprotein (FP) and 27 kDa iron sulfur protein (IP). Purified SDH possesses two types of enzyme activities: 1. redox dye activity, which represents its ability to transfer electrons from succinate to artificial electron acceptors, such as phenazinemethosulfate and ferricyanide; and 2. reconstitutive activity which is its ability to reconstitute with QPs to form SQR. The SDH of bovine SQR has sequence homology to SDHs from *Escherischia coli* SQR, *Bacillus subtilis* SQR and fumarate dehydrogenase of *E. coli* quinol- fumarate reductase (QFR) (8,9). (QFRs belong to a class of enzymes that catalyze the transfer of electrons during anaerobiosis from menaquinol in *E. coli* and rhodoquinol in *Ascaris suum* to the terminal acceptor fumarate.)

The FP subunit of SDH lodges the dicarboxylate binding site. Bovine FP cDNA has been cloned and sequenced (10). The amino acid sequences from different species show several conserved regions a-j (Figure 3). Segment b of the FP polypeptide contains the histidine residue to which FAD is covalently bound via N(3)-8α-riboflavin linkage (11). Segments a, h, and i are probably in contact with the AMP part of FAD (12). FAD is covalently bound to FP before enzyme assembly (13). It has been reported in yeast that FP had to be processed by a matrix processing peptidase before the covalent attachment of FAD (14, 15).

8-α-N3-histidyl-FAD



Iron sulfur clusters

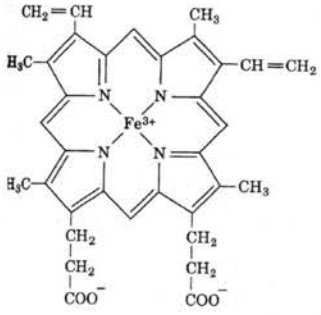


2Fe-2S

3Fe-4S

4Fe-4S

Cytochrome b heme



Ubiquinone

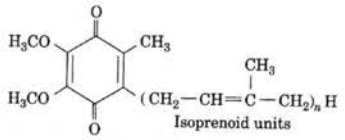


Figure 2. Structures of the Prosthetic Groups in Succinate-Q Reductase.

(from references 11, 55 and 56)

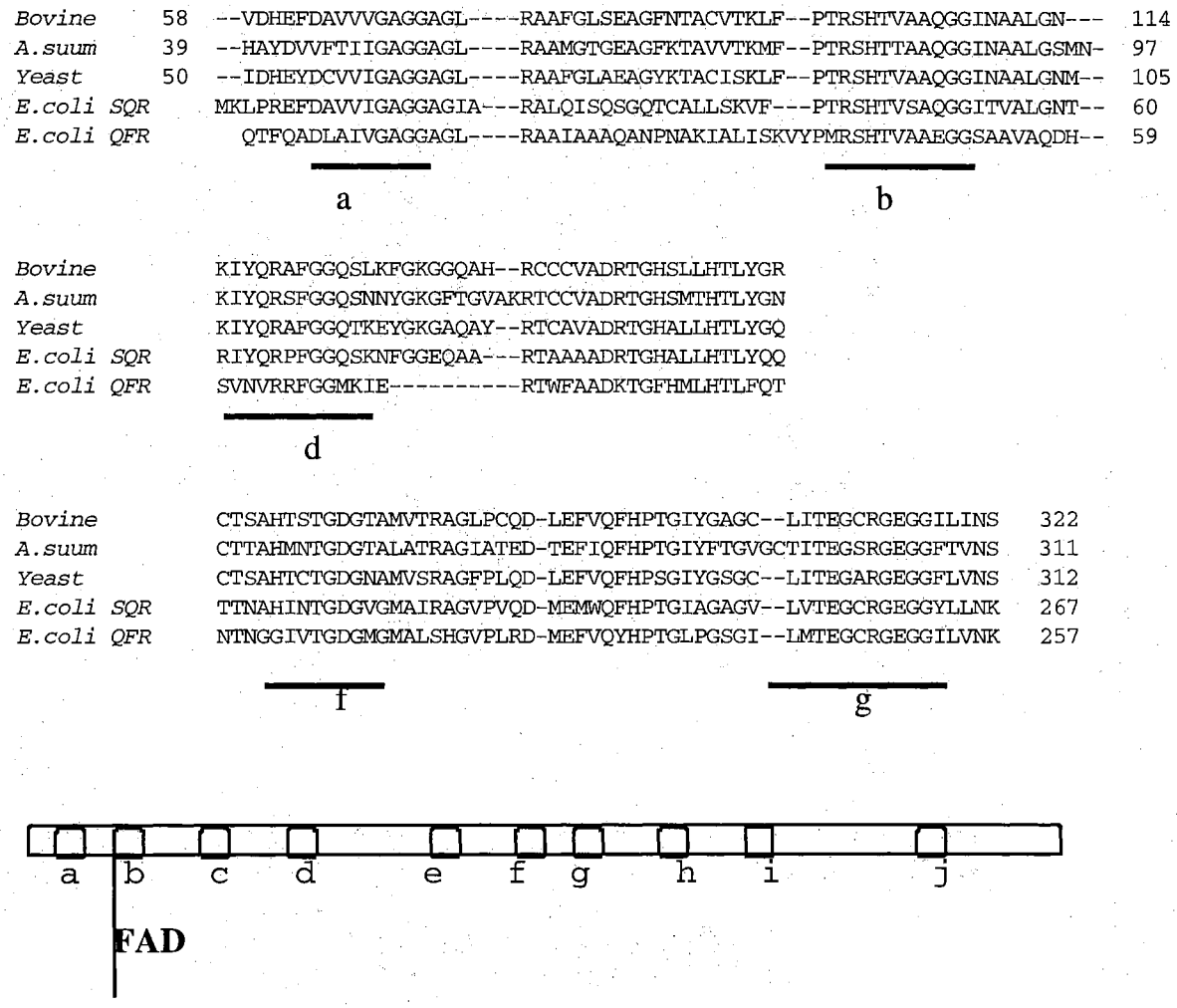


Figure 3. Comparison of Flavoprotein peptide sequences. (Accession #s : Bovine, P31039; *A. suum*, D30650; *E. coli* SQR, P10444; *E. coli* QFR, P00363) (adapted from 57)

The purpose of the covalent bond is to modulate the electronic properties of the flavin sufficiently to enable the enzyme to function as a succino-oxidase. Mutagenesis studies of *E. coli* QFR proved that succinate oxidation is possible only when FAD is covalently bound to FP (16).

FAD is a two electron carrier. The midpoint potentials (Em) of the two consecutive electron transfer steps of FAD in bovine SQR were determined to be -127 mV and -31 mV at pH 7, corresponding to the midpoint potential of -79 mV for the overall reaction (17). The SDH flavin radical state (stability constant = 2.5 x 10^{-2} (17)) is a far more stable redox intermediate compared to the other typical n=2 components, such as NAD^+/NADH or Q/QH_2 in a hydrophobic environment (18).

The IP subunit of SDH harbors the three iron-sulfur clusters, $[2Fe\text{-}2S]^{(2+,1+)}$, $[4Fe\text{-}4S]^{(2+,1+)}$, $[3Fe\text{-}4S]^{(1+,0)}$, called S-1, S-2, S-3 in SQR and FR-1, FR-2, and FR-3 in QFR, respectively. Table 1 shows the different thermodynamic parameters of iron sulfur clusters from bovine SQR, *E. coli* SQR, *E. coli* QFR, and *B. subtilis* SQR (19). It is inferred that the Em of S-3 in SQRs from different organisms is influenced by the type of quinone associated with the enzyme (20). In SQRs that use ubiquinone, S-3 has a higher Em value than enzymes which use menaquinone (21). The amino acid sequence of IP of beef heart SDH is available from peptide sequencing (22). Amino acid sequence comparison of IPs from different organisms show a well conserved N-terminal domain with the motif CxxxxCxxC.....C ligating S-1, resembling a plant type ferredoxin and a C-terminal domain with motifs CxxCxxCxxxCP and CxxxxxCxxxCP resembling bacterial ferredoxins ligating S-2 and S-3. The Em of S-1 is high compared to that of [2Fe-2S] centers of plant ferredoxins. Mutagenesis studies of *E. coli* QFR confirm that S-1 is ligated by 3 cysteines and a water molecule, the latter being hydrogen bonded to an aspartate or cysteine. Information about the spatial organization of the iron-sulfur centers is also available from

Table I.

Midpoint Potentials of the Iron Sulfur Clusters of Complex II

Enzyme	Quinone (Acceptor /Donor)	[2Fe-2S]	4Fe-4S] (mV)	[3Fe-4S]
Bovine SQR	Ubiquinone	0	-260	+60 to +120
E. coli SQR	Ubiquinone	+10	-175	+65
E. coli QFR	Menaquinone	-20 to -79	-320	-50 to -70
B. subtilis SQR	Menaquinone	+80	-240	-25

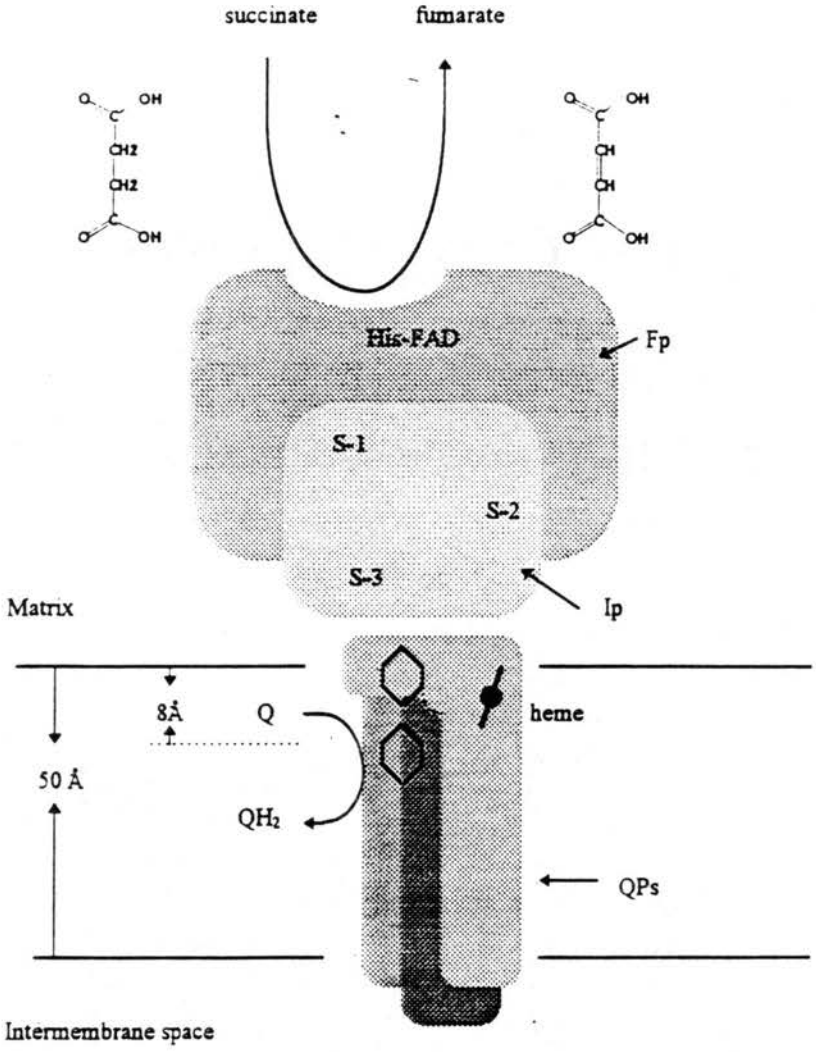


Figure 4. Proposed Topological Model of Succinate-Q Reductase

(Adapted from references 9 and 54)

the epr-detectable magnetic spin-spin interactions. The distance between FAD and S-1 is 12-18 Å, S-1 and S-2 is 9-12 Å, and S-1 and S-3 is 10-20 Å. Strong spin-spin coupling between S-2 and S-3 has also been detected, indicating close mutual distance of these two clusters (9).

The [3Fe-4S] cluster (S-3) of SDH has an important structural role, demonstrated by reconstitution studies of bovine SQR. Purified soluble SDH could be reconstituted with QPs to form SQR only if S-3 was intact (23-25). It was also observed that extraction of ubiquinone from submitochondrial particles affected the redox midpoint potential of S-3 (26). 2-Thenoyltrifluoroacetone (TTFA) is a specific inhibitor of SQR and inhibits electron transfer between S-3 and quinone in mammalian mitochondrial SQR (8). Addition of TTFA increases the signal amplitude of S-3 and shifts the midpoint potential in both pigeon heart (27) and beef heart (28) submitochondrial particles. An interesting mutation P159Q in the FR-3 ligation motif in *E. coli* QFR (29) produced a mutant enzyme that was inactivated by the substrate succinate in the presence of quinone. Based on this finding it has been proposed that the FR-3 is located in the vicinity of quinone binding region of *E. coli* QFR (29).

The function of the hydrophobic subunits of SQR is to provide membrane docking for SDH and Q-binding domains in the complex. The nomenclature "QPs" hails from our laboratory indicating the proteins responsible for Q-binding in SQR. Similar proteins in NQR and QCR are called QPn and QPc respectively. The number of subunits comprising the QPs fraction could be one (SDHC in *B. subtilis,* and *Wolinella succinogenes*), two (SDHC & SDHD, in *E. coli*; SDH3 & SDH4 in *Saccharomyces cerevisiae* and *Paracoccus denitrificans*), or three (QPs1, QPs2, QPs3, in bovine heart). Table II shows a comparison of the membrane anchors from different species.

Table II

Membrane Anchors in Succinate-Q Reductases From Different Species

Name of species	No. of anchor polypeptides	No. of hemes	Properties of cytochrome		α-band[nm]
			Em (mV)	EPR signals	
Bovine SQR	3	1	-185	3.46	560
E. coli SQR	2	1	+36	3.63	556
B. subtilis SQR	1	2	b_H+65,	3.68	558
			b_L-95	3.42	
W. succinogenes QFR	1	2	b_H -20,	--	560
			b_L –200	--	
A. suum (adult) QFR	2	1	-34	3.6	558
P. denitrificans SQR	2	1	-175	--	557
E. coli QFR	2	0	--	--	--
S. cerevisiae SQR	2	0	--	--	--

The membrane docking function of QPs is evidenced by chemical modification studies of QPs that indicated one histidine and two carboxyl groups in the QPs fraction to be essential for interaction with SDH in the formation an active SQR (30, 31).

The involvement of QPs in the Q-binding of bovine SQR is shown by the detection of ubisemiquinone radicals in intact and reconstituted SQR in the presence of excess added quinone (5:1 molar ratio to flavin) (32). No Q-radical was detected in SDH after the addition of Q. The Q-radical in bovine SQR showed a rapidly relaxing g=2.00 semiquinone signal at 150 K as well as split signals arising from spin-spin interactions between two semiquinones at 10 K (32, 33). From the modeling of the dipolar coupling using a point charge approximation, the distance between the two semiquinones was estimated to be 7.7 Å (33). Oriented multilayers of bovine heart mitochondrial membranes were used to show that the semiquinone pair was ordered with quinone-quinone dipolar axis perpendicular to the membrane, in a transmembrane fashion (34). The ubisemiquinone radicals detected in intact or reconstituted SQR do not show power saturation even at 200 mW, and differ from the antimycin sensitive radicals detected in QCR that show power saturation at 1 mW (32). The structural requirements for the Q-binding site in bovine SQR differ from those in QCR (35, 36). When a Q analog is used as electron donor for QCR, a 10 carbon side chain is required for maximum activity, whereas only a 5-carbon side-chain is necessary for a Q-analog to serve as electron acceptor for SQR (36). The structural requirements for quinone derivatives to be reduced by SQR are more specific than for quinol derivatives to be oxidized by QCR (Table III). When Q derivatives are used as the electron acceptor for SQR, the methyl group at the 5-position is less important than the methoxy groups at the 2- and 3- positions. Replacing the 5-methyl group with hydrogen causes a slight increase in activity. However, replacing one or both of the 2- and 3- methoxy groups with a methyl group completely abolishes electron acceptor activity. Replacing the 3-methoxy group with hydrogen results in a complete loss of electron-acceptor activity, while replacing the 2-methoxy with hydrogen results in an

Table III.

Comparison of Electron-Transfer Activity of Q-derivatives (36)

Q derivatives	R2	R3	R5	Activities (%) As acceptor for SQR	As donor for QCR
Q_0C_{10}	MeO	MeO	Me	100	100
2-Me-Q_0C_{10}	Me	MeO	Me	0	15
3-Me-Q_0C_{10}	MeO	Me	Me	0	14
5-Me-PQ_0C_{10}	Me	Me	Me	0	20
2-H-Q_0C_{10}	H	MeO	Me	29	54
3-H-Q_0C_{10}	MeO	H	Me	0	27
2-H-5-Me-PQ_0C_{10}	H	Me	Me	32	22
3-H-5-Me-PQ_0C_{10}	Me	H	Me	0	20
5-H-Q_0C_{10}	MeO	MeO	H	117	91
2-Me-5-H-Q_0C_{10}	Me	MeO	H	18	25
3-Me-5-H-Q_0C_{10}	MeO	Me	H	9	23
PQ_0C_{10}	Me	Me	H	0	27
3,5-Di-H-Q_0C_{10}	MeO	H	H	0	6
2,5-Di-H-Q_0C_{10}	H	MeO	H	30	11
5-MeO-PQ_0C_{10}	Me	Me	MeO	0	20

activity decrease of 70%, suggesting that the 3-methoxy group is more critical than the methoxy group at the 2-position. On the other hand all the 1,4-benzoquinol derivatives showed partial activity when used as electron donors for QCR. The plastoquinone derivatives which show no electron acceptor activity for SQR, are reducible by succinate in the presence of SCR. This reduction is antimycin sensitive and requires endogenous Q, suggesting that the plastoquinone derivatives can only accept electrons from the ubisemiquinone radical at the Qi site of QCR and cannot accept electrons from QPs of SQR (36).

In order to understand the quinone-protein interaction, it is essential to identify the putative Q-binding sites. The presence of two quinone binding sites in *E. coli* QFR has been suggested by mutagenesis studies (37) and by inhibitor kinetic analyses of putative Q-binding site mutants (38). Inhibitor binding studies on bovine SQR also suggest two Q-binding sites (38) in the complex. When a succinate-free, Q-deficient SQR from bovine heart was treated with a [^{3}H]-azido-Q derivative, (3-azido-2-methyl-5-methoxy-6-(3,7-dimethyl-octyl)-1,4-benzoquinone), in the dark followed by illumination with a long wavelength UV radiation, about 50% of bound Q was located on QPs1 subunit and other 50% was equally distributed in QPs2 and QPs3. No radioactivity was found in the SDH subunits indicating that QPs subunits are responsible for Q-binding (39) (Figure 5). The lower azido-Q uptake by QPs2 and QPs3 may result from portions of the Q-binding sites being contributed by these two subunits or from the preferential occupation of these two subunits by the endogenous Q present in the partially Q-deficient SQR.

In order to identify the Q-binding domain in these Q-binding subunits, knowledge of their amino acid sequence is essential. The cDNA information for QPs1 was obtained by our group by immunological screening procedures (40) and the Q-binding domain in QPs1,was identified by isolating and sequencing of an azido-Q-linked peptide as the stretch connecting transmembrane helices 2 and 3 that protrudes from the surface on the matrix

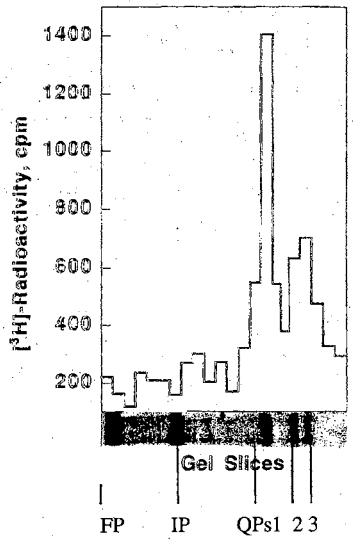


Figure 5. Distribution of [^{3}H]-azido-Q Label Among the Subunits of SQR (39)

side of the inner membrane (39). The availability of sequence information of QPs2 and QPs3 is required for the elucidation of Q-binding domain(s) in these two subunits.

Another interesting aspect among the anchor subunits is the association of heme *b* (2 hemes in *B. subtilis* SQR and *Wolinella succinogenes* QFR, 1 heme in *E. coli* SQR, *A. suum* QFR, Bovine SQR and no heme in *E. coli* QFR) (Table II). EPR and near infrared MCD spectroscopic studies suggested bis-histidine axial ligation of cytochrome b_{560} in bovine SQR (41), *E. coli* SQR (42), *B. subtilis* SQR (43). The histidines important for heme ligation have been identified in *B. subtilis* SQR (44) and *E. coli* SQR (45).

The involvement of cytochrome b_{560} in SQR catalysis has been controversial. Since cytochrome b_{560} in bovine SQR is sub-stoichiometric to FAD and is not reducible by succinate, direct participation of cytochrome b_{560} in the catalytic function of SQR is not likely. Alternatively, it has been proposed that cytochrome b_{560} functions as a mediator between low potential F1/F$^{\cdot}$ and Q/Q$^{\cdot}$ couples in a dual pathway model of electron flow (Figure 6) through cardiac complex II (46, 47). It is also suggested that cytochrome b_{560} is necessary for the binding and simultaneous stabilization of two semiquinones (48).

In spite of the rather unclear role of this cytochrome b_{560} in catalysis there is considerable evidence for its structural role in bovine SQR. The differences observed in the properties of this cytochrome in isolated QPs and in native or reconstituted SQR are listed as follows: (1) the dithionite reduced form of cytochrome b_{560} in QPs fraction has a symmetrical α-absorption peak, which upon reconstitution with SDH becomes broadened showing a shoulder at 553 nm identical to the absorption spectrum of cytochrome b_{560} in SQR. (2) the dithionite reduced form of cytochrome b_{560} in isolated QPs is reactive to carbon monoxide while that in SQR is nonreactive. Addition of SDH to QPs converts cytochrome b_{560} from CO reactive to CO nonreactive. (3) The redox potential of cytochrome b_{560} in QPs is -144 mV, which is higher than that of cytochrome b_{560} in SQR

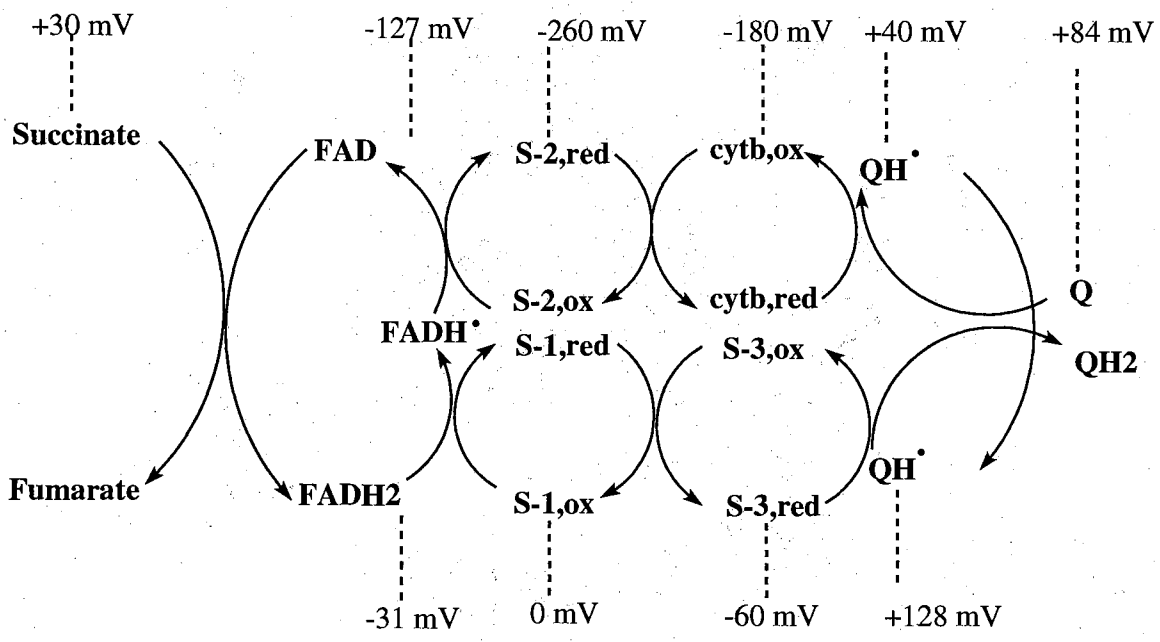


Figure 6. Dual Pathway Model of Electron Transfer Through Succinate-Q Reductase

(-185 mV). Upon addition of SDH the redox potential of about 46% of the cytochrome b_{560} in QPs becomes identical to that in SQR. (4) cytochrome b_{560} in QPs shows two EPR signals, g=3.07, and g=2.91, the former disappears when QPs is reconstituted with SDH and a signal at 3.46 appears corresponding to that in SQR. In addition to this structural role this cytochrome b_{560} may also serve as a low potential electron entrance for fumarate reduction under anaerobic conditions (49).

The cytochrome b_{556} in *E. coli* SQR has been recently characterized (50) and is different from the bovine cytochrome b_{560}. In *E. coli* SQR, the cytochrome b_{556} has identical absorption properties in purified membrane anchor fraction as well as in isolated and reconstituted SQR. The dithionite reduced cytochrome b_{556} shows a symmetrical α-peak and is CO insensitive in the presence or absence of SDH. This suggests that the heme environment in the *E. coli* and bovine enzymes is not identical.

Very recently great progress has been made in our laboratory in the expression of mitochondrial QPs1 in *E. coli* as a GST-Fusion protein. Recombinant GST-QPs1 was isolated in a soluble form and reconstituted with hemin chloride *in vitro* to obtain all the properties of cytochrome b_{560} of QPs except the reconstitution with SDH (51). This work strongly indicates that cytochrome b_{560} in mitochondrial QPs is solely contributed by QPs1. This finding is contrary to that by Nakamura *et al* in their gene deletion studies of *E. coli* SQR that both subunits are necessary for the formation of cytochrome b_{556} (52). This report provides us the incentive to investigate whether the smaller QPs subunits can also provide histidine ligands for cytochrome b_{560} in SQR.

The structure-function study of SQR requires the knowledge of Q-binding and cytochrome *b* ligation of QPs2 and QPs3 and thus the need for amino acid sequence of QPs2 and QPs3. In this thesis I report the cloning of QPs3 cDNA, expression of QPs3 as a GST fusion protein in *E. coli* cells, isolation and characterization of recombinant QPs3.

REFERENCES

1. Nicholls, D. G., and Fergusson, S. J. (1992) Bioenergetics. Academic Press, New York.
2. Hatefi, Y. (1985) *Ann. Rev. Biochem.,* **54**, 1015-1069.
3. Ziegler, D. M., and Doeg, K. A. (1962) *Arch. Biochem. Biophys.*, **97**, 41-50.
4. Yu, L., and Yu, C-A.,(1982) *J. Biol. Chem.*, **257**, 2016-2021.
5. King, T. E. (1963) *J. Biol. Chem.,* **238**, 4037-4051.
6. Davis, K.A. and Hatefi, Y (1971) *Biochemistry* , **10**, 2509-2516.
7. C. A. Yu and L., Yu, (1980) *Biochim. Biophys. Acta.,* **591**, 409-420.
8. Ackrell, B. A. C., Johnson, M., Gunsalus, R. P. & Cecchini, G. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Muller, F., ed) Vol. III 229-297, CRC Press, London.
9. Hederstedt, L. & Ohnishi, T. (1992) in *Molecular Mechanism in Bioenergetics,* (Ernster, L., ed) 163-198, Elesevier Science Publishing Co.
10. Birch-Machin, M. A.,Farnsworth, L., Ackrell, B. A. C., Cochran, B., Jackson, S., Bindoff, L. A., Aitken, A., Diamond, A. G., & Turnbull D. M. (1992) *J. Biol. Chem.,* **267** 11553-11558.
11. Walker, W. H. and Singer, T. P. (1970) *J. Biol. Chem.,* **245**, 4224-4225.
12. Schroder, I., Gunsalus, R. P., Ackrell, B. A., Cochran, B. and Cecchini, G. (1991) *J. Biol. Chem.*, **266**, 13572-13579.
13. Hederstedt, L. (1983) *Eur. J. Biochem.*, **132**, 589-593.
14. Robinson K. M. and Lemire, B. D. (1996) *J. Biol. Chem.*, **271**, 4055- 4060.
15. Robinson K. M. and Lemire, B. D. (1996) *J. Biol. Chem.*, **271**, 4061- 4067.

16. Blaut, M., Whittaker, K., Valdovinos, A., Ackrell, B. A. C. Gunsalus, R. P. and Cecchini, G. (1989) *J. Biol. Chem,* **264**, 13599-13604.

17. Ohnishi, T., King, T. E., Salerno, J. C., Blum, H., Bowyer, J. R. and Maida, T. (1981) *J. Biol. Chem.*, **256**, 5577-5582.

18. Mitchell, P. (1976) *J. Theor. Biol.*, **62,** 327-367.

19. Salerno, J. C. (1991) *Biochem. Soc. Trans.,* **19**, 599-605.

20. Sohnorf U. (1966) Thesis no. 3871, Eidgenossische technische Hochschule, Zurich, Switzerland.

21. Thauer, R. K., Jungermann, K., and Decker K., (1977) *Bacteriol. Rev.*, **41**, 100-180.

22. Yao, Y., Wakabayashi, S., Matsuda, S., Matsubara, H., Yu, L., and Yu, C. A, (1986) in Iron-Sulfur Protein Research (Matsubara, H., Katsube, T., and Wada, K., eds) 240-244, Japan Scientific Societies Press, Tokyo.

23. Baginsky, M. L. And Hatefi, Y. (1969) *J. Biol. Chem.*, **244**, 5313-5319.

24. Ohnishi, T., Lim, J., Winter, D. B. And King, T. E. (1976) *J. Biol. Chem.,* **251**, 2105-2109.

25. Yu, C-A., Yu, L. And King, T. E. (1977) *Biochem. Biophys. Res. Commun.*, **79**, 939-946.

26. Lester, R. L. And Fleischer, S. (1959) *Arch. Biochem. Biophys.*, **80**, 470-473.

27. Ingledew, W. J. And Ohnishi, T. (1977) *Biochem., J.* **164**, 617-620.

28. Salerno, J. C. And Ohnishi, T. (1980) *Biochem., J.* **192**, 769-781.

29. Cecchini, G., Sices, H., Schrodder, I. And Gunsalus, R. P. (1995) *J. Bacteriol.*, **177**, 4587-4592.

30. Paudel, H. K., Yu, L., and Yu, C. A. (1990) *Biochim. Biophys. Acta*, **1056**, 159-165.

31. Xu, J-X, Yu, L., and Yu, C. A., (1987) *Biochemistry,* **26**, 7674-7679.

32. Miki, T., Yu, L., and Yu, C. A. (1992) *Arch. Biochem. Biophys.*, **299**, 61-66.

33. Rudzicka, F. J., Beinert, H. Schelper, K. L., Dunham, K. and Sands, R. H. (1975) *Proc. Natl. Acad. Sci. USA* , **72**, 2886-2890.

34. Salerno, J. C., Harmon, H. J., Blum, H., Leigh, J. S. and Ohnishi, T. (1977) *FEBS letter*s, **82**, 179-182

35. Yu, C-A., Gu, L.-Q., Lin, Y., and Yu, L. (1985) *Biochemistry*, **24**, 3897-3902.

36. Gu, L.-Q., Yu, L. and Yu, C-A. (1990) *Biochim. Biophys. Acta.*, **1015**, 482-492.

37. Westenberg, D. J., Gunsalus, R. P., Ackrell, B. A. C., Sices, H., and Cecchini, G., (1993) *J. Biol. Chem.*, **268**, 815-822.

38. Yankovskaya, V., Sablin, S. O., Ramsay, R. R., Singer, T. P., Ackrell, B. A. C., Cecchini, G., and Miyoshi, H., (1996) *J. Biol. Chem.*, **271**, 21020-21024.

39. Lee, G. Y., He, D-Y., Yu, L., and Yu, C. A. (1995) *J. Biol. chem.*, **270**, 6193-6198.

40. Yu, L., Wei, Y-Y., Usui, S., and Yu, C. A., (1992) *J. Biol. Chem.*, **267**, 24508-24515.

41. Crouse, B. R., Yu, C. A., Yu, L., Johnson, M. K. (1995) *FEBS Lett.*, **367**, 1-4.

42. Peterson, J., Vibat, C. And Gennis, R. B. (1994) *FEBS Lett.*, **355**, 155-156.

43. Friden, H., Cheesman, M. R., Hederstedt, L., Andersson, K. K., and Thompson, A. J. (1990) *Biochim. Biophys. Acta*, **1041**, 207-215.

44. Hägerhäll, C., Friden, H., Aasa, R., and Hederstedt, L. (1995) *Biochemistry*, **34**, 11080-11089.

45. Rose, C. T., Cecchini, G. Nakamura, K., Kita, K., and Gennis, R. B. (1998), *Biochemistry*, **37**, 4148-4159.

46. Cammack, R. in Iron-sulfur Protein Research (Matsubara, H., Katsube, T., and Wada, K., eds),(1986) 40-55, Japan Scientific Societies Press, Tokyo.

47. Rich, P. R., (1984) *Biochem. Biophys. Acta* , **68**, 53-79, 2508-2515.

48. Xu, Y., Salerno, J. C., Wei, Y. H., and King, T. E. (1987) *Biochem. Biophys. Res. Commun.*, **144**, 315-322.

49. Yu, L., Xu, J-X., Haley, P. E., & Yu, C. A. (1987) *J. Biol. Chem.*, **262**, 1137-1143.

50. Yang,X., Yu, L., and Yu, C. A., (1997), *J. Biol. Chem.*, **272**, 9683-9689

51. Gyesoon, L. Y., Zhu, J., Yu L., and Yu C., A. (1998) *Biochim. Biophys. Acta.*, **1363**, 35-46.

52. Nakamura, K., Yamaki, M., Sarada, M., Nakayama, S., Vibat, C. R. T., Gennis R. B., Nakayashiki, T., Inokuchi H., Kojima, S., Kita, K. (1996) *J. Biol. Chem* ., **271** 521-527.

53. Hägerhäll, C. Ph.D Thesis (1990), Department of Microbiology, University of Lund, Sweden.

54. Lee, Gyesoon, Ph.D Thesis (1996) Department of Biochemistry & Molecular Biology, Oklahoma State Univ. Stillwater, OK-74078.

55. Beinert, H., Holm, R. H., Munck, E., (1997) *Science*, **277**, 653-659.

56. Voet, D. and Voet, J. Text Book of Biochemistry, John Wiley and Sons ed.

57. Hägerhäll, C, (1998) *Biochim. Biophys. Acta*, **1320**, 107-141.

CHAPTER I

ISOLATION OF THE cDNA ENCODING QPs3 FROM A BOVINE HEART cDNA LIBRARY

Structure-function studies of SQR requires the knowledge of amino acid sequences of all the membrane anchor subunits. While the largest QPs subunit has been cloned and sequenced (1) and its participation in Q-binding established (2) the involvement of QPs2 and QPs3 in Q-binding is rather uncertain. When a Q-deficient, succinate-free SQR was photoaffinity labeled with azido-Q derivatives, some radioactivity was found in QPs2 and QPs3. Although the radiolabel of QPs2 and QPs3 was 45% and 50% of what was found in QPs1, Q-binding function of these two smaller subunits cannot be ruled out. The identification of Q-binding domains in QPs2 and QPs3 requires knowledge of the amino acid sequences of both subunits. There are two ways to obtain amino acid sequence information. One is by peptide sequencing. This method is rather impractical and time consuming for our purpose. A better approach is to obtain the cDNA of QPs3 and deduce the peptide sequence from nucleotide sequence. The second method is more attractive since our group has reported several cDNA clones of mitochondrial membrane proteins and we have the expertise to do this in our lab. In this chapter, I report the cloning and sequencing of QPs3 cDNA clone from a bovine heart cDNA library.

Experimental Procedures

Materials

Acrylamide, sodium dodecyl sulfate, urea, glycine, bovine serum albumin, Triton X-100, sodium cholate, DCPIP, and β-mercaptoethanol were obtained from Sigma. Phenyl sepharose CL-4B was from Pharmacia. Calcium phosphate was prepared according to Jenner (3), and mixed at a ratio of 3:1 with cellulose powder prior to use in column chromatography.

Restriction enzymes were either from Promega or Life Technologies Inc. Taq DNA polymerase, T4 DNA Polymerase, Erase-a-base system were from Promega. Chymotrypsin, Trypsin, DTT, DNAse I, RNAse A, IPTG, X-gal, gelatin, BCIP, and NBT were from Sigma. Nitrocellulose and nylon membranes were from Schleicher and Schuell. Protein A Horseradish peroxidase conjugate, Goat anti-rabbit IgG-alkaline phosphatase conjugate, SDS-PAGE standards were from Biorad. TA Cloning kit was from Invitrogen. Primers for PCR and Oligonucleotide probes were synthesized at the Recombinant DNA/Protein Resource Facility at Oklahoma State University. Bovine heart cDNA expression libraries constructed with λgt11 vector and λZAP vector were purchased from Clontech and Stratagene respectively. Nonradioactive labeling system was from Life Technlogies Inc. Chemiluminiscent substrate, Lumiphos ^{530} for streptavidin alkaline phosphatase was from Boehrringer Manneheim.

Bacterial Strains, Phages and Plasmids.

E. coli strains used were as follows:- **Y1090r**$^-$*[ΔlacU169Δ(lon, araD)139 strAsupF(trpC:Tn10)]* was used as host for plaque screening and propagation of λgt11 recombinants. **INVαF'** *-F' endA1 recA1 hsdR17 (r_k^-, m_k^+) supE44 thi-1 gyrA96 relA1 ϕ80lacZΔM15 Δ(lacZYA-argF)U169λ*$^-$ was used as a host for PCR2.1 vector propagation. **XL1-Blue MRF'**- *Δ (mcrA) 183, Δ(mcrCB-hsdSMR-mrr) 173, endA1, supE44, thi-1, recA1, gyrA96, relA1, lac[F' proAB, lacI^{q}ΔZm15, Tn10 (tet^r)]* was used as host for plaque screening and propagation of λ Uni-ZAP XR recombinants.

Molecular Cloning and DNA Sequencing

All cloning procedures including isolation and purification of lambda phage DNA were performed according to the methods in Sambrook *et. al* (4). DNA sequencing by the dideoxy method of Sanger was done with a Sequenase™ 2.0 kit from Life Technologies Inc. Automated DNA sequencing was performed at the Recombinant DNA/Protein Resource Facility at Oklahoma State University. Full length cDNA was sequenced either by subcloning of smaller overlapping fragments or by primer walking.

Isolation of Pure QPs3 Protein

Submitochondrial particles (5), SCR (6), and SQR (7) were prepared according to previously reported procedures. SQR from the second calcium phosphate-cellulose column was concentrated to a protein concentration of 4 mg/ml by membrane filtration (centriprep-30, Amicon). Alternately, SQR was precipitated by 43% ammonium sulfate saturation, centrifugation at 48,000 x g for 20 min and dissolved in 50mM phosphate buffer pH 7.8, containing 0.2% sodium cholate and 10% glycerol.

SQR was assayed for its ability to catalyze Q reduction or Q-stimulated DCPIP reduction by succinate. The assays were performed at room temperature in a Shimadzu UV-2101PC. The reaction mixture used for Q-stimulated DCPIP reduction assay contains 38μmol of DCPIP, 100 μmol of sodium/potassium phosphate buffer, pH 7.4, 20 μmol of succinate, 10 nmol of EDTA, 25 nmol of Q_0C_{10} and 0.01% Triton X-100 in the total volume of 1 ml. The reduction of DCPIP was followed by measuring the absorption decrease at 600 nm, using a millimolar extinction coefficient of 21 $mM^{-1}cm^{-1}$

QPs was prepared from SQR by Sepharose CL-4B column chromatography with different concentrations of detergents. Succinate-Q reductase was diluted to 2 mg/ml with 50 mM Tris-HCl, pH 7.4, containing 0.2% sodium cholate. The solution was stirred at room temperature for 30 min and applied to a phenyl-sepharose CL-4B column equilibrated with 50 mM Tris-HCl pH 7.4, containing 0.2% sodium cholate. The column was successively washed with 50 mM Tris-HCl pH7.4, containing 2% sodium cholate, 50 mM

Tris-HCl pH 7.4, containing 2% sodium cholate and 4 M urea followed by the initial buffer to wash out the remaining urea. QPs was eluted from the column with 50 mM Tris-HCl pH 7.4, containing 0.15% SDS.

Pure QPs3 protein was isolated from the QPs fraction by preparative SDS-PAGE using high resolution gel system of Schägger et al (8) except that 8M urea was used in the separating gels instead of glycerol. The separating gels were allowed to polymerize for at least 4 hours. The crude QPs, 1.5 mg/mL, was digested with 1%SDS and 1% β-mercaptoethanol and incubated at 37 °C for 5 hours. The digested sample was loaded on a gel slab in two strips sandwiched with three reference wells (one on each side and one at the center) loaded with the digested samples treated with fluorescamine. The electrophoresis was at 17 volts until the samples entered the separating gels after which the gel was run at 45 volts for 18 hours. The protein bands (in the reference wells) were visualized under UV. The SDS-PAGE pattern of the fluorescamine treated sample was identical to that of untreated sample as established by Coomassie blue staining. The QPs3 bands were sliced from the gels and then eluted from the gel slices with an electroelutor from Bio-Rad. The purity of subunit was judged by SDS-PAGE and coomassie blue staining.

Production and Purification of Antibodies Against QPs3

The eluted QPs3 was concentrated by membrane filtration, using centricon-10, to a protein concentration of 2 mg/ml and precipitated with cold acetone (-20 °C). The precipitates were washed with 50% acetone dried under argon, and suspended in PBS buffer to a protein concentration of 1.5 mg/mL. About 150 ug of QPs3 protein in 0.5 mL was emulsified with Freund's adjuvant and injected into a NZW rabbit's back at several subcutaneous sites. Boosters were given weekly and blood was drawn from the ear. The serum was characterized by dot blot to monitor the appearance of antibodies. After the fourth booster blood was collected by cardiac puncture and serum was separated by clotting followed by centrifugation at 10,000 x g. Purification of antibodies was done as follows.

Sera were brought to 50% ammonium sulfate saturation with a neutralized saturated ammonium sulfate solution and centrifuged at 28,000 x g for 30 min. The precipitate was dissolved in 20 mM Tris-HCl, pH 8.0, containing 28 mM NaCl, and dialyzed overnight against the same buffer. The dialyzed solution was centrifuged to remove undissolved particles and the clear supernatant was applied to a DEAE-affigel blue column equilibrated with 20 mM Tris-HCl, pH 8.0, containing 50 mM NaCl. This step primarily removes serum albumin which binds to the column and is separated from the IgG that is in the effluent. The effluent fractions with $OD_{280} > 0.08$ were pooled and concentrated using an Ultrafiltration cell with Amicon PM-30 membrane followed by Centriprep-30 to a final volume of 2-3 ml and protein of >25 mg/mL. This IgG preparation contained a small population of IgM and further purification of IgG was done by molecular sieving using Sephacryl S-300 SF column equilibrated with phosphate buffered saline (PBS). The purified IgG was concentrated to a protein of >5 mg/mL and stored at -80°C in the presence of 0.01% sodium azide.

Immunological Screening of a Bovine Heart cDNA Library with Anti-QPs3 Antibodies.

The beef heart cDNA expression library in λgt11 was screened with anti-QPs3 antibodies according to reported methods (9, 10). Anti-QPs3 antibodies were preincubated with *E. coli* lysate (about 500 μg lysate protein to 1 μg antibody) for 2 hours at room temperature. The lysate treated antibody was filtered through a 0.2 μm membrane and used as a primary antibody along with goat anti-rabbit IgG alkaline phosphatase conjugate as the secondary antibody in the screening procedures. BCIP and NBT were used for the alkaline phosphatase colorimetric detection.

Screening of cDNA Libraries by Plaque Hybridization.

Two oligonucleotide probes with biotin label at the 5' end were synthesized based on the partial N-terminal sequence obtained from microsequencing (see results section). Each of the probe was a guessmer encoding the N-terminal amino acids of QPs3. Probe 1. was 5' GTAGTCCATGGCTGAGCATGGCTTCAGGTAGGCAGCAGGGAT3'

complementary to the coding sequence of residues 24-37 (I-P-A-A-Y-L-N-P-C-S-A-M-D-Y). Probe 2 was 5' GCTGCCTCCCTGCACTGGACAGGCGAGCGTGTGGTCTCTGT 3' coding residues 5-14 (A-A-S-L-W-T-G-E-R-V-V-S). The screening and detection procedures were essentially as described by Martin *et al.* (11).

Reductive Carboxymethylation and Succinylation of QPs3.

The electrophoretically eluted QPs3 protein was concentrated by membrane filtration with centricon-10, to a final protein concentration of about 3 mg/ml and precipitated with 50% cold acetone (-20 °C). The protein precipitate was washed with cold water and dried under argon. The precipitate thus obtained was homogenized in 0.1M Tris, 6M guanidine HCl, 1mM EDTA, pH 8.3. Dithiothreitol was added to the suspension to a final concentration of 2 mM. The mixture was made anaerobic by passing argon through it and then incubated at 37 °C for 1 hour. Iodoacetic acid neutralized with NaOH, was added to give a final concentration of 5 mM. The reaction mixture was flushed with argon and the container was sealed and alkylation was continued at 37 °C under dark conditions for 1 h. Additional treatment with 1 mM dithiothreitol and incubation with 2 mM iodoacetate under the same conditions resulted in complete alkylation.

The suspension of alkylated protein was treated with successive increments of succinic anhydride with constant stirring while maintaining the pH of the solution between 7 and 8 by dropwise addition of 0.1 N NaOH. Each portion of the anhydride was allowed to completely react (as indicated by the cessation of acid production) before the next addition. After maximal clarification of the suspension was obtained, β-mercaptoethanol was added to a final concentration of 1% (v/v), and the solution was dialyzed against 50 mM ammonium bicarbonate overnight.

Proteolytic Cleavage of QPs3.

The carboxymethylated and succinylated QPs3 protein was collected from the dialyzed sample by centrifugation at 100,000 x g for 20 min. The collected precipitates were suspended in 50 mM ammonium bicarbonate buffer, containing 1 M urea to a final

protein concentration of 1 mg/ml and digested with chymotrypsin at 37 °C for 2 h, using a chymotrypsin to QPs3 ratio of 1:50 (w/w). After the 2-h incubation, a second addition of chymotrypsin was made (1:100), and the digestion continued at 37 °C for 24 h.

Isolation of QPs3 Peptides

One hundred microliter aliquots of the chymotrypsin digested QPs3 protein were separated by reverse phase high performance liquid chromatography (HPLC) on a synchropak RP-8 column (0.46 x 25 cm) using a gradient formed from 90% acetonitrile in 0.1% trifluoroacetic acid and 0.1% trifluoroacetic acid with a flow rate of 0.8 ml /min. Absorbance was monitored at 214 nm. The fractions at retention times of, 18.96, 32.15, and 34.21 were collected, dried, dissolved in 20% acetic acid and rechromatographed in the same system and used for sequence analyses at the Molecular Biology Resource Facility, Saint Francis Hospital of Tulsa Medical Research Institute, University of Oklahoma Health Sciences Center, under the supervision of Dr. Ken Jackson.

Results And Discussion

Immunological Specificity of Anti-QPs3 Antibodies

Anti-QPs3 antibodies obtained are immunologically specific as determined by western blots using QCR, SQR, purified QPs1, QPs2 and QPs3 as antigens (Figure 7). Anti-QPs3 antibodies bind to the fourth and fifth subunits of SQR with equal intensity and to the purified QPs2 and QPs3. No binding to subunits of the QCR was observed. The cross reaction of anti-QPs3 with QPs2 is not due to the cross contamination of QPs2 in QPs3 preparation used as antigen because the purity of QPs3 is evident from the preparation showing a single protein band corresponding to the fifth subunit of SQR in high resolution SDS-PAGE. The N-terminal amino acid sequence analysis showed only one peptide in the isolated sample. It should be noted that anti-QPs2 antibodies also reacted

equally well with QPs2 and QPs3 (12). Thus, the cross reaction may result from the sharing of common epitopes by these two proteins.

Anti-QPs3 antibodies also reacted with unidentified high molecular weight *E. coli* Lysate proteins (>30 KDa) but did not react with any subunit of purified *E. coli* SQR. For this reason in all our library screening procedures anti-QPs3 antibodies were preincubated with *E. coli* lysate to remove the cross-reacting antibodies and thus avoid false positives in our screening experiments.

Immunological Screening of cDNA Expression Libraries Using Anti-QPs3 Antibodies

Since the amino acid sequences of the membrane-anchoring subunits in SQRs from different species show little conservation, homology probing method could not be used to obtain QPs3 cDNA. The availability of anti-QPs3 antibodies in our laboratory together with our previous success in immunological screening of a beef heart cDNA expression library in λgt11 to obtain cDNA for the Rieske iron sulfur protein (10), the QPc-9.5 kDa (13) of QCR and QPs1 (1) of SQR, encouraged us to attempt the immunological screening method to isolate the cDNA for QPs3. However no positive clone could be obtained. This failure to obtain cDNA for QPs3 by the immunological screening method was probably due to the low titer of antibodies against QPs3 rather than the lack of QPs3 cDNA in the cDNA library used because we also failed to obtain a positive clone from other beef heart cDNA libraries, such as in λZAP (from Stratagene).

Partial Amino Terminal Sequence of QPs3

When purified QPs3 subunit (Figure 7) was sequenced a N-terminal sequence of 43 residues was obtained:- NH_2-S-G-S-K-A-A-S-L-H-W-T-G-E-R-V-V-S-V-L-L-L-G-L-I-P-A-A-Y-L-N-P-C-S-A-M-D-Y-S-L-A-A-T-L-. Every sequencing cycle was clear without any background signal and surprisingly the noise was very low in the chromatograms. The success of the peptide sequencing was due to two important factors. The modification of QPs purification yielded pure protein in sufficient quantities. By using a hydrophobic column we were able to obtain an enriched QPs fraction. Since the SDH subunits are more

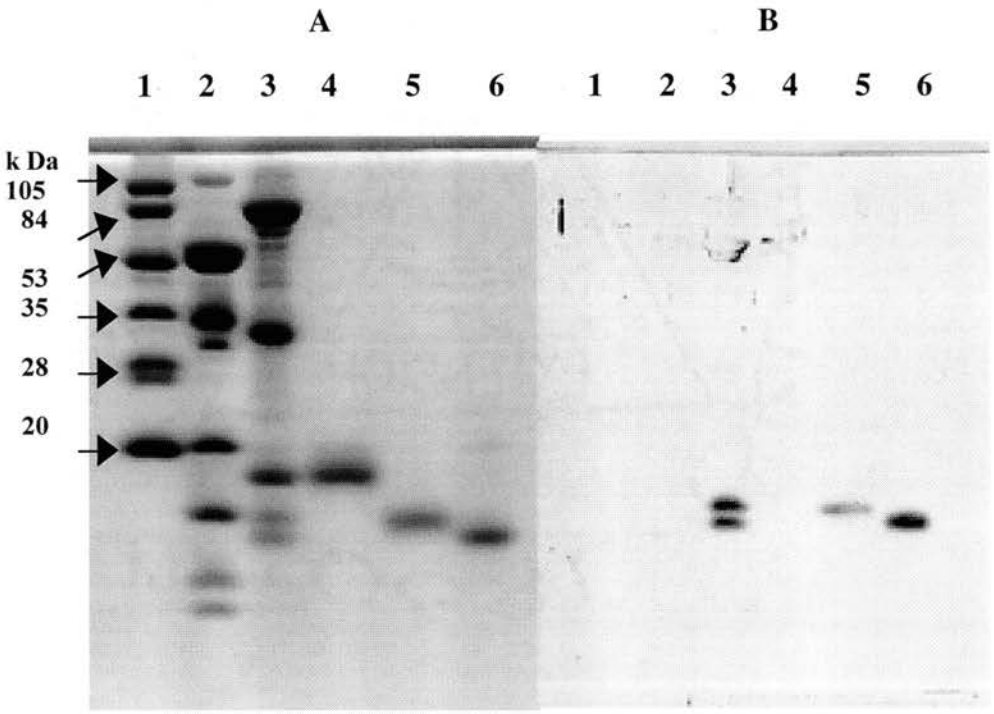


Figure 7. Panel A shows the SDS-PAGE of molecular weight markers (lane1), ubiquinol cytochrome c reductase (lane 2), succinate-Q reductase (lane 3), isolated QPs1 (lane 4), isolated QPs2 (lane 5), isolated QPs3 (lane 6); Panel B shows the Western Blot of A reacted with anti-QPs3 antibodies as the primary antibody and Protein-A horseradish peroxidase conjugate as the secondary antibody.

hydrophilic they did not bind to the hydrophobic column. Further the precasting and prerunning of acrylamide gels and the use of free radical scavenger, thioglycolate in the running buffers helped to prevent blocking of NH_2 terminus of the protein and thus made the microsequencing possible.

Previously a short stretch of QPs3 sequence has been reported as NH_2-SDSKAASL by Dr. Capaldi's group (14). The amino acid at the second position is different from our sequence. This discrepancy is solved by the deduced amino acid sequence from our QPs3 cDNA sequence.

Isolation of the cDNA Encoding QPs3 From the Beef Heart cDNA Library in λZAP by PCR Cloning

Enzymatic amplification of specific cDNA inserts from λgt11 libraries has been reported (15). The design of synthetic guessmers requires knowledge of a partial amino acid sequence of the target protein. The success in obtaining a long and clear stretch of amino acid residues from microsequencing enabled the use of PCR cloning method to isolate the QPs3 cDNA from a beef heart cDNA library in λZAP. A 110-bp cDNA fragment was amplified from a beef heart cDNA library in λZAP ($4x10^6$ pfu) by PCR using two synthetic guessmers, 5'GCTGCCTCCCTGCACTGGAC3' (the sense primer) and 5'GCAGCCAGGGAGTAGTCCAT3' (the antisense primer). The sense primer represents the guessed sequence for residues 5-11 (A^5-A-S-L-H-W-T^{11}) with the degenerate third base of the codon of T^{11} being omitted. The antisense primer represents the guessed sequence for residues 41-35 (A^{41}-A-L-S-Y-D-M^{35}). Since W and M have no degeneracy in their genetic codes, the presence of W^{10} and M^{35} in the QPs3 partial sequence enables us to design these two PCR guessmers with specificity at the 3' end: five specific bases in the 3' end of the sense guessmer and three in the antisense guessmer. The protocol for the isolation of QPs3 cDNA by PCR is summarized in Figure 8.

PCR amplification was performed in a minicycler from M. J. Research. The thermal cycle was set-up as follows: step 1, 95 °C for 3 min, step 2,. 94 °C for 1 min for denaturation; step 3,. 37 °C for 2 min for annealing; and step 4,. 70 °C for 90 second for extension. A total of 30 cycles were performed with a final extension step of 7 minutes.

A $4x10^6$ pfu of a cDNA library constructed in Uni-ZAP XR vector was used as template in the PCR reaction consisting of 20 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 30 mM KCl, 0.05% Tween 20, 100 ug of autoclaved gelatin, 100 μM of dNTP, 2 units of Taq polymerase, and 200 pmol of guessmers. The resulting 110-bp PCR product was cloned into a PCR vector (TA cloning kit) and sequenced. The DNA sequence of this 110-bp PCR product translated to match the amino acid residues between Trp^{10} and Met^{35} of the chemically determined partial N-terminal sequence of QPs3.

Based on the nucleotide sequence for residues 10-35 of QPs3, two gene specific primers, 5'TTGCTCCTGGGCCTAATTCC3', corresponding to residues 21-27 (sense primer), and 5'AGGAGCAAAACACTGAACAAC3', corresponding to residues 22-17 (antisense primer) were synthesized and used with the vector ZAP's primers, T7 and T3, respectively, in the subsequent PCR reactions to yield 5' RACE and 3' RACE products. These two PCR products were cloned into the PCR II vector and sequenced. The 3' RACE product is confirmed by matching the deduced amino acid residues 21-42 of QPs3 with chemically determined partial N-terminal amino acid sequence, and residues 59-66 and 44-49 with chymotryptic peptides of QPs3 with retention times of 18.96 and 31.15 min, respectively, on HPLC chromatogram.

Sequence Analysis of QPs3

Figure 9 shows the nucleotide sequence and the deduced amino acid sequence of QPs3. The QPs3 cDNA is 1330 base pairs long with an open reading frame of 474 base pairs that encodes 158 amino acid residues: 103 of which, starting with serine, belong to mature QPs3, 55 of which, starting with methionine, constitute an N-terminal

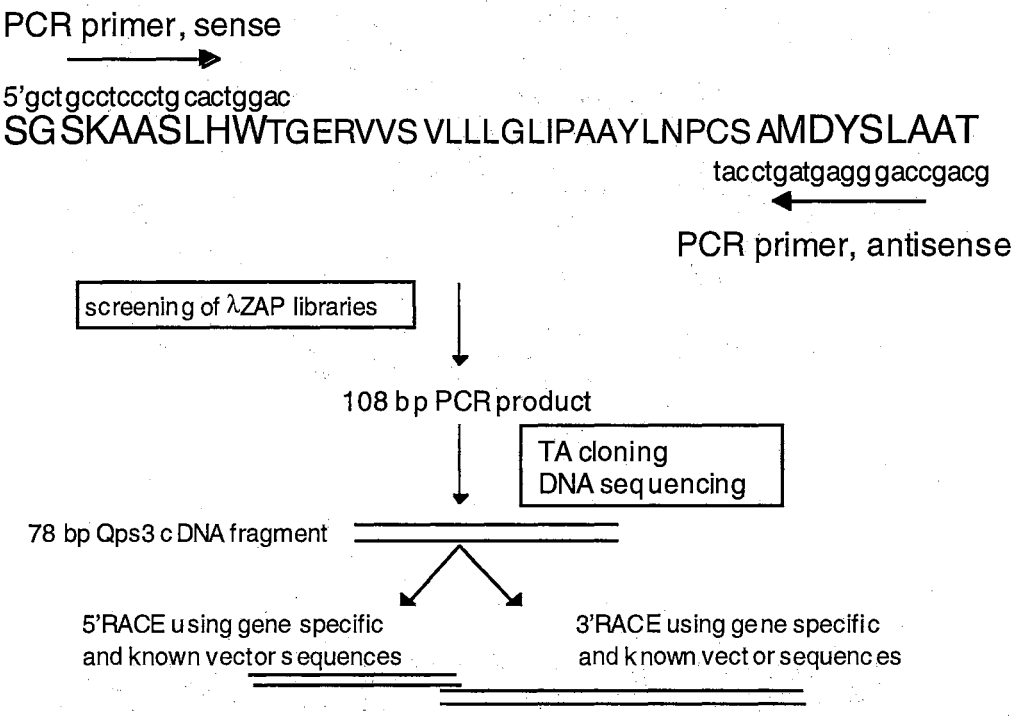


Figure. 8 The Protocol To Isolate QPs3 cDNA by PCR Cloning

```
GAATTCGGCACGAG ATG GCT CTC TGG AGG CTA AGT GTC CTC TGC GGC GCC AAA GAA GGG CGA
               met ala leu trp arg leu ser val leu cys gly ala lys glu gly arg

GCT CTG TTC CTC CGA ACC CCA GTG GTC AGA CCA GCT CTT GTC TCA GCA TTT CTC CAG GAC
ala leu phe leu arg thr pro val val arg pro ala leu val ser ala phe leu gln asp

CGA CCT GCT CAA GGA TGG TGT GGA ACA CAG CAT ATT CAC CTG TCA CCC AGC CAC CAT
arg pro ala gln gly trp cys gly thr gln his ile his leu ser pro ser his his
1                                       11
TCT GGT TCC AAG GCT GCA TCT CTC CAC TGG ACT GGT GAG AGG GTT GTC AGT GTT TTG CTC
ser gly ser lys ala ala ser leu his trp thr gly glu arg val val ser val leu leu
21                                      31
CTG GGC CTA ATT CCA GCT GCA TAT TTG AAT CCG TGT TCT GCG ATG GAC TAC TCT CTG GCT
leu gly leu ile pro ala ala tyr leu asn pro cys ser ala met asp tyr ser leu ala
41                                      51
GCA ACC CTC ACT CTT CAC AGT CAC TGG GGC ATT GGA CAA GTT GTT ACT GAC TAT GTT CAT
ala thr leu thr leu his ser his trp gly ile gly gln val val thr asp tyr val his
61                                      71
GGA GAT GCA GTG CAG AAA GCT GCC AAG ACA GGC CTG TTG GTG CTC TCG GCT TTC ACC TTT
gly asp ala val gln lys ala ala lys thr gly leu leu val leu ser ala phe thr phe
81                                      91
GCT GGG CTC TGT TAC TTC AAC TAT CAT GAC GTG GGC ATC TGC AAA GCT GTG GCT ATG CTG
ala gly leu cys tyr phe asn tyr his asp val gly ile cys lys ala val ala met leu
101
TGG AAG CTC TGA
trp lys leu *

CCTTTTAGACTTAATACCTTGAGAATTGATTGTACACCTCCTTGCCTCTGCTCTTGTCATGCCATTTCAACTCACAAATAA
GAAGGAAAACATTGGGTGAGACAAACCTCCTCTTCCTAATCACCTGATTGTTTTTAGAATTTAATCTTTTGAAGAAAGATT
TGGAGAGAAGTTGTATTCTTTAAGAAATTATAAGACTGAATTTTGTACTTCCTGGGGAGGTAATGGAGTGTCTCAGCTTTT
CATAAGTCTCATAGTATAACTGAACATTATATATGAGCTTTTGGGCTTTTAATTGATCAAACTCTTAAAGGAATTCAGCTT
TATTACTCCGATATCTGATCAAACGTCTGTGTTTGGCTAGGATGGATGGAGAATGGGAAAGACTTAATTCATAAGTAAAGA
CTGCAGAAAATTAGGCAGTGTTTAGTTTTTGTAAACCTCCCCCTCTGTTCAGTTGATACCAGTTACTGATGGTTACATGTC
TTAGGGAAATTTGAAAATTAGAAATGCTGATATTTCACATTACTGATTTCTAAATCCTAGAAAGAAGAGCCTGGAGAGTTT
CTGAATGTAGAGAAGTTCCATTTAGGGAAGAGGTCCCTTTATAGATGTTTCCAAATTGTTGCAGATTCTAAACCAAGACTT
AATCCTCAAATGTGTTTATTTTAACTTGACCTAAAATAAGTCTGTCCCACAAATATAAAATTATAAGTGATAAGTGTTGGT
TTCCCACTGGGAATTTCTAATGTGAAAATGTATTCCCATGAAGGTAATTTTTAAAAAAATAAAATGCTGCATAATAAAAAT
TTATTCTACTCTTTAAAAAAAAAAAAAAA
```

Figure 9. The nucleotide sequence of cDNA encoding bovine QPs3 and the deduced amino acid sequence. The first amino terminal residue serine of mature QPs3 is marked as 1. The cDNA sequence has been submitted to the GENBANK database with accession number U50987

presequence. In addition, the cDNA has 820 nucleotides of 3' non-coding sequence and contains a poly(A) tail.

The presequence of QPs3 is rich in the basic amino acid arginine and contains the hydroxyl amino acid serine. This is characteristic of the cleavable amino terminal presequences that are essential for the import of mitochondrial proteins encoded by nuclear DNA (16). Since the QPs3 presequence lacks arginine at the -2 position relative to the mature amino terminus, QPs3 may be matured by a two-step cleavage process (17). Mitochondrial proteins with leader peptides containing the R-X-(F)-X-X-(S) motif, where R= arginine at the -10 position, X=other amino acid at -9 ; (F)= hydrophobic residues at -8 ; and (S)= serine, threonine or glycine at -5 , are thought to cleave first by a matrix processing protease between residues at -9 and -8, since arginine at position -10 is at position-2 relative to the cleaved bond. The remaining octapeptide is subsequently removed by an intermediate-specific protease. Although the QPs3 presequence lacks the arginine^{-10} in the common motif of this two-step cleavage, the maturation of QPs3 may still follow the same process because mutation of arginine^{-10} in the human ornithine transcarbamylase precursor or rat malate dehydrogenase precursor, to alanine (18, 19) does not alter the two step maturation of these two proteins.

The molecular weight of mature QPs3, determined from the deduced amino acid sequence, is 10,989 daltons, which is fairly close to the 9 kDa estimated from SDS-PAGE. Figure 10 compares the amino acid sequence of bovine QPs3 with those of *S. cerevisiae* SDH4 (20), adult *A. suum cytbS* (21), *E. coli* SDHD (22) and FRDD (23). Bovine QPs3 shares no sequence homology with gene products of *S. cerevisiae sdh*4, *E. coli sdh*D and *frd*D, but has 50% sequence identity with the *A. suum* (adult) cytochrome *b* small subunit of fumarate reductase (20). It is noteworthy that the similarity between bovine QPs3 and *A. suum* cybS is greatest in the region from Leu-23 to His-60 of QPs3 (68%). This region contains a conserved histidine residue that matches His-71 of *E. coli* SDHD which

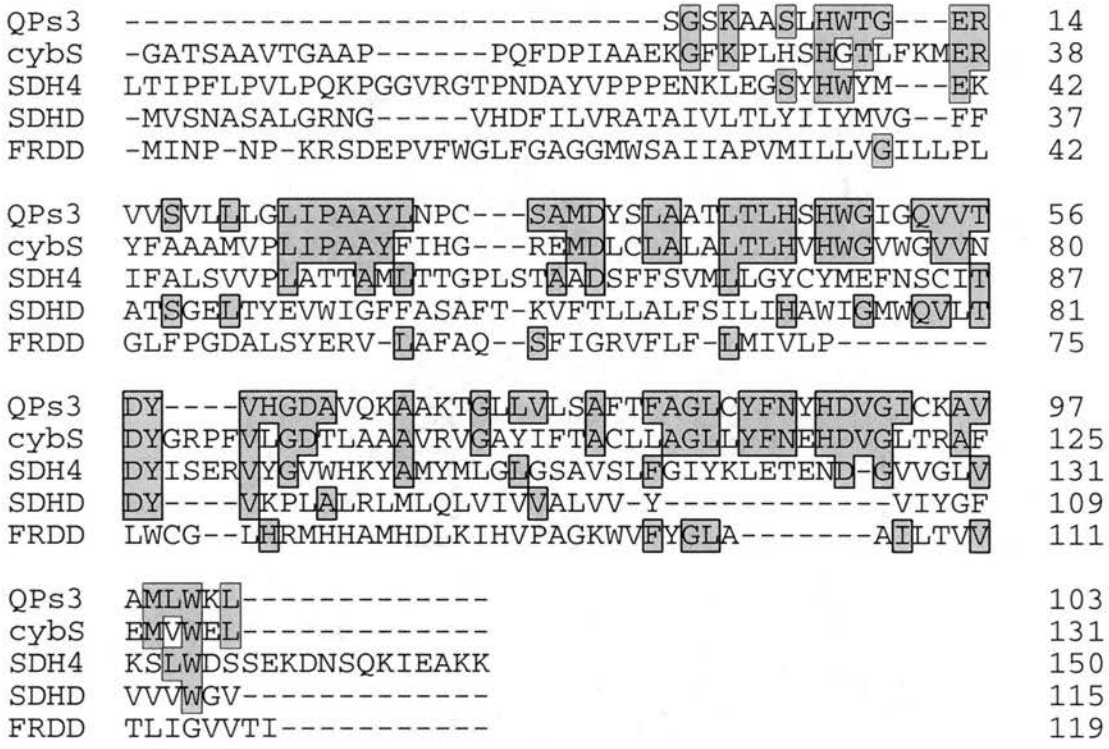


Figure 10. Comparison of the peptide sequences of the smallest membrane anchor subunits of succinate-Q reductases from different species. Amino acid residues to those identical in QPs3 are boxed.

provides the heme *b* ligand (24). His-81 of *E. coli* FRDD that was reported to be involved in Q-binding is also in this region (25). The QPs3 cDNA was found to have 85% sequence identity to the DNA sequence of a Homo Sapiens cDNA clone (R91018) according to a BLAST search of the Expressed Sequence Tag (EST) database.

Chapter Summary

The cDNA encoding the smallest membrane anchoring subunit (QPs3) of bovine heart mitochondrial SQR was cloned and sequenced. This cDNA is 1330 base pairs long with an open reading frame of 474 base pairs that encodes the 103 amino acid residues of mature QPs3 and a 55 amino acid residue presequence. The cDNA has a 820 base pair long 3' untranslated region, including a poly(A) tail. The molecular mass of QPs3 is calculated as 10,989 Da. QPs3 has no sequence identity to SDHD of *E. coli* SQR in contrast to QPs1 which shares more than 40% sequence identity with SDHC. QPs3, however shares more than 50% identity with the smallest anchor subunit of FRD from *A. suum* (adult). QPs3 cDNA is also found to be related to a human cDNA clone reported in the EST database.

References

1. Yu, L., Wei, Y-Y., Usui, S., and Yu, C. A. (1992) *J. Biol. Chem.*, **267**, 24508-24515.
2. Lee, G. Y., He, D-Y., Yu, L., and Yu, C. A. (1995) *J. Biol. chem.*, **270**, 6193-6198.
3. Jenner, E. L. (June 5 1973) US Patent 3737516.
4. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A laboratory Manual,* Cold Spring Harbor Press, NY.
5. Harmon, H. J., and Crane, F. L.. (1976) *Biochim. Biophys. Acta*, **440**, 45-58.

6. Yu C. A., and Yu, L. (9180) *Biochemistry*, **19**, 5715-5720.

7. Yu C. A., and Yu L. (1982) *J. Biol. Chem.*, **257**, 2016-2021.

8. Schagger, H., Link, T. A., Engel, W. D., and von Jagow, G. (1986) *Methods Enzymol.*, **126**, 224-237.

9. Mierendorf, R. C., Percy, C., and Young, R. (1987) *Methods Enzymol.*, **152**, 458-469.

10. Usui, S., Yu, L., and Yu, C. A. (1990) *Biochem. Biophys. Res. Commun.*, **167**, 575-579.

11. Martin R., Hoover, C., Grimme, S., Grogan, C., Holtke, J., and Kessler, C., (1990) *Biotechniques*, vol 9, 762-769.

12. Gyesoon L. Ph. D Thesis, (1996), Department of Biochemistry and Molecular Biology, O.S.U. Stillwater, OK-74078.

13. Yu, L., Deng, K-P., Yu, C. A. (1995) *J. Biol. Chem.*, **270**, 25634-638.

14. Cochran, B., Capaldi, R. A., and Ackrell, B. A. C. (1994) *Biochim. Biophys. Acta*, **1188**, 162-166.

15. Friedman, K. D., Rosen, N. L., Newman, P. J., and Montgomery, R. (1988) *Nucleic Acids Res.*, **16**, 8718.

16. Hurt, E. C., and Van Loon, A. P. (1986) *Trends Biochem., Sci.* **11**, 204-207.

17. Hendrick, P. J., Hodges, P. E. and Rosenberg, L. E. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 4056-4060.

18. Horwich, A. L., Kalousek, F. Fenton. W. A., Pollock, R. A., and Rosenberg, L. E. (1986) *Cell* , **44**, 451-459.

19. Chu, T. W., Grant, P. M., and Strauss, A. W. (1987) *J. Biol. Chem.*, **262**, 12906-12811.

20. Bullis, B. L., and Lemire, B. D. (1994) *J. Biol. Chem.*, **269**, 6543-6549.

21. Saruta, F., Hirawake, H., Takamiya, S., Ma, Y-C., Aoki, T., Sekimizu, K., Kojima, S., and Kita, K. (1996) *Biochim. Biophys. Acta* , **1276**, 1-5.

22. Wood, D., Darlison, M. G., Wilde, R. J., and Guest, J. R. (1984) *Biochem. J.*, **222**, 519-534.

23. Cole, S. T. (1982) *Eur. J. Biochem.*, **122**, 479-484.

24. Rose, C. T., Cecchini, G., Nakamura, K., Kita, K., and Gennis, R. B. (1998), *Biochemistry* , **37**, 3148-3159.

25. Westenberg D.J., Gunsalus R. P., Ackrell B.A., Sices H. and Cecchini G. (1993) *J. Biol. Chem.*, **268**, 815-822.

Experimental Procedures

Materials

Glutaraldehyde, enzyme grade horseradish peroxidase, bovine serum albumin, dichlorophenolindophenol, Triton X-100, sodium cholate, were purchased from Sigma. Protein A-horseradish peroxidase conjugate, Low range SDS-PAGE molecular weight markers, DEAE-Affigel blue, were from Biorad. Sephacryl separation matrices were from Pharmacia. Peptides were synthesized by the Recombinant DNA/Protein Resource Facility at Oklahoma State University.

Enzyme Preparations and Assays

Intact mitochondria (5), mitoplasts (6), submitochondrial particles (7) and SQR (8) were prepared and assayed by reported methods. SQR was assayed for its ability to catalyze Q reduction or Q-stimulated DCPIP reduction by succinate. The assays were performed at room temperature in a Shimadzu UV-2101PC. The reaction mixture used for Q-stimulated DCPIP reduction assay contains 38μmol of DCPIP, 100 μmol of sodium/potassium phosphate buffer, pH 7.4, 20 μmol of succinate, 10 nmol of EDTA, 25 nmol of Q_0C_{10} and 0.01% Triton X-100 in the total volume of 1 ml. The reduction of DCPIP was followed by measuring the absorption decrease at 600 nm, using a millimolar extinction coefficient of 21 $mM^{-1}cm^{-1}$. Protein concentration was determined by the Lowry method (9). Western blotting was performed according to a published method (10).

Production and Purification of Antibodies Against QPs3 N-terminal and Connecting Peptides

Three polypeptides, one containing 14 amino acid residues (NH_2-S-G-S-K-A-A-S-L-H-W-T-G-E-R-COOH) corresponding to residues 1-14 of QPs3 (the N-terminal peptide, see Figure 13), another peptide containing 11 amino acid residues (NH_2-T-D-Y-V-H-G-D-A-V-Q-K-COOH) corresponding to residues 56-66 (the connecting peptide, the loop between helices 2 and 3, see Figure 13) and a third containing 14 residues corresponding to

the C-terminal residues 90-103 (NH_2-H-D-V-G-I-C-K-A-V-A-M-L-W-K-L-COOH) were synthesized. These peptides were conjugated to carrier protein (Scheme I) ovalbumin using the homobifunctional reagent, glutaraldehyde, as described below. The solubility of the synthetic peptide was tested in water and PBS buffer, pH 7.8. The peptide solution was mixed with ovalbumin in the weight ratio 1:1 and molar ratio of 50 moles peptide/1 mole carrier. The mixture was slowly stirred on ice and an equal volume of 2% solution of glutaraldehyde was added dropwise. The mixture was stirred at 0 °C for about 2 hours after which about 3 mg of sodium borohydride for every mL solution was added in small increments and the mixture was continuously stirred for 30 min. The coupled peptide-carrier solution was dialyzed against PBS buffer with frequent changes of buffer and concentrated with centricon and stored at 4 °C for future use. The coupling reaction was verified by running an aliquot of coupled antigen on a 12 % SDS-PAGE and comparing with the same amount of carrier protein only. About 150 μg of coupled protein was used as antigen to raise monospecific polyclonal antibodies in rabbits. Boosters were given weekly for 5 weeks, and sera were collected by cardiac puncture. Purification of antibodies was performed as described in chapter I. Control antibodies were prepared in the same manner from serum from the respective rabbit taken prior to immunization. Characterization of antibodies was done by western blots using SQR and pure QPs subunits as antigens.

Preparation of Fab' Fragment-Horseradish Peroxidase Conjugates of Anti-QPs3 Antibodies

F(ab')2 and F(ab') fragments were prepared by pepsin digestion of IgG according to the methods of Stanworth and Turner (11) and Taniguchi et al. (12), respectively. Specifically, two mg of horseradish peroxidase (type II, Sigma) dissolved in 0.3 ml of 0.1 M sodium phosphate, pH 7.0, was mixed with 20 μl of a coupling reagent, 4-(N-maleimidomethyl)-cyclohexane-1-carboxylic acid N-hydroxysuccinimide ester in N,N-dimethylformamide, and incubated at 30°C for 45 min. After centrifugation at 10,000 x g

the supernatant solution was applied to a Sephadex G-25 column equilibrated with 0.1 M sodium phosphate, pH 6.0, to remove excess coupling reagent. Peroxidase with maleimide groups was collected and concentrated to a protein concentration of 4 mg/ml. Fab' fragment was prepared by reducing the cystine groups of F(ab)2 to cysteine using a 100 times molar excess of 2-mercaptoethanol. After 2 hours of 30 °C incubation the mixture was applied to a Sephadex G-25 column equilibrated with 0.1 M sodium phosphate, pH 6.0, containing 5 mM EDTA to remove the 2-mercaptoethanol. Fab' was collected and adjusted to a protein concentration of 5 mg/ml. The freshly prepared Fab' fragment (2.5 mg) was mixed with the derivatized peroxidase (2 mg) and incubated at 4°C for 20 hours. Fab'-HRP conjugate was separated on a Sephacryl S-200 HR column in 0.1 M sodium phosphate, pH 6.5. The horseradish peroxidase activity of the purified conjugate was assayed, using a TMB peroxidase substrate kit (Bio-Rad), according to the manufacturer's instructions.

Results and Discussions

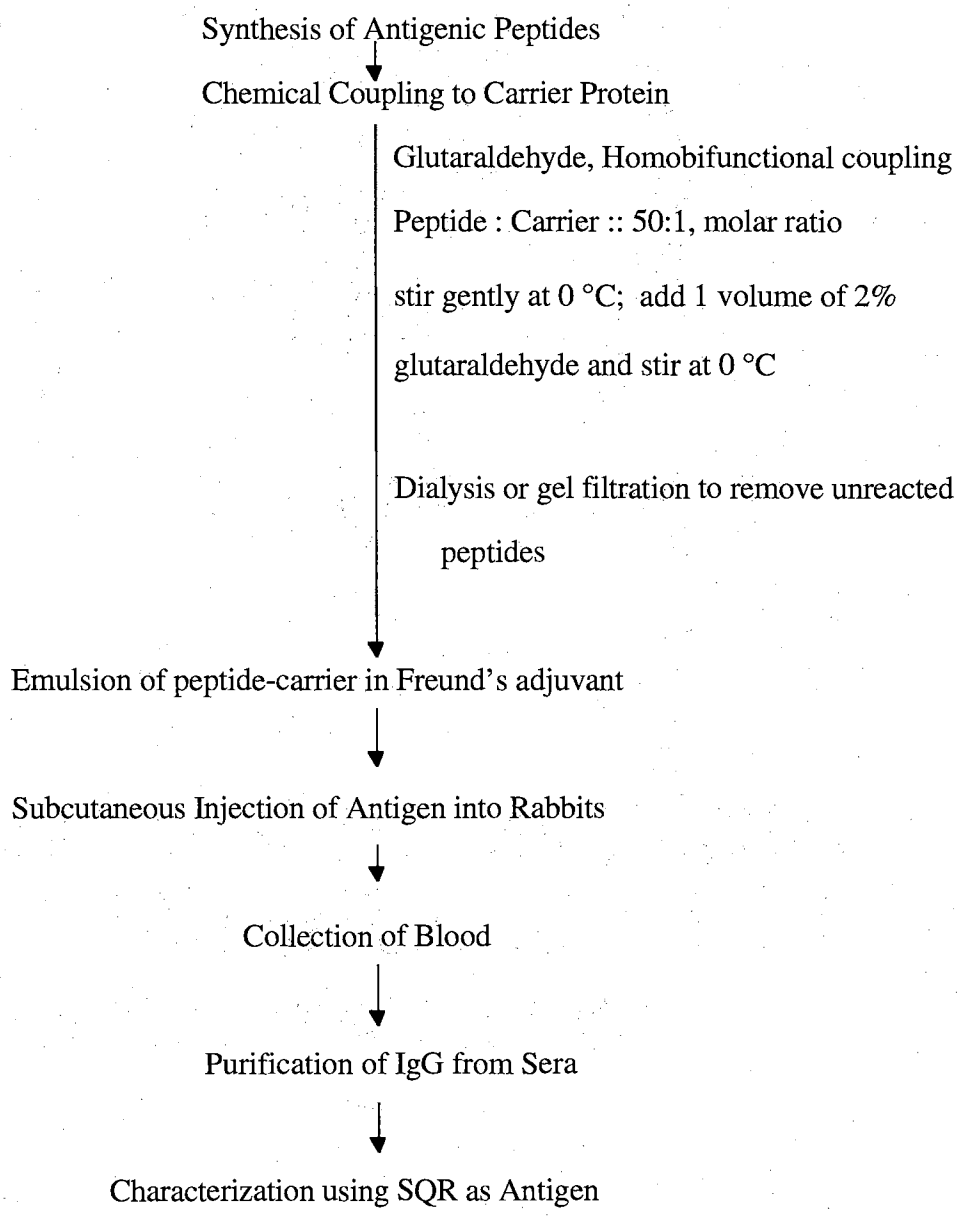
Preparation of QPs3 Peptide Antigens and Immunological Specificity of Anti-QPs3 Peptide-Antibodies

The preparation of peptide antigen by coupling to glutaraldehyde and the preparation of Fab'-horseradish peroxidase is outlined in Schemes I and II, respectively. The advantages of using Fab'-HRP conjugate are two-fold: (I) the antibody fragment conjugate has easy access to the epitopes of the antigen in our membrane preparation because of its smaller molecular size than the whole IgG; (ii) the amount of conjugate bound to the antigen can be estimated by assaying the horseradish peroxidase activity.

The N-terminal peptide and the connecting peptide could be successfully coupled to ovalbumin (Coupled antigen-ovalbumin showed a decreased mobility on SDS-PAGE than ovalbumin alone) and were injected into rabbits to produce monospecific polyclonal anti-

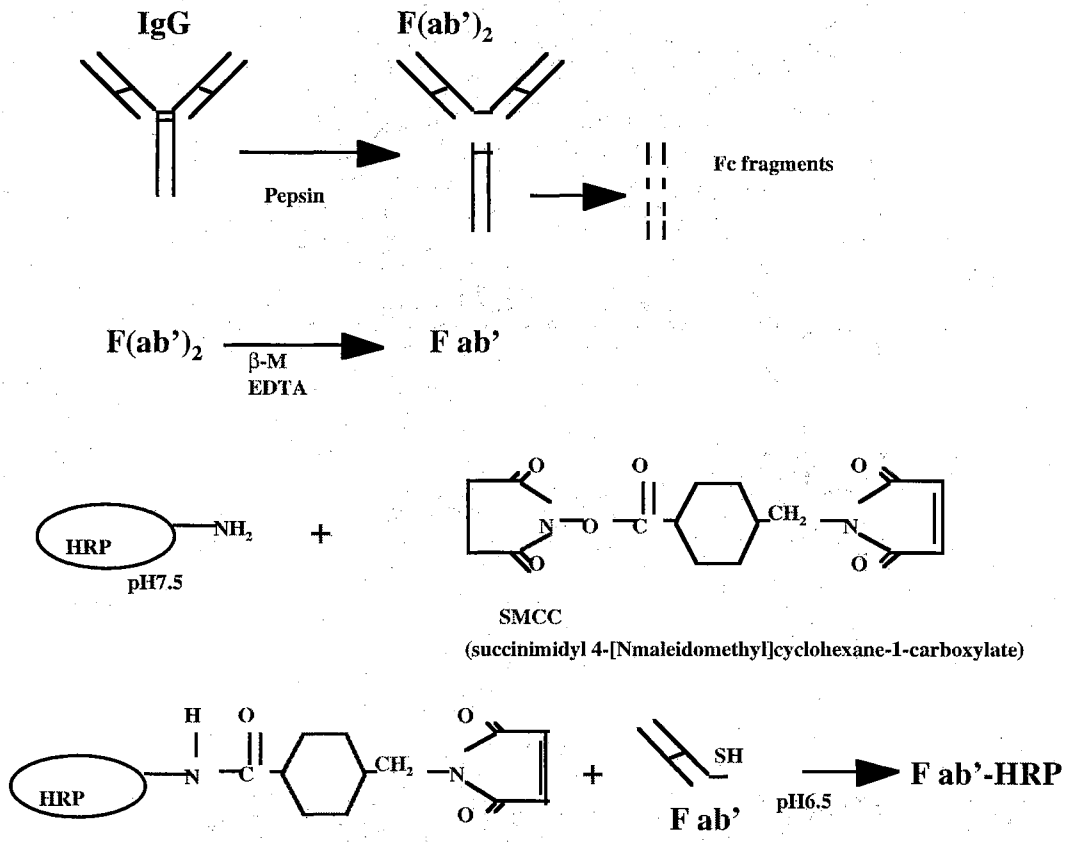
Scheme I

Production of Monospecific Polyclonal Antibodies Against QPs3 Peptides.



Scheme II.

Preparation of Fab'-Horseradish Peroxidase Conjugate



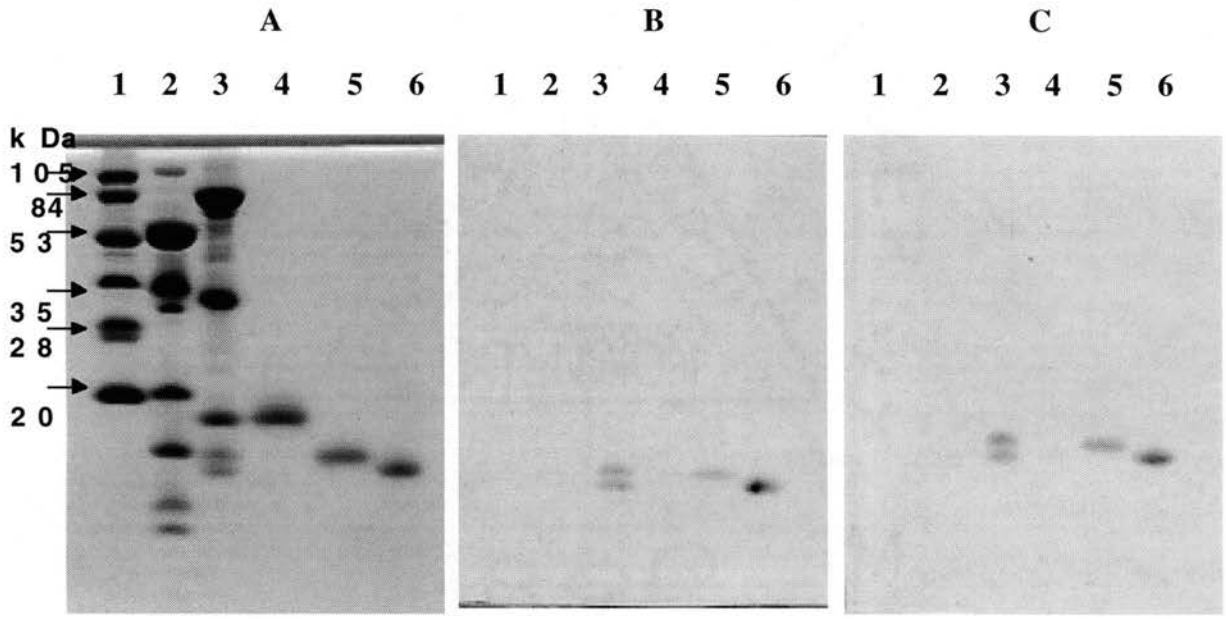


Figure 11. Western Blot Analyses of Antibodies Against N-terminal and Connecting Peptides of QPs3. Panel A, SDS-PAGE of molecular weight standards (lane 1), ubiquinol-cytochrome *c* reductase (50 µg, lane 2), succinate-Q reductase (26 µg, lane 3), purified QPs1 (7 µg, lane 4), QPs2 (6 µg, lane 5), and QPs3 (8 µg, lane 6). The proteins on the gel of panel A were electrophoretically transferred to a nitrocellulose membrane without staining and then incubated with, antibodies against the N-terminal peptide (panel B), and antibodies against the connecting peptide (panel C).Protein A Horseradish peroxidase conjugate was used as second antibody.

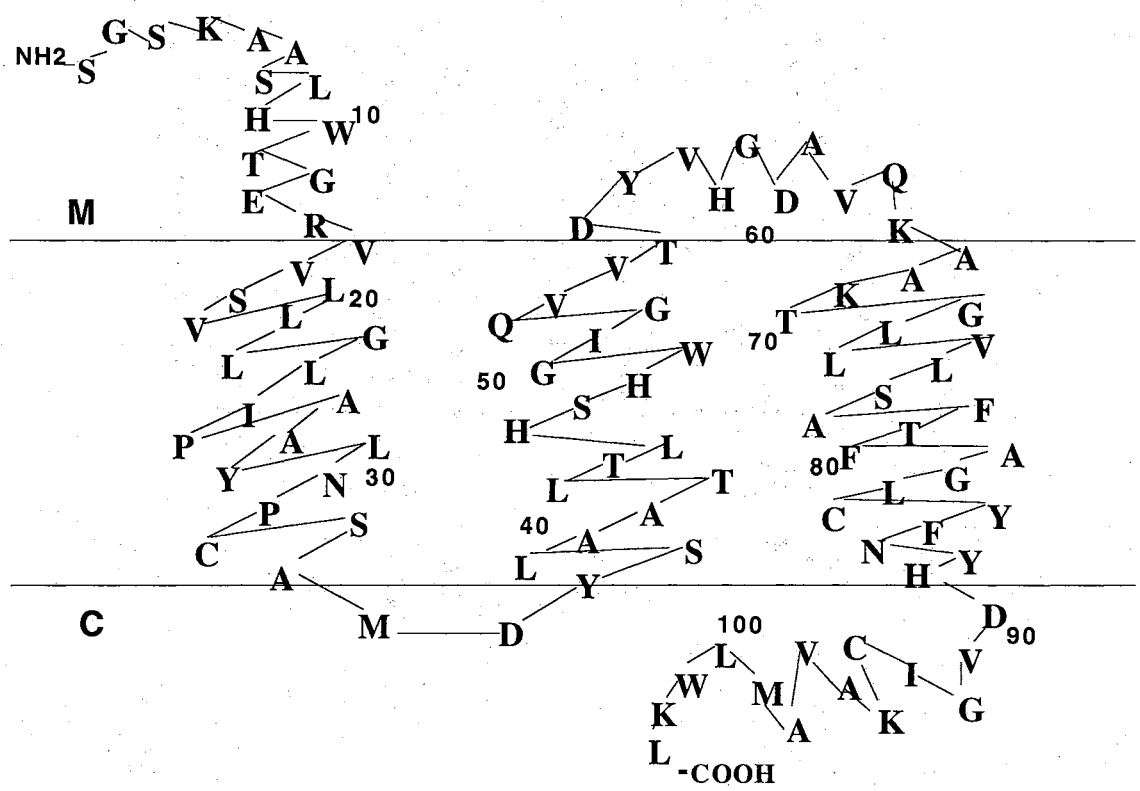
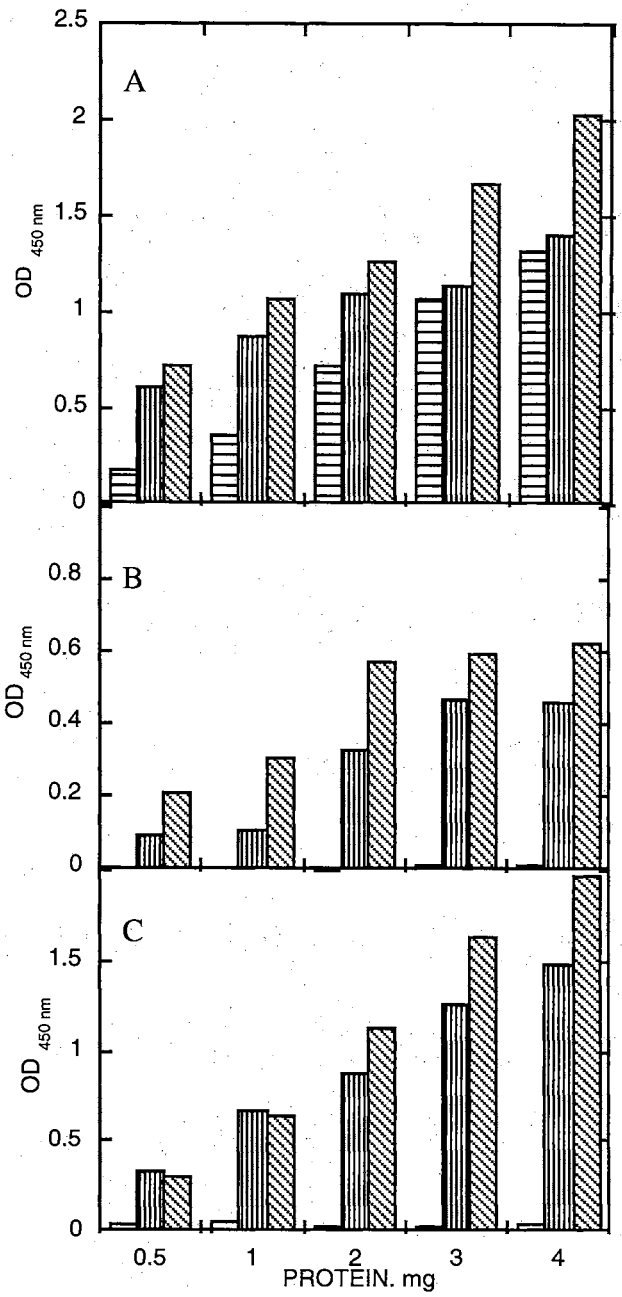


Figure 12. The Proposed Structural Model of QPs3 in the Inner Mitochondrial Membrane

Figure 13. Binding of Fab' fragment-horseradish peroxidase conjugates prepared from antibodies against QPs3, the N-terminal and connecting peptide of QPs3 with mitoplasts, submitochondrial particles, and alkali treated submitochondrial particles. The indicated amounts of mitoplasts (▤), submitochondrial particles (▥), and alkaline-treated submitochondrial particles (▧) were mixed with 10 milliunits of Fab-horseradish peroxidase conjugates prepared from anti-QPs3 antibodies (A), anti-N-terminal peptide antibodies (B), and anti-connecting peptide antibodies (C) in 50 mM sodium phosphate, pH 7.4, containing 0.25 M sucrose and incubated at 4 °C for 3 h. The mixtures were centrifuged at 30,000 x g for 15 min, and the precipitate was suspended in 50 mM sodium phosphate, pH 7.4, containing 0.25 M sucrose. This procedure was repeated three more times before aliquots of the suspension were taken and horseradish peroxidase activity was assayed. Preimmune Fab'-horseradish peroxidase conjugate treated with mitoplasts, submitochondrial particles and alkali-treated SMP in the identical manner was used as control. The activities indicated are after subtracting the control activity.


A
B
C
OD 450 nm
2.5
2
1.5
1
0.5
0
0.8
0.6
0.4
0.2
0
1.5
1
0.5
0
0.5
1
2
3
4
PROTEIN. mg

QPs3 antibodies. However, the C-terminal peptide was insoluble in buffers above pH 6.0 and hence could not be successfully coupled to the carrier.

The antibodies against QPs3, N-terminal peptide and connecting peptide were tested for their specificity in a western blot using antigens namely, QCR, SQR, and purified QPs1, QPs2, and QPs3 (Figures 7 and 11)

It should be mentioned that during the course of immunological studies of QPs3 we observed that antibodies against QPs3, the N-terminal, and the connecting peptides cross react with QPs2 (see lanes 3, 5, & 6 in Panels B of figure 6 and, C, & D of Figure 11). They do not react with QPs1 or proteins in QCR (see Figure 11). These results suggest that QPs2 and QPs3 have several common epitopes.

The Proposed Structure of QPs3 in the Mitochondrial Inner Membrane

Figure 12 shows the proposed structure of QPs3 in the inner mitochondrial membrane. This structural model, was constructed based on hydropathy plots of the amino acid sequence of QPs3, predicted tendencies to form α-helices and β-sheets, and the binding of Fab' fragment-horseradish peroxidase conjugates, prepared from antibodies against synthetic peptides corresponding to residues 1-14 and 55-66 of QPs3, in mitoplasts, submitochondrial particles (SMP), and alkali-treated submitochondrial particles. In this model QPs3 has three transmembrane helices corresponding to residues 15-34 (helix I), 37-56 (helix II) and 67-89 (helix III). The N-terminus region, residues 1-14, and the loop connecting helices II and III, residues 57-66, are extruded from the M-side of the inner mitochondrial membrane. The loop connecting helices I and II, residues 36-37, and the C-terminus region, residues 90-103, are on the C-side of the membrane.

The sidedness of the membrane in this model was determined immunologically with Fab'-horseradish peroxidase conjugates prepared from anti-QPs3, anti-N-terminus peptide (residues 1-14), and anti-connecting peptide (residues 57-66) antibodies, in bovine heart mitoplasts (digitonin-treated intact mitochondria), submitochondrial particles (SMP) (reverse orientation), and alkaline treated SMP (SMP devoid of succinate dehydrogenase).

The peroxidase activity assays of these three particles are shown in Figure 13. Since the peroxidase activity observed with preimmune Fab'-horseradish peroxidase-treated preparations is assumed to be due to nonspecific binding, it is subtracted from that of the anti-QPs3, anti-N-terminus peptide, or anti-connecting peptide Fab'-horseradish peroxidase-treated preparations. The intactness of mitoplasts and submitochondrial particle preparations was established by the absence and presence of rotenone-sensitive NADH-Q reductase activity.

When mitoplasts and SMP preparations were treated with anti-QPs3 Fab' fragment-peroxidase conjugates, peroxidase activity was detected in both preparations. The slightly higher activity in treated SMP suggests that QPs3 is a transmembranous protein with slightly more mass exposed on the matrix side of the membrane. When an alkali-treated SMP preparation is treated with anti-QPs3 Fab' fragment-horseradish peroxidase conjugate, only a slight increase in peroxidase activity is observed, indicating that few epitopes on the matrix side of QPs3 are covered by succinate dehydrogenase.

When mitoplasts and SMP preparations are treated with Fab' fragment-horseradish peroxidase conjugates prepared from anti-N-terminal and connecting peptides antibodies, peroxidase activity was observed only on SMP, indicating that the N-terminal end and the loop connecting helices II and III are exposed on the M-side of the mitochondrial inner membrane.

Chapter Summary

The topology of QPs3 in the inner mitochondrial membrane was studied using an immunological approach. The structure of QPs3 in the inner mitochondrial membrane is proposed based on the hydropathy profile of the amino acid sequence, predicted tendencies to form α-helices, and β-sheets, and on immunobinding of Fab' fragment-horseradish peroxidase conjugates prepared frrom antibodies against two synthetic peptides, corresponding to the N-terminus region and the loop connecting helices 2 and 3 of QPs3,

in mitoplasts and submitochondrial particles. Accordingly QPs3 has three transmembrane segments with its amino terminus exposed to the matrix side, and carboxy terminus exposed to the cytoplasmic side.

References

1. Merli, A., Capaldi, R. A., Ackrell, B. A. C. and Kearney, E. B. (1979) *Biochemistry*, **18**, 1393-1400.
2. Juhasz, P., Papayannopuolos, I. A. and Biemann, K. (1992) in *Proceedings of the 12 th American Peptide Symposium,* (Smith, J. A. and Rivier, J. E., Eds) 558-559, Escon Sci Pub. The Netherlands.
3. Ackrell, B. A. C., Johnson, M., Gunsalus, R. P. and Cecchini, G. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Muller, F., ed) Vol. III 229-297, CRC Press, London.
4. Kyte, J. and Doolittle, R. F. (1982) *J. Mol. Biol.*, **157**, 105-132.
5. Harmon, H. J., and Crane, F. L. (1976) *Biochim. Biophys. Acta*, **440**, 45-58.
6. Greenawalt, J. W. (1974) *Methods Enzymol.*, **31**, 310-330.
7. Harmon, H. J. (1982) *J. Bioenerg. Biomembr.*, **14**, 377-386.
8. Yu, C. A., and Yu, L. (1980b) *Biochemistry*, **19**, 5715-5720.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., (1951) *J. Biol. Chem.*, **193**, 265-275.
10. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 4350-4354.
11. Stanworth, D. R., and Turner, M. W., (1978) in *Handbook of Experimental Immunology*, 3rd ed. (Weir, D. M., ed) Vol I 6.1-6.102, Blackwell Scientific Publications Oxford.
12. Taniguchi, S., Tamoto, K., and Koyama J. (1978) *J. Biochem.* (Tokyo) **84**, 907-915

CHAPTER III

IDENTIFICATION OF THE UBIQUINONE-BINDING DOMAIN IN QPs3 OF SUCCINATE-Q REDUCTASE

When a succinate-free, partially Q-deficient SQR is treated with [^{3}H]azido-Q derivatives in the dark followed by illumination with a long wavelength UV light , about 50% of bound Q is located on QPs1 and the other 50% is equally distributed between QPs2 and QPs3 (1). Although lesser amounts of azido-Q were found in QPs2 and QPs3, involvement of these two subunits in the Q-binding site cannot be ruled out. The low azido-Q uptake by QPs2 and QPs3 may result from (i) only part of a Q-binding site being formed by these two subunits; (ii) preferential binding of these two subunits by endogenous Q present in the partially Q-deficient SQR used in these photoaffinity labeling studies; or (iii) QPs2 and QPs3 being the same peptide, i.e., either QPs2 is incompletely processed QPs3 or QPs3 is a C-terminal truncate of QPs2. If the latter is true, the amount of azido-Q uptake by QPs2 or QPs3 equals that by QPs1. Isolation of an azido-Q-linked peptide from azido-Q-labeled QPs2 or QPs3, obtained from azido-Q-labeled succinate-Q reductase, will help determine the Q-binding role of QPs2 or QPs3. In this chapter, I report the isolation and partial N-terminal sequence of azido-Q-linked peptides from labeled QPs3 and QPs2, and the location of the Q-binding domain in the proposed model of QPs3.

Experimental Procedures

Materials

Bovine serum albumin, DCPIP, Triton X-100, sodium cholate, Phenyl Sepharose CL-4B, TTFA, and deoxycholic acid were obtained from Sigma. n-Dodecyl-β-D-maltoside was from Anatrace. Insta-gel liquid scintillation cocktail was from Packard Instrument Co.

The ubiquinone derivatives, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (Q_0C_{10}) and 3-azido-2-methyl-5-methoxy- and 3-azido-2-methyl-5-methoxy[^{3}H]-6-decyl-1,4-benzoquinone {azido-Q and [^{3}H]azido-Q} were synthesized in our laboratory according to previously reported methods (1) and are briefly described below. Calcium phosphate was prepared according to Jenner and mixed at a 3:1 ratio with cellulose powder prior to use in column chromatography.

Synthesis of 3-Azido-2-Methyl-5-Methoxy-6-Geranyl-and 3-Azido-2-Methyl-5-Methoxy-6-(3,7-Diethyl[^{3}H]-Octyl)-1,4-Benzoquinones (azido-Q and [^{3}H]Azido-Q)

Six grams of 4-methyl-3-nitroanisole were mixed with 1g of 10% Pd-C in 95 ml of methanol. After the addition of 5 ml of methanol-HCl (2.47 M), the reaction was carried out for 40 min at 2 atm hydrogen pressure in a Parr shaker. The methanol solvent was evaporated in vacuo and the deep greenish oil was dissolved in 600 ml of cold 0.5 M sulfuric acid. Then 250 ml of a cold solution containing 27 g sodium dichromate and 20 ml concentrated sufuric acid was added slowly with stirring, the temperature being maintained at about 5 °C. After stirring for 4 hours at room temperature the mixture was extracted with 200 ml methanol four times. The combined deep brown extract was stirred with increasing amounts of florisil until the color of the solution became yellowish; florisil was removed by filtration. Upon removal of methanol, 0.5 g of yellow crystals of 2-methyl-5-methoxy-1, 4-benzoquinone were obtained.

Sixty mg of 2-methyl-5-methoxy-1, 4-benzoquinone was dissolved in 20 ml of diethyl ether and shaken with 0.5 g sodium dithionite in 4 ml water until the solution

became colorless. The mixture was extracted with diethyl ether. The reduced benzoquinone, obtained as colorless crystals upon removal of solvent, was dissolved in 4 ml of dioxane, mixed with 80 ml of geraniol, and treated with 100 ml of $BF_3O(C_2H_5)_2$ for 30 min with stirring. The mixture was stirred for 3 hours at room temperature, treated with 12 ml of water, and extracted with diethylether. The ether extract was dried over Na_2SO_4 and oxidized with 0.1 g of Ag_2O for 1 hour with stirring. The solution was filtered and the filtrate was purified by silica gel G plates developed with hexane/diethylether (3.5:1). 2-Methyl-5-methoxy-6-geranyl-1,4-benzoquinone has an R_F value of 0.67. A by-product, 2-methoxy-5-methyl-6-geranyl-1,4-benzoquinone, with an R_F value of 0.22, was also detected on the thin layer chromatogram. 2-Methyl-5-methoxy-6-geranyl-1,4-benzoquinone was eluted with ether and upon removal of solvent, 9 mg of orange oil was obtained. 1H-NMR ($CDCl_3$): 6.42,(q,1), 5.06 (t,2), 4.01 (s,3), 3.18 (d,2), 2.02 (m,7), 1.66 (t,9). UV_{max} (95% ethanol): Ox., 263 nm; Rd., 288 nm; high resolution mass spectra, m/e, 288.1695.

For the synthesis of 3-azido-2-methyl-5-methoxy-6-geranyl-1,4-benzoquinone, 25 mg of 2-methyl-5-methoxy-6-geranyl-1,4-benzoquinone was dissolved in 1 ml of acetic acid (90%), and incubated at 75 °C with constant stirring. The subsequent steps were performed in the dark. Three additions of sodium azide solution (17 mg sodium azide in water) were made over a period of 3 hours. Then the mixture was stirred for 7 hours at 75 °C, concentrated, and purified by thin layer chromatography using the same solvent. The sample was eluted from the plate with ether. Upon removal of ether, 0.5 mg of pure 3-azido-2-methyl-5-methoxy-6-geranyl-1,4-benzoquinone was obtained. 1H-NMR ($CDCl_3$): 1.66,(t,9), 1.98 (m,7), 3.16 (d,2), 3.98 (s,3), 5.04 (t,2), 1.66 (t,9). UV_{max} (95% ethanol): Ox., 306 nm; Rd., 298 nm.

The 3-azido-2-methyl-5-methoxy-6-(3,7-dimethyl[^{3}H]-octyl)-1,4-benzoquinone was obtained via hydrogenation of 2-methyl-5-methoxy-6-geranyl-1,4-benzoquinone with tritiated hydrogen gas followed by azodilization with sodium azide. The tritiated gas was

generated from tritiated water by addition of metallic sodium in one arm of the H-shaped apparatus after evacuation. Metallic sodium was hooked by a movable wire through a silicone plug. The amount of tritiated water used was calculated to be enough to generate a pressure of 3 atm of tritiated hydrogen in the apparatus. 2-Methyl-5-methoxy-6-geranyl-1,4-benzoquinone and Pd/C were mixed and placed in the other arm of the H-shaped apparatus and the whole system was subjected to evacuation. During the evacuation the arm containing tritiated water was placed in a dry ice- acetone bath to decrease evaporation. After evacuation the metallic sodium was slowly lowered into the water to generate the tritiated hydrogen gas. The hydrogenation was completed in 3 hours at room temperature with vigorous shaking. Specific radioactivity for the 3-azido-2-methyl-5-methoxy-6-(3,7-dimethyl[^{3}H]-octyl)-1,4-benzoquinone was 55,000 cpm / nmol, determined in a Beckman liquid scintillation counter.

Enzyme Preparation and Assays

Submitochondrial particles (2), and SQR (3) were prepared and assayed as previously reported. SQR was assayed, at room temperature, for its ability to catalyze TTFA sensitive Q-stimulated DCPIP reduction by succinate, using a Shimadzu UV-2101PC. The reaction mixture (1 ml) contained 40 µmol of DCPIP, 100 µmol of sodium potassium phosphate buffer, pH 7.4, 20 µmol of succinate, 10 nmol of EDTA, 25 nmol of Q_0C_{10} and 0.01% of Triton X-100. The reduction of DCPIP was followed by measuring the absorption decrease at 600 nm, using a millimolar extinction coefficient of 21 $mmol^{-1}cm^{-1}$. The concentration of TTFA used was 10^{-4} M.

Isolation of [^{3}H]azido-Q-labeled QPs2 and QPs3 from [^{3}H]azido-Q SQR

Purified SQR was photoaffinity labeled with the [^{3}H]azido-Q derivative as reported previously (1). The azido-Q-labeled reductase was precipitated by 43% saturation of ammonium sulfate, separated by centrifugation at 48,000 x g for 20 min, dissolved in 20 mM Tris-Cl, pH 7.8, and dialyzed against double-distilled water overnight, with one

change of water. [^{3}H]azido-Q-labeled QPs3 was purified to homogeneity from this dialyzed, labeled succinate-Q reductase. Succinate-Q reductase was diluted to 2 mg/ml with 50 mM Tris-HCl, pH 7.4, containing 0.2% sodium cholate. The solution was stirred at room temperature for 30 min and applied to a phenyl-sepharose CL-4B column equilibrated with 50 mM Tris-HCl, pH 7.4, containing 0.2% sodium cholate. The column was, in sequence, washed with 50 mM Tris-HCl, pH 7.4, containing 0.2% sodium cholate, 50 mM Tris-HCl, pH 7.4, containing 2% sodium cholate and 4 M urea, and 50 mM Tris-HCl, pH 7.4, containing 0.2% sodium cholate. QPs was eluted from the column with 50 mM Tris-HCl, pH 7.4, containing 0.15% SDS. Pure QPs3 was obtained from QPs by preparative SDS-PAGE essentially according to the previously reported method (Chapter I).

Reductive Carboxymethylation and Succinylation of Azido-Q labeled QPs2 and QPs3

The electrophoretically eluted [^{3}H]azido-Q-labeled QPs2 and QPs3 were concentrated by membrane filtration with centricon-10, to a final protein concentration of about 3 mg/ml and precipitated with 50% cold acetone (-20 °C). The protein precipitate was washed with cold water and dried under argon. The precipitate thus obtained was homogenized in 0.1M Tris, 6M guanidine HCl, 1mM EDTA, pH 8.3. Dithiothreitol was added to the suspension to a final concentration of 2 mM. The mixture was made anaerobic by passing argon through it and then incubated at 37 °C for 1 hour. Iodoacetic acid neutralized with NaOH, was added to give a final concentration of 5 mM. The reaction mixture was flushed with argon and the container was sealed and alkylation was continued at 37 °C under dark conditions for 1 h. Additional treatment with 1 mM dithiothreitol and incubation with 2 mM iodoacetate under same conditions resulted in complete alkylation.

The suspension of alkylated protein was treated with successive increments of succinic anhydride with constant stirring while maintaining the pH of the solution between 7 and 8 by dropwise addition of 0.1 N NaOH. Each portion of the anhydride was allowed to completely react (as indicated by the cessation of acid production) before the next

addition. After maximal clarification of the suspension was obtained, β-mercaptoethanol was added to a final concentration of 1% (v/v), and the solution was dialyzed against 50 mM ammonium bicarbonate overnight.

Chymotrypsin Digestion of Carboxymethylated and Succinylated [^{3}H]azido-linked QPs2 and QPs3

The reductive carboxymethylated and succinylated QPs2 and QPs3, 1 mg/ml, suspended in 50 mM ammonium bicarbonate buffer, containing 1 M urea were treated with chymotrypsin at 37 °C for 2 h, using a chymotrypsin to QPs3 ratio of 1:50 (w/w). After the 2-h incubation, a second addition of chymotrypsin was made (1:100), and the digestion continued at 37 °C for 24 h.

Isolation of [^{3}H]Azido-Q-Linked Peptides

One hundred microliter aliquots of the chymotrypsin digested, reductive carboxymethylated, succinylated, [^{3}H]azido-Q-labeled QPs3 and [^{3}H]azido-Q-labeled QPs2 were separated by reverse phase high performance liquid chromatography (HPLC) on a Synchropak RP-8 column (0.46 x 25 cm) using a gradient formed from 90% acetonitrile in 0.1% trifluoroacetic acid and 0.1% trifluoroacetic acid with a flow rate of 0.8 ml /min. 0.8 ml fractions were collected and monitored for radioactivity and absorbance at 214 nm. Fractions with radioactivity were collected, dried, and sequenced. Those containing no radioactivity, with retention times of 18.96 and 31.15 were also collected, dried, and sequenced.

Amino Acid Sequence Determination

These analyses were done at the Molecular Biology Resource Facility, Saint Francis Hospital of Tulsa Medical Research Institute, University of Oklahoma Health Sciences Center, under the supervision of Dr. Ken Jackson.

Results and Discussion

Isolation and Characterization of the Ubiquinone-Binding Peptides of QPs3.

[^{3}H]azido-Q-labeled QPs3 was isolated from [^{3}H]azido-Q labeled succinate-Q reductase by a procedure involving phenyl-sepharose CL-4B column chromatography, preparative SDS-PAGE , electrophoretic elution of proteins from gel slices, and acetone precipitation (Scheme III). The use of a hydrophobic column, phenyl-sepharose CL-4B, and elution with different detergents resuled in the isolation of [^{3}H]-azido-Q-labeled QPs from [^{3}H]azido-Q labeled succinate-Q reductase. Since the FP and IP subunits of succinate dehydrogenase are less hydrophobic than those of QPs subunits, they were eluted with detergents having less hydrophobicity than that used for eluting QPs. This column chromatographic step also removed most of the non-protein bound [^{3}H]azido-Q from QPs. Pure [^{3}H]azido-Q-labeled QPs2 and QPs3 were isolated from the labeled QPs by preparative SDS-PAGE using a high resolution gel system in the presence of 8 M urea. The use of preparative SDS-PAGE not only separated QPs3 from other QPs subunits, it also further removed non-protein bound azido Q adducts from QPs3. QPs2 and QPs3 in gel slices were obtained by electrophoretic eluting. The SDS present in the eluted protein solution was removed by 50% acetone precipitation.

Although the isolated [^{3}H]azido-Q-labeled QPs3 is pure and free of free azido-Q, it is highly aggregated and resistant to proteolytic enzyme digestion. Inclusion of 0.1% SDS and 2 M urea in the digestion mixture did not increase proteolysis. Modification of isolated azido-Q labeled QPs3 by reductive carboxymethylation followed by succinylation rendered the protein susceptible to chymotrypsin digestion. Reductive carboxymethylated and succinylated QPs3 was not completely soluble in aqueous solution; the solution became clear only after chymotrypsin digestion. A similar situation was observed with azido-Q labeled-cytochrome *b* (4).

Scheme III

Isolation of Azido Q-labeled QPs3 and QPs2 Peptides

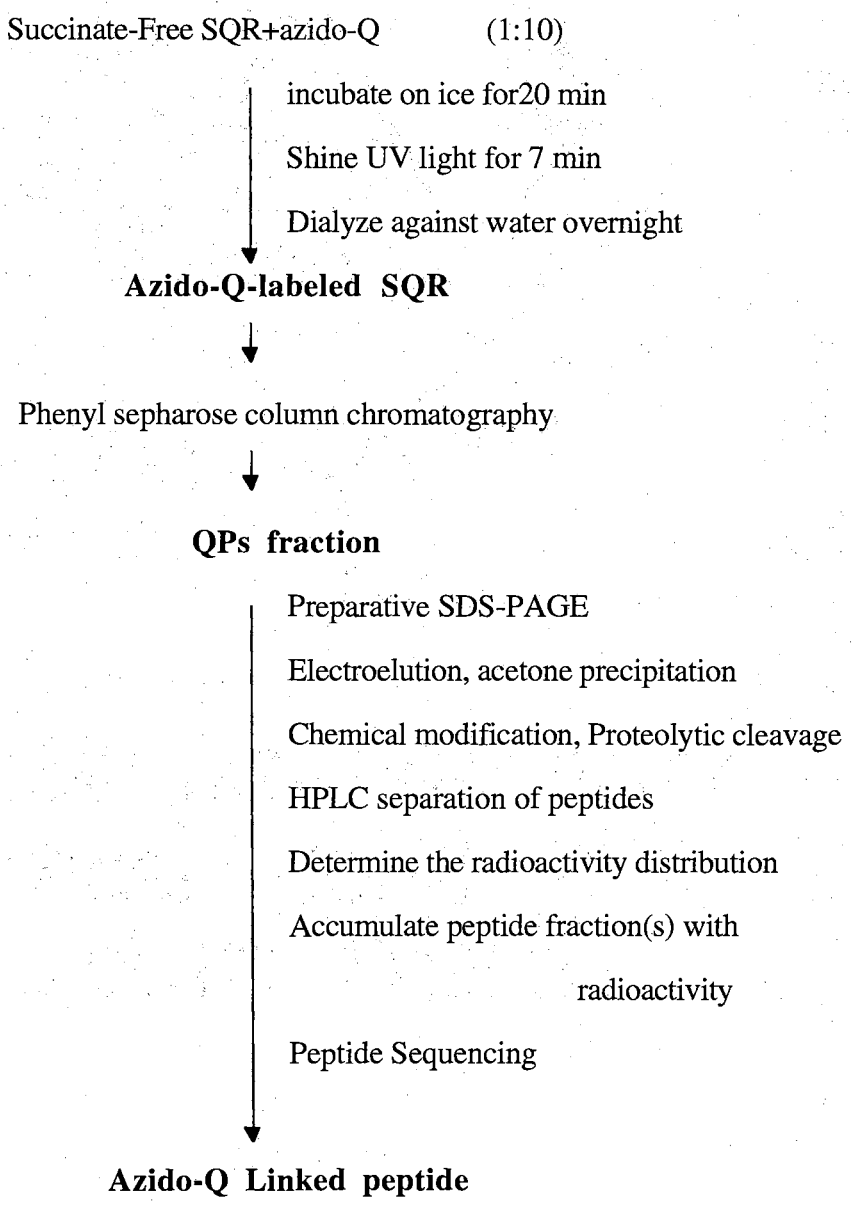
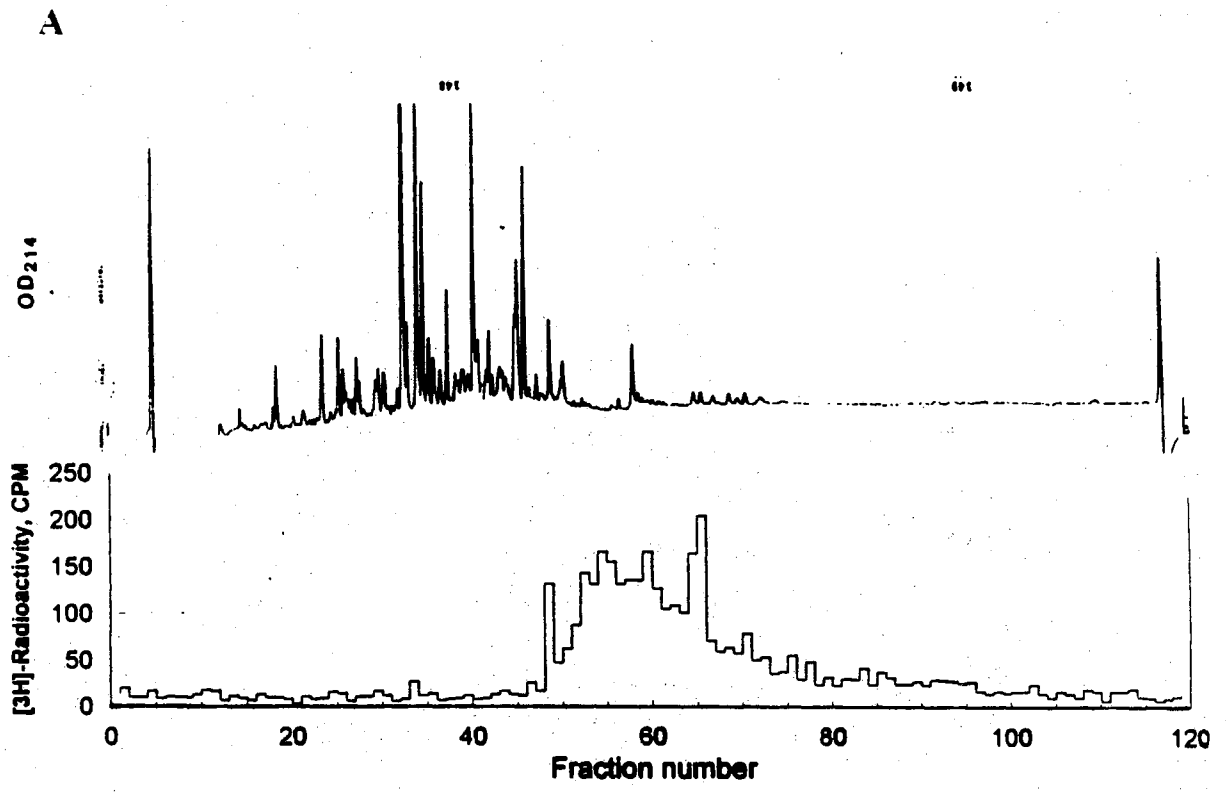


Figure 14 shows the distribution of radioactivity among the chymotryptic peptides of QPs3, and QPs2 separated by HPLC. Most of the radioactivity was found in fraction 66 (P-66). The HPLC chromatograms and radioactivity distribution of the chymotryptic peptides of QPs2 are identical to those of QPs3.

When P-66 from QPs3 was sequenced, a partial N-terminal sequence of Leu-Asn-Pro-Cys-Ser-Ala-Met-Asp-Tyr, corresponding to residues 29-37 in QPs3, was obtained. An identical sequence was obtained for the radioactivity containing fraction from QPs2. Thus the Q-binding domain in the proposed structure of QPs3, is probably located at the end of transmembrane helix 1, near the C-side of the membrane. The sequencing of a [^{3}H]-azido-Q-labeled QPs1 peptide yielded a sequence of Gly-Leu-Thr-Ile-Ser-Gln, which is a region exposed on the matrix side of the membrane.

The finding that the Q-binding domains in QPs3 and QPs1 of bovine succinate-Q reductase are on the opposite sides of the membrane is consistent with a two-Q binding site hypothesis formulated from inhibitor studies of this enzyme complex (5). The presence of two quinone-binding sites in *E. coli* fumarate reductase is suggested by mutational studies (6) and by inhibitor kinetic analysis of putative Q-binding site mutants (5). When these two quinone binding sites in *E. coli* fumarate reductase are incorporated into a proposed mechanism of Q reduction in photoreaction centers (7, 8), Glu-29, Ala-32, His-82, Trp-86 of FrdC and His-80 of FrdD are considered participants in a Q_B-type site, and FrdD phe-57, Glu-59, and Ser-60 in an apolar Q_A-type site (6). According to the proposed structure of *E. coli* FrdC and FrdD, the Q_B-type site is located at the cytoplasmic side and the Q_A-type site at periplasmic side. If this reasoning is applied to beef heart mitochondrial succinate-Q reductase, the Q-binding domain identified in QPs1 (1) would be the Q_B-type site and the domain in QPs3 would be the Q_A-type site. More detailed information on Q-binding must await determination of the three-dimensional structure of succinate-Q reductase.


A
OD214
[3H]-Radioactivity, CPM
250
200
150
100
50
0
0
20
40
60
80
100
120
Fraction number

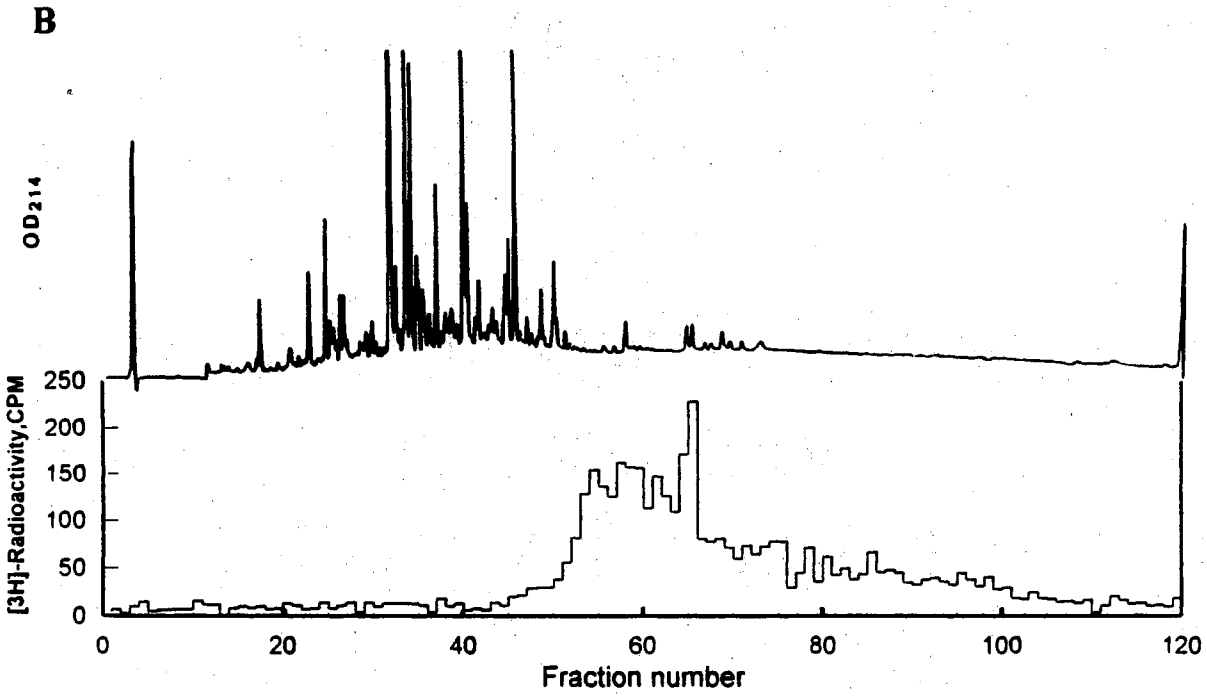

B
OD214
[3H]-Radioactivity,CPM
250
200
150
100
50
0
0
20
40
60
80
100
120
Fraction number

Figure 14. ^{3}H radioactivity distribution on HPLC chromatograms of chymotrypsin-digested, [^{3}H]-azido-Q-labeled QPs2 (Panel A) and [^{3}H]-azido-Q-labeled QPs3 (Panel B). The reductive carboxymethylated, succinylated, [^{3}H]-azido-Q labeled QPs2/QPs3, 1 mg/ml, 3.6 x 10^{4} cpm/mg, was digested with chymotrypsin, and 100-µl aliquots of the digested solution were subjected to HPLC as described under "Experimental Procedures". 100-µl aliquots were withdrawn from each tube for radioactivity determination.

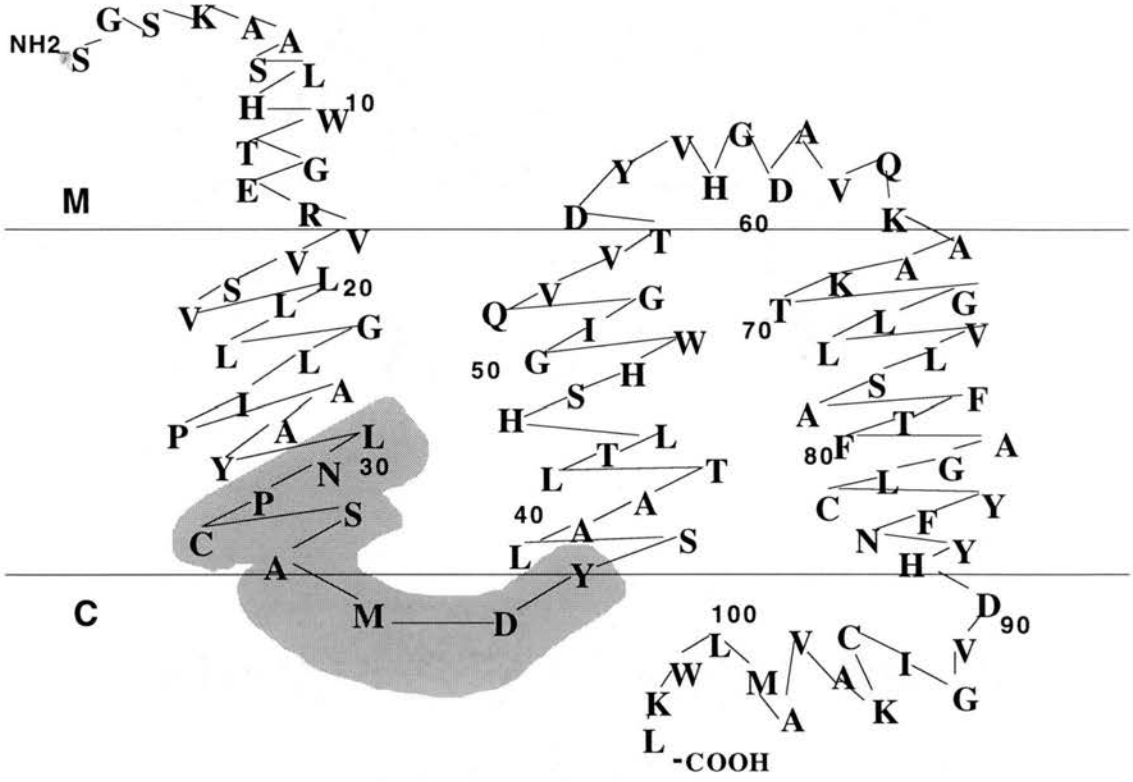


Figure 15. Location of The Q-binding Site in QPs3 of Bovine Succinate -Q Reductase

Possible relationship between QPs2 and QPs3

During the course of immunological studies of QPs3 we observed that antibodies against QPs3, the N-terminal, and the connecting peptides of QPs3 cross react with QPs2 (see figures 7 and 11). This immunocross reaction of QPs2 and QPs3 is expected because the QPs3 sequence is contained in QPs2. This is evident from the following observations: (i) when a electrophoretically pure QPs2 preparation is subjected to protein sequencing, a major peptide with a partial N-terminal amino acid sequence of Ser-Pro-Ser-His-His-Ser-Gly-Ser-Lys-Ala- is obtained. The same sequence was obtained for QPs2 when the protein was obtained by two different methods, 1. electroeluted protein from gels, 2. electroblotted protein band on PVDF membrane. This sequence contains five amino acid residues from the C-terminus of the presequence and five amino acid residues from the N-terminus of mature QPs3, suggesting that QPs2 is incompletely processed Qps3; (ii) when chymotrypsin digested QPs2 and QPs3 are subjected to HPLC separation, identical chromatograms are obtained (iii) when peptides with identical retention times, from the respective QPs2 and QPs3 HPLC chromatograms, are sequenced, identical sequences are obtained; (iv) the [^{3}H]-azido-Q labeled peptide from QPs2 is identical to that from QPs3. (v) The antibody raised against recombinant QPs3 subunit crossreacted with QPs2 (Figure 18). At present we do not know whether QPs2 is the same or a different gene product than QPs3. This requires further investigation.

Chapter Summary

Previous photoaffinity labeling studies of SQR using 3-azido-2-methyl-5-methoxy-[^{3}H]-6-decyl-1,4-benzoquinone ([^{3}H]-azido-Q), identified QPs3 as one of the putative Q-binding proteins in this reductase. An azido-Q-linked peptide with a retention time of 66 min is obtained by high performance liquid chroatography of carboxymethylated and succinylated [^{3}H]-azido-Q-labeled QPs3 purified from labeled SQR by a procedure

involving phenyl-sepharose 4B column chromatography, preparative SDS-PAGE, and acetone precipitation. The amino acid sequence of this peptide is NH_2-L-N-P-C-S-A-M-D-Y-COOH, corresponding to the residues #29-37. The quinone binding domain in the proposed model of QPs3 is probably located at the end of transmembrane helix I toward the C-side of the mitochondrial inner membrane. The finding that the Q-binding domains on QPs1 and QPs3 are located on opposite sides of the membrane is consistent with a two Q-binding site hypothesis formulated from inhibitor studies of this enzyme complex.

References

1. Lee, G. Y., He, D-Y., Yu, L., and Yu, C. A. (1995) *J. Biol. Chem.*, **270**, 6193-6198.
2. Harmon, H. J., and Crane, F. L., (1976) *Biochim. Biophys. Acta.*, **440**, 45-58.
3. Yu, C. A., and Yu, L. (1982), *J. Biol. Chem.*, **257**, 2016-2021.
4. He, D-Y., Yu, L., and Yu, C. A. (1994) *J. Biol. Chem.*, **269**, 2292-2298.
5. Yankovskaya, V., Sablin, S. O., Ramsay, R. R., Singer, T. P., Ackrell, B. A., Cecchini, G., and Miyoshi, H. (1996) *J. Biol. Chem.*, **271**, 21020-21024.
6. Westenberg D.J., Gunsalus R. P., Ackrell B.A., Sices H. and Cecchini G. (1993) *J. Biol. Chem.*, **268**, 815-822.
7. Allen, J., Feher, G., Yeates, T. O., Komiya, H., and Rees, D. C. (1988) *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 8487-8491.
8. Michel, H., Epp, O., and Deisenhofer, J (1986) *EMBO J.*, **5**, 2445-2451.

CHAPTER IV

EXPRESSION AND PROPERTIES OF QPs3 OF BOVINE HEART SUCCINATE-Q REDUCTASE

The availability of functionally active QPs3 is essential in order to better understand the structure-function relationship of QPs3 in SQR, especially on its role in Q-binding, heme ligation of cytochrome b_{560}, and succinate dehydrogenase docking. The failure of isolating QPs3 either from SQR or QPs coupled with the availability of the cDNA for QPs3 in our laboratory, has encouraged us to develop a method to over-express QPs3 in *E. coli* as a glutathione S-transferase fusion protein, using the pGEX expression vector system (1), which allows one-step affinty purification of the recombinant fusion protein with glutathione-agarose gel. In this chapter, I report the construction of a pGEX expression vector for QPs3, pGEX/QPs3, growth conditions for overexpression of the active soluble form of GST-QPs3 fusion protein in *E. coli*, isolation and characterization of recombinant QPs3.

Experimental Procedures

Materials

Restriction enzymes, T4 polynucleotide kinase, T4 DNA polymerase, T4 DNA ligase, were either obtained from Promega or from Life Technologies Inc. Plasmid preparation and fragment isolation kits were obtained from Qiagen. Nitrocellulose membranes were from Schleicher & Schuell. Bovine serum albumin, isopropyl-β-D-thiogalactopyranoside (IPTG), D-glucose, ampicillin, tetracycline, δ-aminolevulunic acid, ferrous sulfate, gelatin, hemin chloride, sorbitol, betaine, glutathione, (reduced form),

glutathione agarose beads, thrombin, leupeptin, phenylmethylsulfonylfluoride (PMSF) and 2,6-dichlorophenol indophenol (DCPIP) were from Sigma. Agarose, acrylamide, bisacrylamide, HRP color development reagent, protein A horseradish peroxidase conjugate and Bradford reagent were obtained from Biorad. LB agar, LB broth base, Select peptone 140 and Select yeast extract were from Life technologies Inc. Dodecyl maltoside was from Anatrace. Oligonucleotides were synthesized by the Recombinant DNA/ Protein Resource Facility at Oklahoma State University.

Bacterial Strains and Plasmids

E. coli strain INVαF' was used as host for PCR2.1 vector (Invitrogen); *E. coli* JM109, or DH5α was used as host for pSelect (Promega) and pGEX2T (Pharmacia).

DNA Manipulation and DNA Sequencing

General molecular genetic techniques were performed according to procedures described in Sambrook et al (2). DNA sequencing was performed with an Applied Biosystems model 373 automatic DNA sequencer at the DNA/Protein Resource Facility at Oklahoma State University.

Isolation of Recombinant GST-QPs3 fusion protein

400 ml of an overnight culture of *E. coli* JM109 / pGEX/QPs3 was used to inoculate 12 liters of SOC medium containing 440 mM sorbitol, and 2.5 mM betaine and 60 mg/liter ampicillin. The culture was grown in a fermentor chamber with aeration until the OD_{660nm} reached 0.9 (about 3.5 hours). The culture was cooled until it reached 25°C and IPTG was added to a final concentration of 0.4 mM. Growth was continued until 3.5 hours post-induction and then harvested by centrifugation at 8,000 x g for 15 min. About 48 g (wet weight) of cells were obtained and resuspended in 3 ml / g of PBS buffer (20 mM Na/K phosphate pH 7.3, containing 150 mM NaCl). Cells were disrupted by sonication at 0 °C at 30 milliwatts for a total of 80 seconds: four 20-second pulses separated by 3 to 4 min intervals. During sonication PMSF and leupeptin were added to a final concentration of 1 mM, and 0.01 mg/ml respectively. Triton X-100 was added to the

broken cell suspension to final concentration of 1% (w/v). The mixture was stirred on ice for 1 hour and centrifuged at 30,000 x g and the supernatant was mixed with an equal volume of glutathione agarose gel equilibrated with PBS. The mixture was stirred at 4 °C for 1 hour after which the gel was packed into a column and washed with the equilibrating buffer extensively at a flow rate of 0.8 ml/min. The bound fusion protein was eluted with 50 mM Tris-HCl pH 8.0, containing 5 mM reduced glutathione and 0.25 M sucrose. The OD_{280} of fractions were determined and the fractions containing the fusion protein were pooled and dialyzed against 50 mM Tris-HCl, pH 8.0, containing 0.25 M sucrose for at least 8 hours with 2 changes of buffer. The dialyzed sample was concentrated with a centriprep (Amicon), to a protein concentration of 5 mg/ml, mixed with glycerol to a final concentration of 10% and frozen at -80 °C until further use. QPs3 protein was released from GST-QPs3 by thrombin digestion (1μg/500μg) and isolated by gel filtration using a superose-12 column.

Enzyme Preparations and General Biochemical Techniques

QPs (3) was prepared as reported previously. Absorption spectra and enzyme assays were performed at room temperature in a Shimadzu UV-2101PC. Protein content was determined by the Lowry method or Bradford assay using a kit from Biorad. The heme content was determined by pyridine hemochromagen spectra using a millimolar extinction coefficient of 34.6 for the absorbance at 557 nm minus 600 nm (4). SDS-PAGE, was done according to Laemmli (5) or Schägger *et al.* (6) The EPR measurements were made with a Bruker ER-200D equipped with an Air Product Heli-Tran System.

Results And Discussion

Construction of Expression Vector for QPs3

Our previous success in overexpression of functionally active subunit IV of the *R. sphaeroides* cytochrome bc_1 complex (7), the QPc-9.5 kDa of bovine QCR (8), and QPs1 of bovine SQR in *E. coli* using the pGEX system (9), encouraged us to use the pGEX system to express QPs3 in *E. coli*. The expression vector for QPs3, pGEX/QPs3, was constructed by in-frame fusion of QPs3 gene into the glutathione S-transferase gene in pGEX-2T plasmid (Figure 16). Since *Bam*HI and *Eco*RI sites are unique sites in the pGEX-2T vector and are missing in mature QPs3 cDNA, the in-frame fusion of the QPs3 gene with the GST gene in pGEX-2T plasmid was achieved by generating a *Bam*HI-*Eco*RI fragment encoding mature QPs3 and subsequently ligating it into the *Bam*HI and *Eco*RI sites of the pGEX-2T plasmid. The 331 bp *Bam*H1-*Eco*R1 cDNA fragment encoding mature QPs3 was amplified from a bovine heart cDNA library by PCR using two synthetic primers, 5'GGATCCTCTGGTTCCAAG3' (the sense primer) and 5'GAATTCTAAAAGGTCAGAGC3' (the antisense primer). This fragment was cloned into PCR2.1 vector and confirmed by DNA sequencing before being subcloned into the *Bam*H1 and *Eco*R1 site of pGEX2T vector to generate pGEX/QPs3. *E. coli* transformants producing the GST-QPs3 fusion protein were identified by immunological screening of colonies with anti-peptide QPs3 antibodies (10). Both *E.coli* strains JM109 and DH5α were found to be suitable hosts for pGEX/QPs3.

Production and Purification of Recombinant QPs3

Production of recombinant GST-QPs3 fusion protein was IPTG concentration, induction growth time and induction growth medium dependent. The yield increased as the IPTG concentration and induction growth time were increased, reaching a maximum when cells were harvested 3 h after 0.4 mM IPTG growth induction (Figure 17). When IPTG

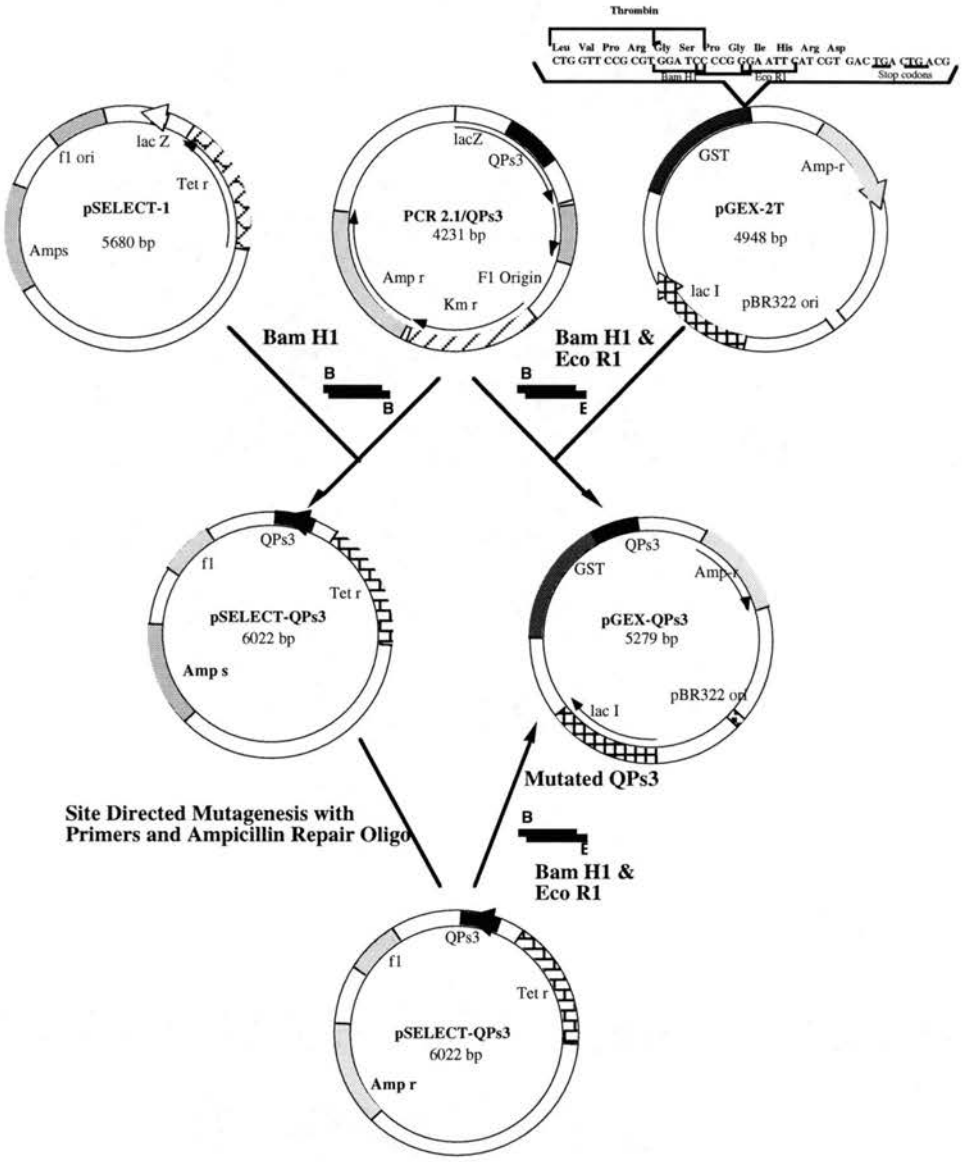


Figure 16. Construction of The Expression Vector pGEX/QPs3 for Wild Type and Mutants

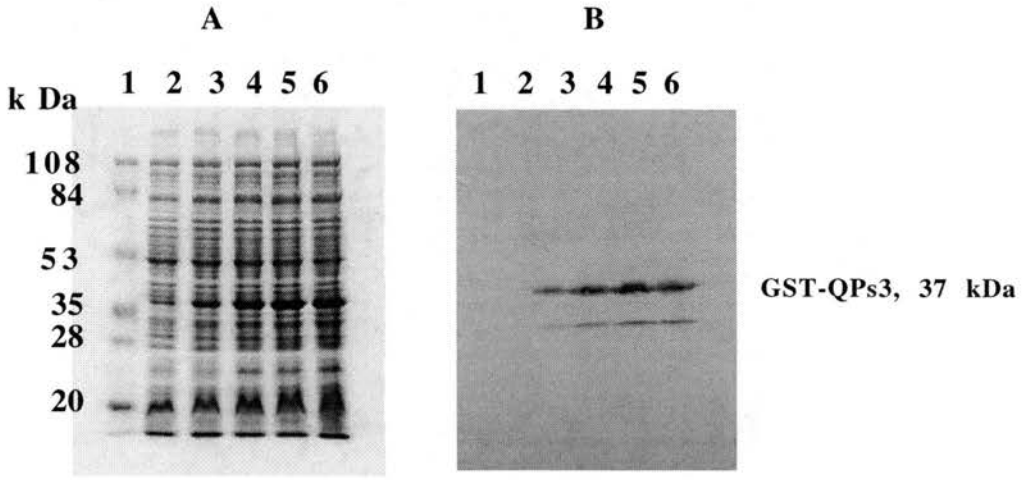


Figure 17. The Time Course of IPTG Induction of GST-QPs3 Expression in *E. coli* JM109.

A. SDS-PAGE,(12% acrylamide, laemmli's system) *lane 1* is prestained SDS-PAGE standards. *Lanes 2-6* represent an aliquot of whole cells at 0, 1, 2, 3, and 4 hours after the addition of IPTG.

B. Western blot of A. The proteins on A were electrophoretically transferred to nitrocellulose membrane. Monospecific polyclonal antibodies that were raised against QPs3 peptides were used as the primary antibody. Protein A-horseradish peroxidase conjugate was used as a second antibody

concentration was increased to 1 mM, no change in expression yield was observed When cells were grown for more than 5 h, the total yield decreased and degradative products increased as determined by western blotting using anti-QPs3 peptide antibodies. The yield of recombinant GST-QPs3 fusion protein could be increased in *E. coli* by using an induction growth medium containing magnesium. When LB or peptone-phosphate enriched medium was used, production of GST-QPs3 fusion protein in *E. coli* accounted for less than 1% of the total cellular protein. However, including 5 mM magnesium in the enriched medium or using SOC medium increased the yield of GST-QPs3 fusion protein in *E. coli* to about 15% (Table IV). Athough the yield of fusion protein in *E. coli* was slightly higher with enriched medium supplemented with magnesium, the SOC medium was preferred because of lesser degradation products with the latter.

E. coli is generally well known for rendering foreign proteins expressed in its cell as insoluble aggregates or inclusion bodies. When the recombinant fusion protein was mostly synthesized as insoluble aggregates, its affinity to glutathione gel was reduced and our one step purification is limited. Hence it is necessary to choose conditions for expression so that the fusion protein was soluble or detergent soluble and properly folded to recognize the binding to the affinity gel. Although methods are available to convert the denatured inclusion body to soluble form, it is worthwhile to change growth conditions to make the cells to produce active soluble fusion protein. The increase in soluble yield by including betaine and sorbitol in the culture medium and growing the induced cells at 27 °C instead of 37 °C has been reported for GST-QPc-9.5 kDa fusion protein (8), GST-QPs1 (9) and DMAPP:AMP transferase (11). Although the reason for this increase is not known, it has been suggested (11) that increased internal concentrations of compatable osmolytes, like betaine, cause a thermodynamically unfavourable "preferential hydration" which minimizes solvent-protein contact and stabilizes protein structures. When the growth

Table IV.

The Yield of Recombinant GST-QPs3 Fusion Protein in The Soluble and Inclusion Body Fractions of *E. coli* JM109 / pGEX-QPs3 Cells in Different Growth Media at Room Temperature

Growth Medium	% Total Fusion Protein*	% Soluble Fraction	% Inclusion body
SOC	7.5	1.0	6.5
SOC + betaine + sorbitol	9.2	3.5	5.5
Enriched Medium	0.3	–	0.3
Enriched + Magnesium	7.5	1.5	6.0
Enriched + Magnesium + Sorbitol + Betaine	15	5.5	9.8

* The % refers to the intensity of the 37 k Da fusion protein band as compared with the total intensities of cellular protein bands on SDS-PAGE

medium was supplemented with 0.44 M sorbitol and 2.5 mM betaine there was a three fold increase in the yield from the soluble fraction (12-13% of total GST-QPs3 without betaine-sorbitol to 34-36%). The IPTG induction growth in SOC medium containing 2.5 mM betaine and 440 mM sorbitol at 25 °C for 3.5 hours was found to be optimal for the production of soluble recombinant GST-QPs3 fusion protein.

The SDS-PAGE analysis of fusion protein obtained from the affinity column showed the 37 kDa fusion protein as the major band (Figure 18 panel A lane 3). When the fusion protein was treated with thrombin at a ratio of 1:500 w/w at 25 °C in the presence of 0.01% lauryl maltoside most of QPs3 in the fusion protein could be cleaved in about one hour. The recombinant QPs3 thus obtained was dispersed in detergent with an estimated molecular weight of over 500 kDa and could be recovered from the digestion mixture in the void volume of superose 12 column (Pharmacia). When the thrombin released QPs3 was subjected to SDS-PAGE, it was detected as a single band corresponding to the fifth subunit in succinate-Q reductase (Figure 18, panel A, lane 5). The protocol to obtain recombinant QPs3 is detailed in Scheme IV.

Quinone-binding Properties of Recombinant QPs3.

Since QPs3 has been identified as one of the Q-binding proteins in SQR (8) it is of interest to see whether or not recombinant QPs3 can bind Q. It was reported that addition of $Q_0C_{10}Br$ to recombinant QPc-9.5 kDa subunit (Q-binding subunit of QCR) causes a spectral blue shift. The spectral shift was found to be protein concentration dependent and reached a saturation concentration (8, 12). When $Q_0C_{10}Br$ in 95% ethanol was added in small increments to recombinant QPs3 in a 20 mM sodium phosphate buffer containing 0.25 M sucrose, 150 mM NaCl, 2.5 mM $CaCl_2$, 10% ethanol 0.01% lauryl maltoside and 5% glycerol, a spectral blue shift of Q was observed (Figure 19). Titration of recombinant QPs3 with Q showed a saturation point at around 1.1 moles of Q per mole protein indicating that the recombinant QPs3 has specific Q-binding ability and thus is possibily functionally active.

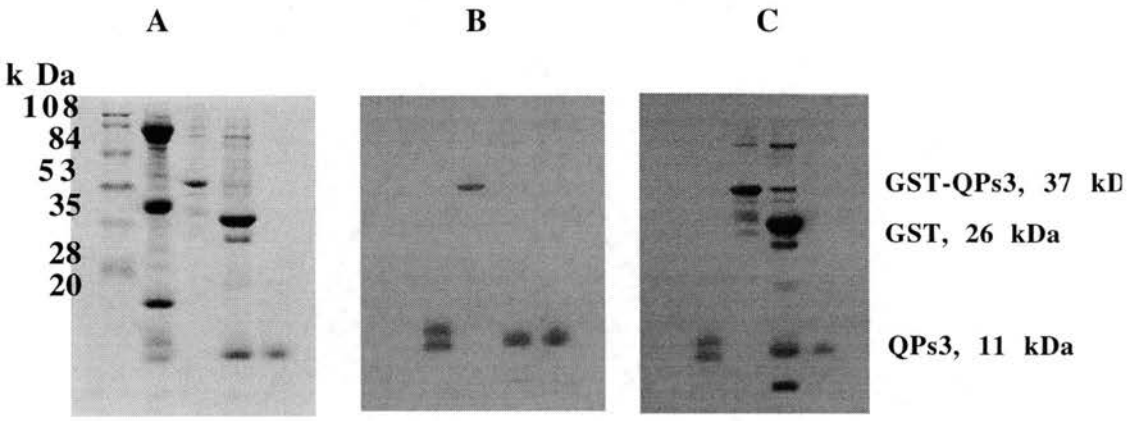
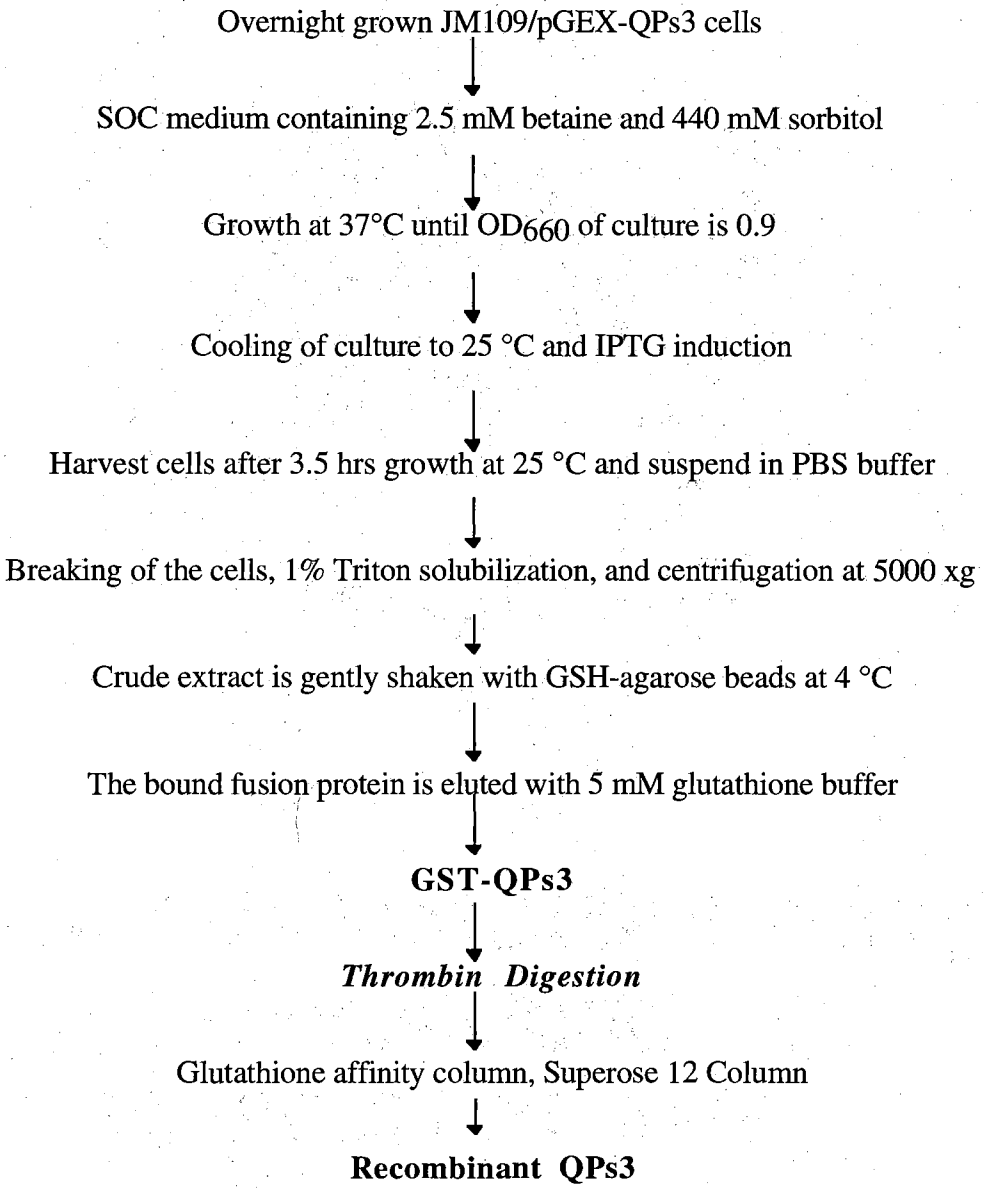


Figure 18. Characterization of antiGST-QPs3 antibodies and identity of recombinant QPs3. A: SDS-PAGE. *Lane 1* represents prestained molecular weight markers; *lane 2* Bovine heart mitochondrial succinate-ubiquinone reductase; *lane 3* GST-QPs3 from the affinity column; lane 4, GST-QPs3 after thrombin digestion; *lane 5* purified recombinant QPs3. The high resolution SDS-PAGE system of Schagger *et al* was used. The proteins in A were electrophoretically transfered to nitrocellulose membrane, and reacted against anti-connecting peptide antibodies in Panel B and anti-GST-QPs3 antibodies in Panel C. Protein A horseradish peroxidase conjugate was used as second antibody.

Scheme IV

The protocol for isolation of the Recombinant QPs3 protein from *E. coli* JM109/pGEX-QPs3



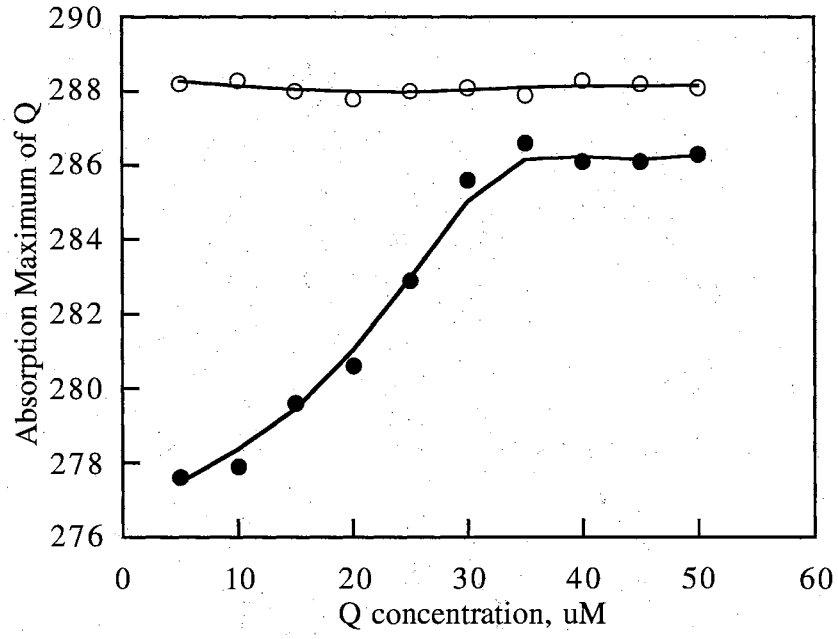


Figure 19. Effect of recombinant QPs3 on the absorption maxima of Q. One ml aliquots of 20 mM sodium phosphate buffer pH 7.3 containing 150 mM NaCl, 2.5 mM$CaCl_2$ 10% ethanol and 0.01 % lauryl maltoside was added to 50 µl 50 mM Tris-HCl pH 8.0 buffer containing of GST (o), or recombinant wild type QPs3 (●). The final protein concentration was 300 µg/ml. Each sample was prepared in duplicate and $Q_0C_{10}Br$ in 95% alcohol was added in 1 µl increments to one sample cuvette to obtain the indicated concentrations. The same amount of alcohol alone was added to the reference cuvette. After each addition the sample was incubated for 5 min and a difference spectra between the $Q_0C_{10}Br$-added and alcohol-added samples was recorded from 320 nm to 250 nm.

Restoration of Cytochrome b_{560} to Recombinant QPs3 by Hemin-Chloride

The QPs fraction of mitochondrial SQR is associated with a cytochrome b_{560}, that is found in substoichiometric ratio with respect to FAD. The cytochrome b_{560} is not reducible by succinate and shows altered spectral characteristics and CO sensitivity in the presence and absence of SDH (to recall see page 15 of this thesis). Previous near infra red MCD (magnetic circular dichroism) spectroscopic studies of SQR and isolated mitochondrial QPs indicate a bis-histidine ligand for cytochrome b_{560} (13). In general, mitochondrial QPs1 is believed to be the cytochrome b_{560} of SQR. This has been recently substantiated by *in vitro* heme reconstitution of recombinant GST-QPs1 to form cytochrome b_{560} (12). However in the *E.coli* SQR, cytochrome b_{556} is ligated by histidine 84 from SDHC and and histidine 71 from SDHD subunits (14). In this case both subunits are essential for heme ligation. Although recombinant QPs1 can form cytochrome b_{560}, QPs2 and/or QPs3 could also have putative histidine ligands for cytochrome b_{560} To test whether QPs3 has putative histidine ligands we attempted *in vitro* heme reconstitution of recombinant QPs3.

Purified recombinant QPs3 did not contain any cytochrome b_{560}. The addition of aminolevulunic acid, the committed precursor for heme synthesis, to the IPTG-induced *E. coli* cultures did not result in any increase in the cytochrome b_{560} content of QPs3. Since GST-QPs3 was in a soluble form unlike the rQPs3 which is in a detergent dispersed form, it was more suitable for our heme reconstitution experiments. When hemin chloride was added to GST-QPs3, the maximum absorption peak (Soret band) of the oxidized form of heme shifted from 398 nm to 411 nm with increasing absorbance. The spectral shift was reached after one hour incubation at room temperature. At the end of the incubation time when the sample was reduced with dithionite, a characteristic cytochrome b_{560} spectrum with Soret peak at 426 nm, α-absorption at 560 nm, a broad β-absorption at 530

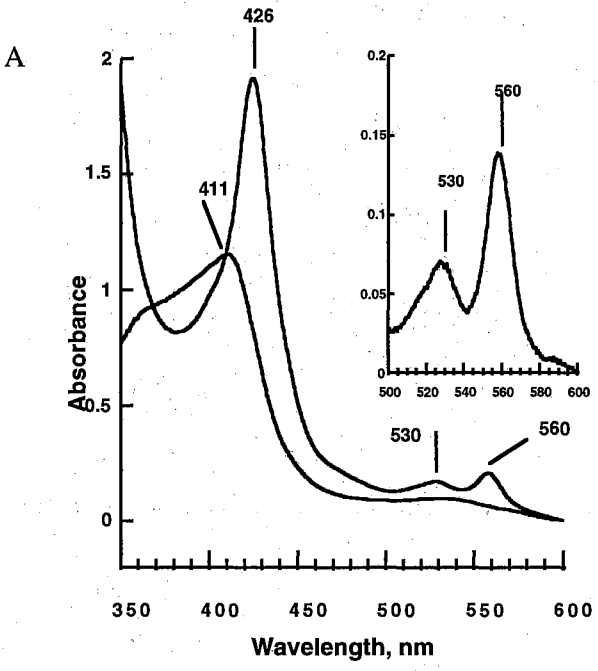

A
426
411
530
560
530
560
Absorbance
Wavelength, nm
B
Absorbance
Wavelength, nm

Figure 20. Restoration of cytochrome b_{560} in heme reconstituted recombinant GST-QPs3. 3 μl of 6 mM hemin chloride in DMSO was added to 1.5 mg of GST-QPs3 (Panel A) and 1.5 mg of GST (Panel B) in 1 ml, in Tris-HCl pH 8.0 buffer, containing 0.25 M sucrose. The oxidized (—) and the dithionite reduced (---) spectra were recorded at room temperature. The inset represents the difference spectrum of dithionite-reduced versus the oxidized form in the α and β regions.

nm was generated (Figure 20 panel A). These spectral characteristics are identical to those of cytochrome b_{560} of a reconstitutively active QPs preparation. When GST protein isolated from *E. coli* cells JM109/pGEX2T was incubated with hemin chloride in a similar manner as GST-QPs3, no cytochrome b_{560} spectrum was generated (Figure 20 panel B). This indicates that the two heme ligands are solely provided by QPs3.

EPR Characterization of GST-QPs3 Protein after Heme Reconstitution

Figure 21 shows the EPR characteristics of mitochondrial QPs, GST with hemin chloride, and heme reconstituted GST-QPs3 protein before and after thrombin treatment. Reconstituted cytochrome b_{560} in GST-QPs3 shows an EPR peak at g=2.91 corresponding to one of the two EPR signals (g=2.91 and g=3.07) observed for cytochrome b_{560} in isolated QPs. The g=2.91 peak was regarded as a reconstitutively inactive peak since only the g=3.07 peak disappears upon the reconstitution of QPs with SDH to form SQR. However this second peak is still due to a bis histidyl ligation of heme according to previously reported studies in *Desulfovibrio vulgaris* (15, 16). In this species the cytochrome c_3 has 4 hemes (g=3.16, 2.95, 2.95, and 2.71) all of which have histidines as the fifth and sixth ligands. The ligands have been identified by crystallographic studies. The g=2.95 signal is attributed to the imidazoles being almost coplanar lying close to a methine-methine vector (15).

The restoration of cytochrome b_{560} was heme concentration dependent (Figure 22). When 0.4 mg of GST-QPs3 was incubated with different amounts of hemin chloride corresponding to molar ratios ranging from 0 to 1.5 of heme to protein, the peak height at 560 nm increased up to a ratio of 0.75 mole of heme to 1 mole protein. On the addition of more heme there was a presence of broad shoulder between 570 and 580 indicating the presence of free heme. Beyond the molar ratio of 0.75 heme per mole protein the increase in 560 nm absorbance was seen only in the sample that was not treated with thrombin (Figure 22).

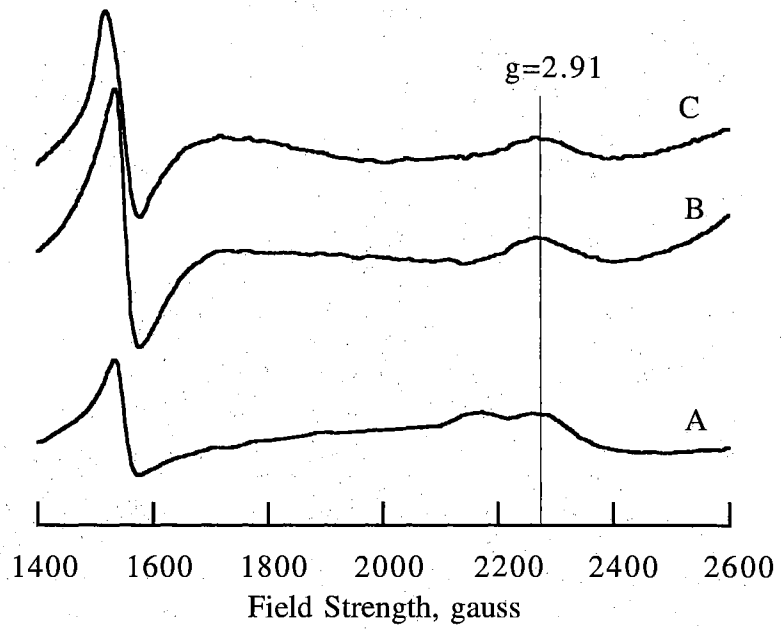


Figure 21. EPR spectra of heme reconstituted Wild type GST-QPs3 protein before and after thrombin treatment:- Trace A- mitochondrial QPs, trace B-GST-QPs3 with hemin Chloride, trace C- GST-QPs3 with hemin chloride and thrombin. 15 µl of hemin chloride in DMSO was added to 0.6 ml of Tris-HCl buffer containing the protein samples (10 mg / ml) and incubated at room temperature for 1 hr prior to EPR measurement. Thrombin was added at 0.01 unit/µg of protein. The EPR settings were: modulation frequency, 100 KHz; modulation amplitude, 20 G; time constant, 0.5 s; microwave frequency, 9.42 GHz; microwave power, 20 milliwatts; scan rate, 200 s; temperature 10 k.

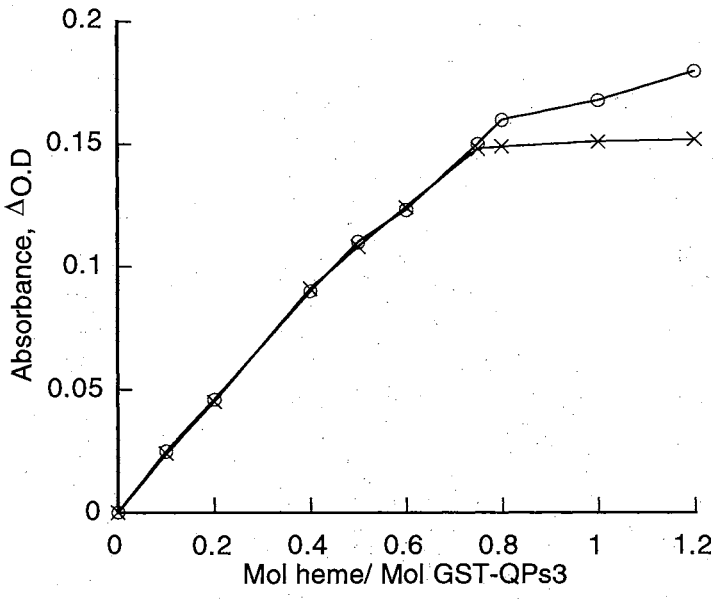


Figure 22. Effect of heme concentration on the reconstitution of cytochrome b_{560}

One ml aliquots of purified GST-QPs3 fusion protein, 0.85 mg/ml, in PBS buffer were added to 3 µl of DMSO containing indicated concentrations of hemin chloride, incubated at room temperature for one hour, before determining the difference spectra between the oxidized and the reduced forms with (x) and without (o) thrombin digestion.

When heme reconstituted, thrombin-treated GST-QPs3 was passed through a superose 12 column, and each fraction was analyzed for its absorption spectra, SDS-PAGE, and Western blotting, cytochrome b_{560} was found only in the QPs3 containing fractions. No cytochrome was detected in the GST fractions. These results further confirm the fact that QPs3 contains putative heme ligands of cytochrome b_{560}. The identification of ligands of cytochrome b_{560} by site-directed mutagenesis is described in the succeeding Chapter.

Chapter Summary

QPs3 of bovine SQR was over-expressed in *E. coli* as a glutathione S-transferase (GST) fusion protein using the constructed expression vector, pGEX2T/QPs3. The yield of recombinant fusion protein was growth conditions dependent. The maximum yield was obtained from cells harvested 3.5 hours post-induction growth at 25 °C on SOC medium containing 440 mM sorbitol, 2.5 mM betaine. QPs3 was released from the fusion protein by proteolytic cleavage with thrombin. Isolated recombinant QPs3 showed one protein band in SDS-PAGE corresponding to the fifth subunit of SQR. Recombinant QPs3 showed Q-binding that is specific since its titration against quinone resulted in a spectral blue shift that reached saturation. Recombinant QPs3 contained no cytochrome b_{560}. However, addition of hemin chloride restored the spectral characteristics of cytochrome b_{560}. Reconstituted cytochrome b_{560} showed a epr signal at g=2.91 which corresponded to one of the epr signals of cytochrome b_{560} in a reconstitutively active QPs preparation. The epr and spectral properties were not affected by the thrombin treatment suggesting that both the ligands of the cytochrome b_{560}, probably histidines, are provided by QPs3.

References

1. Smith D. B., and Johnson K. S., (1988) *Gene*, **67**, 31-40.

2. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A laboratory Manual, Cold Spring Harbor Press, NY.

3. Yu, L., Xu, J-X., Haley, P. E., and Yu C. A. (1987) *J. Biol. Chem.*, **262**, 1137-1143.

4. Paul, K. G., Theorell, H., and Akesson, A., (1955) *Acta. Chem. Scand.*, **7** 1284-1287.

5. Laemmli, U. K. (1970) *Nature,* **227**, 680-685.

6. Schagger, H., Link, T. A., Engel, W. D., and vonJagow, G. (1986) *Methods Enzymol.*, **126**, 224-237.

7. Chen, Y. R., Yu, C. A., and Yu, L., (1996) *J. Biol. Chem.*, **271**, 2057-2062.

8. Yu, L., Deng, K-P., Yu C. A., (1995) *J. Biol. Chem.*, **270** 25634-38.

9. Gyesoon, Zhu, J. Z., Yu, L., and Yu, C. A. (1998) *Biochim. Biophys. Acta,* **1363** 35-46.

10. Shenoy, S. K. Yu, L., and Yu, C. A. (1997), *J. Biol. Chem.*, **272**, 17867-17872.

11. Blackwell J. R., and Horgan, R. (1991) *FEBS Lett.*, **295**, 10-12.

12. Yu, C. A., and Yu, L. (1982) *J. Biol. Chem.,* **257**, 6127-6131.

13. Crouse, B. R., Yu, C. A., Yu, L., Johnson, M. K. (1995) *FEBS Lett.*, **367**, 1-4.

14. Rose, C. T., Cecchini, G. Nakamura, K., Kita, K., and Gennis, R. B. (1997), *Biochemistry,* **37,** 4148-4159.

15. Palmer, G. (1985), *Biochemical Society Transactions*, 548-560.

16. Higuichi, Y., Kusunoki, M., Matsura, Y., Yasuoka, N., and Kakudo, M. (1984), *J. Mol. Biol.,* **172**, 109-139.

CHAPTER V

IDENTIFICATION OF AMINO ACID RESIDUES RESPONSIBLE FOR Q-BINDING AND HEME LIGATION OF QPs3 OF BOVINE HEART SUCCINATE-Q REDUCTASE

The availability of a functionally active recombinant QPs3 (chapter IV) provides us the opportunity to undertake structure-function studies of this subunit. The Q-binding site of this subunit was identified as the stretch of amino acids from leucine 29 to tyrosine 37 by photoaffinity labeling and isolation and sequencing of an azido-Q linked QPs3 peptide (1). Heme reconstitution of GST-QPs3 showed that heme binding was specific and the cytochrome spectrum generated was due to ligands provided by QPs3. In this chapter I report the identification of the amino acid residues responsible for Q-binding and heme ligation by site directed mutagenesis and *in vitro* Q-binding and heme reconstitution assays of the mutated QPs3 protein.

Experimental Procedures

Materials

Restriction enzymes, T4 polynucleotide kinase, T4 DNA polymerase, T4 DNA ligase, were either obtained from Promega or from Life Technologies Inc. Plasmid preparation and fragment isolation kits were obtained from Qiagen. Nitrocellulose membranes were from Schleicher & Schuell. Bovine serum albumin, isopropyl-β-D-thiogalactopyranoside (IPTG), D-glucose, ampicillin, tetracycline, δ-aminolevulunic acid,

ferrous sulfate, gelatin, hemin chloride, sorbitol, betaine, glutathione, (reduced form), glutathione agarose beads, thrombin, leupeptin, phenylmethylsulfonylfluoride (PMSF) and 2,6-dichlorophenol indophenol (DCPIP) were from Sigma. Agarose, acrylamide, bisacrylamide, HRP color development reagent, protein A horseradish peroxidase conjugate and Bradford reagent were obtained from Biorad. LB agar, LB broth base, Select peptone 140 and Select yeast extract were from Life Technologies Inc. Dodecyl maltoside was from Anatrace. Oligonucleotides were synthesized by the DNA/ Protein Resource Facility at Oklahoma State University.

Bacterial Strains and Plasmids

E. coli strain INVαF' was used as host for PCR2.1 vector (Invitrogen); *E. coli* JM109, or DH5α was used as host for pSelect (Promega) and pGEX2T (Pharmacia).

DNA Manipulation and DNA Sequencing

General molecular genetic techniques were performed according to procedures described in Sambrook et al (2). DNA sequencing was performed with an Applied Biosystems model 373 automatic DNA sequencer at the DNA/Protein Resource Facility at Oklahoma State University.

Construction of Mutants and Expression of Mutated QPs3 as GST Fusion Proteins in *E. coli*

A 342 base pair EcoRI fragment was excised from PCR2.1/QPs3 plasmid and cloned into the EcoRI site of pSELECT-1 (tetracycline resistant and ampicillin sensitive) vector. The single-stranded pSELECT/QPs3 was used as the template in the mutagenesis reactions. The mutagenic primers used are as follows:

H9N- 5'GCATCTCTCAACTGGACTGGT; H9Y- 5'GCATCTCTCTACTGGACTGGT

H9D- 5'GCATCTCTCGACTGGACTGGT; H46N- 5'CTCACTCTTAACAGTCACTGG

H46Y- 5'CTCACTCTTTACAGTCACTGG; H46D- 5'CTCACTCTTGACAGTCACTGG

H48N- 5'CTTCACAGTAACTGGGGCATT; H48Y- 5'CTTCACAGTTACTGGGGCATT

H48D- 5'CTTCACAGTGACTGGGGCATT; H60N- 5'GACTATGTTAATGGAGATGCA

H60Y- 5'GACTATGTTTATGGAGATGCA; H60D- 5'GACTATGTTGATGGAGATGCA

H89N- 5'TTCAACTATAATGACGTGGGC; H89Y- 5'TTCAACTATTATGACGTGGGC

H89D- 5'TTCAACTATGATGACGTGGGC; S33A- 5'AATCCGTGTGCTGCGATGGAC;

D36A- 5'CTGCGATGGCCTACTCTC; Y37A- 5'CGATGGACGCCTCTCTGGC

Each of these oligos was used in combination with an ampicillin repair oligo and annealed to the single stranded pSELECT/QPs3. This mutagenesis procedure produced greater than 50% mutants. The double mutant H46,60 N was constructed by annealing the H46N oligonucleotide primer to single stranded H60N-pSELECT-QPs3. The presence of mutation was confirmed by DNA sequencing both in pSELECT and pGEX vectors carrying the mutated QPs3 gene. The transformants expressing the fusion protein were identified by immunological screening of bacterial colonies with antibodies raised against QPs3 connecting peptide (1). Isolation of recombinant mutant GST-QPs3 fusion protein was done in a similar manner as the wild type.

The heme content was determined by pyridine hemochromagen spectra using a millimolar extinction coefficient of 34.6 for the absorbance at 557 nm minus 600 nm (3). SDS-PAGE, was done according to Laemmli (4) or Schägger *et al.* (5) The EPR measurements were made with a Bruker ER-200D equipped with an Air Product Heli-Tran System.

Results And Discussion

Identification of Amino Acid Residues Involved in Q-binding of QPs3

The photoaffinity labeling techniques used to locate the Q-binding sites did not allow the identification of a specific Q-binding residue due to the longer life and nonspecific nature of the nitrene radical. The availability of a functionally active recombinant QPs3 provides us the opportunity to identify the putative Q-binding residues by a molecular genetics approach.

From crystallographic studies of the reaction center (6) it is known that two types of residues are important for Q-binding and Q-mediated electron transfer. The first type is of those amino acids that can form hydrogen bonds with the carbonyl oxygen of the benzoquinone ring. The second type is aromatic residues whose conjugated ring can stabilize the 1,4 benzoquinone ring by π-π interaction. The Q-binding domain of QPs3 shows serine33, and aspartate36 as candidates for hydrogen bonding, and tyrosine37 as a candidate for aromatic interaction. Each of these residues were individually altered to alanine and the mutant recombinant QPs3 isolated and analyzed for the Q-binding ability. D36A mutant showed similar Q-binding (Figure 23) as the wild type suggesting that this residue may not be important for Q-binding; however S33A and Y36A did not show the spectral blue shift and these two residues are probably involved in Q-binding in bovine SQR. Definitive assignment has to await the three-dimensional structure of this reductase.

Identification of Heme Ligands of Reconstituted Cytochrome b_{560} of Recombinant QPs3

QPs3 has five histidine residues at positions 9, 46, 48, 60 and 89 in its amino acid sequence. In order to identify the histidine residues ligating the heme we altered each of the histidine residues to asparagine, tyrosine and aspartate, by site directed mutagenesis and assayed the mutant QPs3 for their ability to reconstitute cytochrome *b*560 with hemin chloride. We failed to obtain expressed H46D, H48D, H60D, H60Y H89D and H89Y mutant QPs3 proteins since they could be expressed only as inclusion bodies. All the other nine mutants (H9D, H9N, H9Y, H46N, H46Y, H48N, H48Y, H60N, and H89N) were isolated in the same manner as the wild type GST-QPs3. The expression level and yield of H9Y, H46N, H48Y were higher than the wild type, but the elution of H48Y required 10 mM glutathione instead of 5 mM glutathione and pH of the eluting buffer at 8.5 rather than 8.0. A final yield ranging from 4 to 5 mg fusion protein per liter culture was obtained in these cases.

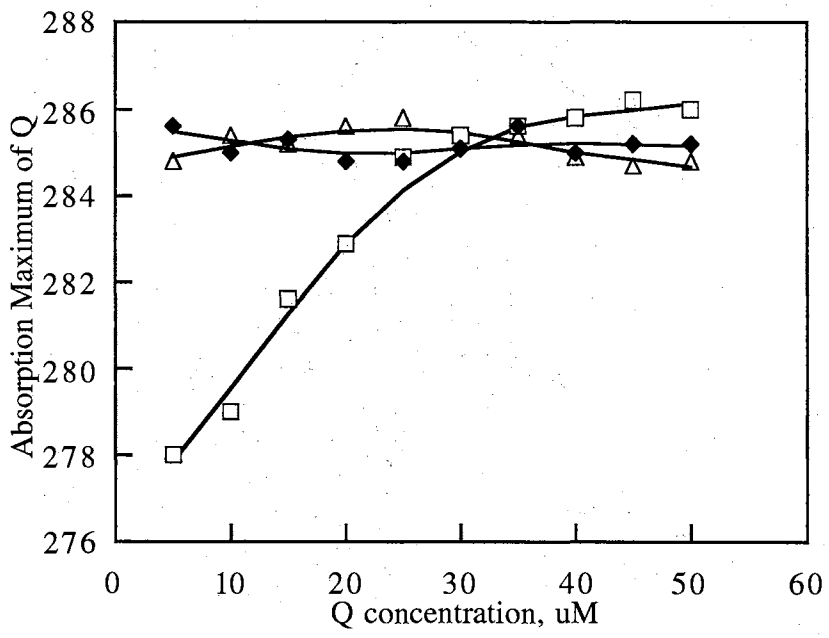


Figure 23. Effect of mutant recombinant QPs3 on the absorption maxima of Q. One ml aliquots of 20 mM sodium phosphate buffer pH 7.3 containing 150 mM NaCl, 2.5 mM$CaCl_2$ 10% ethanol and 0.01 % lauryl maltoside was added to 50 µl 50 mM Tris-HCl pH 8.0 buffer containing the respective mutant recombinant QPs3:- S33A(Δ), D36A (□), and Y37A (◆). The final protein concentration was 300 µg/ml. Each sample was prepared in duplicate and $Q_0C_{10}Br$ in 95% alcohol was added in 1 µl increments to one sample cuvette to obtain the indicated concentrations. The same amount of alcohol alone was added to the reference cuvette. After each addition the sample was incubated for 5 min and a difference spectra between the $Q_0C_{10}Br$-added and alcohol-added samples was recorded from 320 nm to 250 nm.

Heme reconstitution of QPs3 mutant proteins

Hemin chloride dissolved in DMSO was added to each of the isolated mutant QPs3 at the ratio of 0.75 heme to protein and the spectral properties were analyzed after thrombin treatment. The heme added H9D, H9N, H9Y, H48N, and H89N mutant proteins showed spectral characteristics essentially equivalent to the wild type (Figure 24) indicating that these histidines are not involved in heme ligation. However the spectra of H46Y and H60N showed slight changes in the soret region of the spectrum; the absorption maxima shifted from 424 nm to 438 nm (Figure 24). In the case of H46N mutant there was no restoration of cytochrome b_{560} in the heme added samples. When a double mutant H46,60N in which both the histidines at position 46 and 60 of the polypeptide were replaced by asparagine, was reconstituted with hemin chloride, the alpha peak of the dithionite-reduced minus oxidized spectrum was broadened and looked similar to heme added GST suggesting that both H46 and H60 of QPs3 are involved in the ligation of heme.

EPR Characterization of Mutant GST-QPs3 Proteins after Heme Reconstitution

The mutants H9D, H9N, H9Y, H89N showed epr spectra comparable to the wild type (Figure 25). This further confirms that these residues are not essential for heme ligation. The mutants H46N, H46Y, H60N and H46,60N did not produce epr signal at g=2.91and this denotes that the cytochrome b_{560} was not formed in this mutant and H46 and H60 provide the ligands to heme in cytochrome b_{560}.

H46 is identified as a conserved residue among all the analogous QPs3 membrane anchor proteins and is replaced by tyrosine residue in yeast SDH4 subunit; a cytochrome b_{560} has not so far been characterized in yeast SQR (7). This residue also corresponds to histidine 71 of *E.coli* SDHD which has been recently identified as ligand to cytochrome b_{556} in *E. coli* SQR (8). On the other hand H60 is not a highly conserved residue among analogous QPs3 subunits

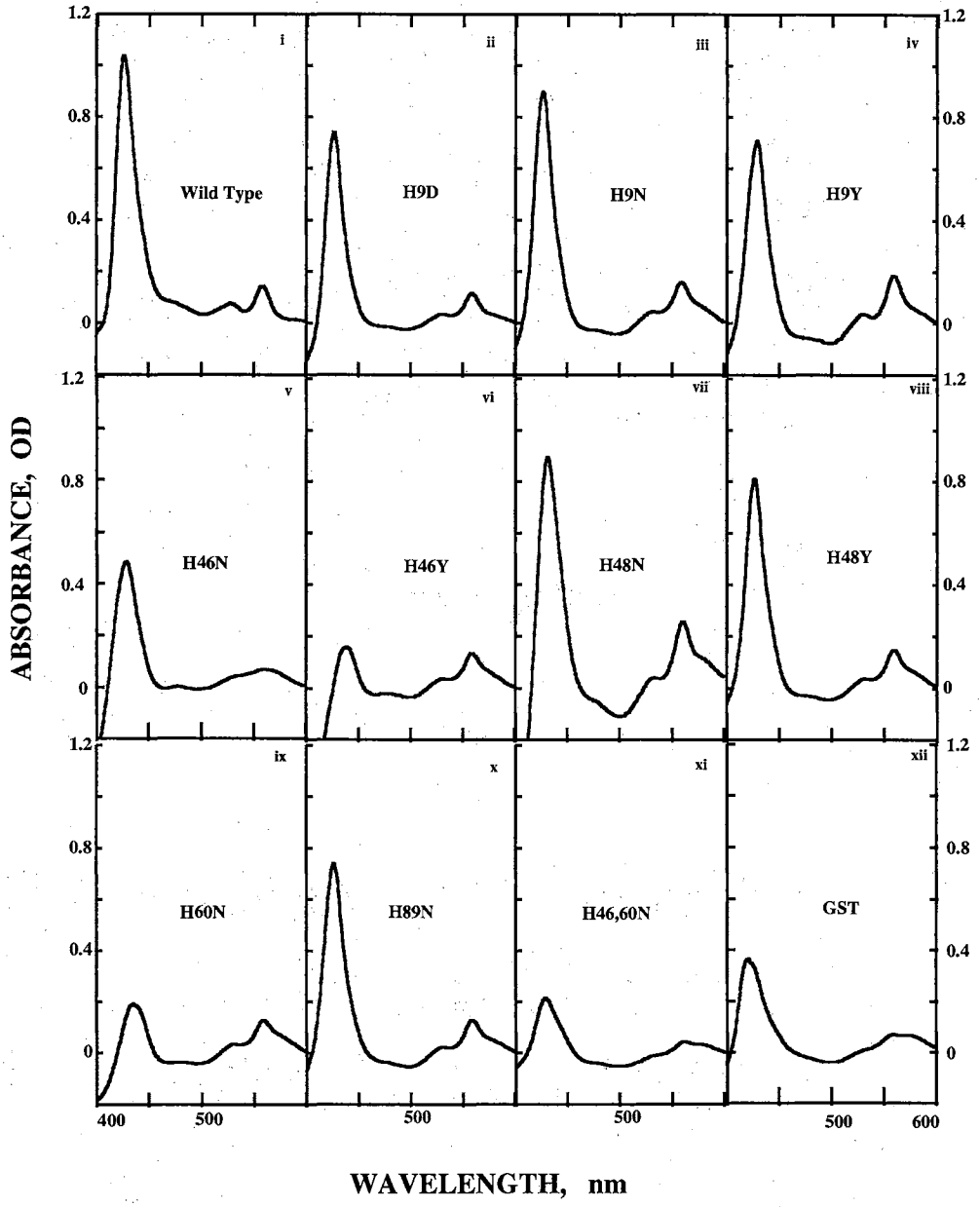

i
Wild Type
ii
H9D
iii
H9N
iv
H9Y
v
H46N
vi
H46Y
vii
H48N
viii
H48Y
ix
H60N
x
H89N
xi
H46,60N
xii
GST
1.2
0.8
0.4
0
400
500
600
ABSORBANCE, OD
WAVELENGTH, nm

Figure 24. Dithionite-reduced minus oxidized spectra of QPs3 Wild Type and Mutant proteins The Panels are represented as follows: (i) Wild type, (ii) H9D, (iii) H9N, (iv) H9Y (v) H46N (vi) H46Y (vii) H48N (viii) H48Y (ix) H60N (x) H89N (xi) H4660N (xii) GST

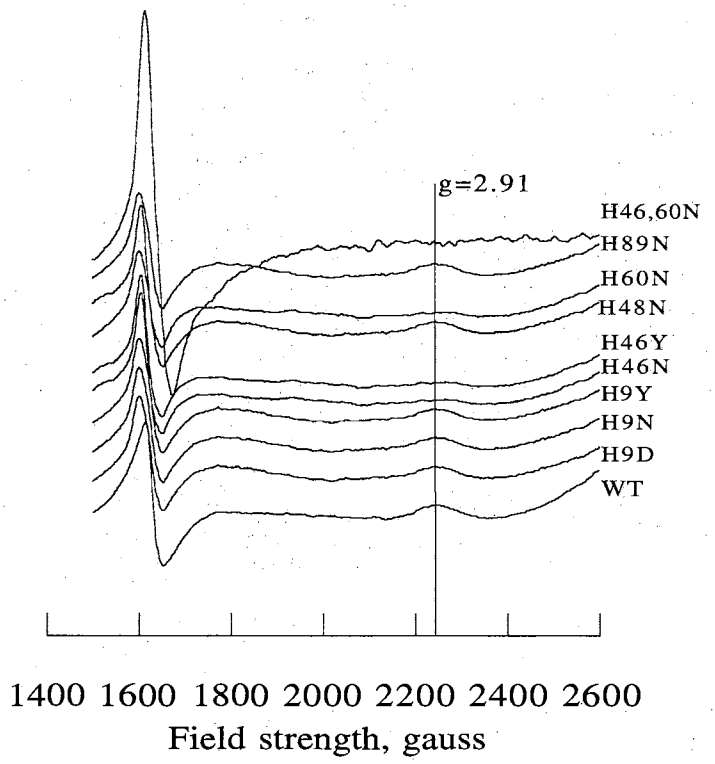


Figure 25. EPR spectra of heme reconstituted Wild type and mutant GST-QPs3 proteins after thrombin treatment. 15 μl of hemin chloride in DMSO was added to 0.6 ml of Tris-HCl buffer containing the respective protein samples (10 mg / ml) and incubated at room temperature for 1 hr prior to EPR measurement. Thrombin was added at 0.01 unit/μg of protein. The EPR settings were: modulation frequency, 100 KHz; modulation amplitude, 20 G; time constant, 0.5 s; microwave frequency, 9.42 GHz; microwave power, 20 milliwatts; scan rate, 200 s; temperature 10 k.

From the *in vitro* heme reconstitution studies of recombinant QPs1 and QPs3 subunits, it is evident that both can individually ligate heme to form cytochrome b_{560}. It is possible that in the isolated SQR or QPs, both QPs1 and QPs3 could ligate their own heme. If there could be two hemes in SQR, each ligated by QPs1 and QPs3 then the cytochrome b_{560} content in isolated SQR should not be substoichiometric to flavin.

In vitro reconstitution experiments using isolated SDH with either or both recombinant QPs subunits did not yield a TTFA sensitive SQR. The inability of obtaining a reconstitutively active fraction could be because QPs1 and QPs3 subunits were purified from parallel sources. In order to improve the interaction between the two subunits both subnits can be coexpressed and the recombinant QPs can be tested for reconstitution with SDH. Alternatively the failure to obtain SQR activity could be due to the lacking of QPs2. Work in our laboratory is being directed toward knowing the identity of QPs2.

Chapter Summary

By a site-directed mutagenesis approach, histidines at position 46 and 60 of QPs3 are identified to be responsible for heme ligation of cytochrome *b* of recombinant QPs3. Two residues, serine33 and tyrosine37 are identified to be crucial for Q-binding, since, when these amino acids were mutated to alanine the Q-binding properties of the recombinant QPs3 were altered from that of the wild type.

References

1. Shenoy, S. K. Yu, L., and Yu, C. A. (1997), *J. Biol. Chem.*, **272**, 17867-17872.
2. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A laboratory Manual, Cold Spring Harbor Press, NY.

3. Paul, K. G., Theorell, H., and Akesson, A., (1955) *Acta. Chem. Scand.*, **7** 1284-1287.
4. Laemmli, U. K. (1970) *Nature,* **227**, 680-685.
5. Schagger, H., Link, T. A., Engel, W. D., and vonJagow, G. (1986) *Methods Enzymol.*, **126**, 224-237.
6. Stowell, . H., McPhillips, T. M., Rees, D. C., Soltis, S. M., Abresch, E., and Feher, G., (1997) *Science*, **276**, 812-816.
7. Rose, C. T., Cecchini, G. Nakamura, K., Kita, K., and Gennis, R. B. (1997), *Biochem*istry, **37,** 4148-4159.
8. Hagerhall C., (1998), *Biochim. Biophys. Acta*, **1320**, 107-141.

v

VITA

SUDHA K. SHENOY

Candidate for the Degree of

Doctor of Philosophy

Thesis: STRUCTURE-FUNCTION STUDIES OF QPs3 OF BOVINE HEART MITOCHONDRIAL SUCCINATE-Q REDUCTASE

Major Field: Biochemistry and Molecular Biology

Biographical:

Personal Data: Born in India On May fifth 1962, the daughter of Tara Kamath and Gopalakrishna Kamath

Education: Graduated from Adarsh Vidyalaya Matriculation School, Madras, India in May 1978; received Bachelor's degree in Zoology from the University of Madras in May 1982; received Master's degree in Zoology from the University of Madras in May 1986. Completed the requirements for the Ph.D. degree in the field of Biochemistry and Molecular Biology at Oklahoma State University in December, 1998.

Abstracts Presented at Meetings:

Cloning and sequencing of mitochondrial succinate-Q reductase (1996) *The FASEB Journal* , 10, A1492; Expression and properties of QPs3 of mitochondrial succinate-Q reductase (1997) *Biophysical Journal,* 72, A319; Reactivation, cloning, and expression of Core proteins of mitochondrial *bc*1 (1997) *The FASEB Journal* , 11, A1280; Cloning and functional expression of Core proteins of bovine heart mitochondrial *bc*1 complex (1998) *Biophysical Journal*

Publications: **Shenoy S. K.**, Yu, L. and Yu, C. A. (1997) Smallest membrane anchor subunit QPs3 of bovine heart succinate-ubiquinone reductase: Cloning, sequencing, topology and Q-binding *J. Biol. Chem.*, 272, 17867-17872; Chen,Y.-R., **Shenoy, S. K.**, Yu, C. A., and Yu, L. (1995) Identification of amino acid residues involved in structural and ubiquinone-binding functions of subunit IV of the cytochrome bc_1 complex from *R.sphaeroides.*, *J. Biol. Chem.*, 270, 11496-11501.

Professional Membership:

American Association for the Advancement of Science; Oklahoma Academy of Science.