QUINONE-BINDING PROTEINS OF BOVINE HEART MITOCHONDRIAL SUCCINATE-UBIQUINONE REDUCTASE

By

GYESOON YOON LEE

Bachelor of Pharmacy
Kyung-Hee University
Seoul, Korea
1984

Master of Pharmacy
Kyung-Hee University
Seoul, Korea
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
May, 1996

QUINONE-BINDING PROTEINS OF BOVINE HEART MITOCHONDRIAL SUCCINATE-UBIQUINONE REDUCTASE

Thesis Approved:

Linda Ju

Thesis Adviser

H. Glen Spivey

Ulrich Melcher

Chang-an Yu

Robert Emp

Thomas C. Collins

Dean of the Graduate College

ACKNOWLEDGEMENTS

First of all, I thank God with my whole heart for His guiding and helping me to finish my Ph.D. program. Since I began studying Biochemistry as a special student in 1990, He has given me a hope whenever I could not have any. Finally He led me to accomplish my hope.

I would like to express my deep gratitude to Dr. Linda Yu, my advisor and mentor. She directed me to find out my binding site in the field of Biochemistry and Molecular Biology as a scientist, taught me how to find clones (informations) which were needed for my research life, and finally helped me to express them and make them functional in the Science society. She did not only advise my research, but also let me learn how to step on my new life as a woman Biochemist through her life. Also I am very thankful to Dr. Chang-An Yu. His endless encouragement and strict but patient and warm guidance through my graduate studies has made it possible for me to be at this stage. It is God's blessing to have met Dr. Linda and Chang-An Yu.

In addition, I want to express my gratitude to Dr. Ulrich Melcher, Dr. H. Olin Spivey, Dr. Robert Burnap who are my committee members. Their teaching through my course works, advices, and critical suggestions are really helpful to my study.

Surely my husband is deserved to share my honor. He recognized my potential, motivated me to study, and gave me endless encourage, and immeasurable love and

support. I am, also, really indebted to my parent and parent-in-law, especially mother-in-law. Their love, prayer, and support were the source of my strength, and, of course, I could not finish my study without my mother-in-law's help of taking care of my kids during the last one year, which I can never repay. Last, I give the biggest hug to my son, Samuel, and daughter, Peace. I dedicate my dissertation to my husband, Insung Lee, with my love.

TABLE OF CONTENTS

Chapter Page

Chapter Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

Figure Page

LIST OF SCHEMES

Scheme Page

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
BSA	bovine serum albumin
CAPS	3-[cyclohexylamino]-1-propanesulfonic acid
CO	carbon monoxide
CcO	cytochrome *c* oxidase
DCPIP	dichlorophenolindophenol
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EPR	electroparamagnetic resonance
FAD	flavin adenine dinucleotide
F	flavin
$F^{\cdot}$	radical of flavin
$FH^{\cdot}$	protonated form of flavin radical
Fp	flavoprotein
FPLC	fast protein (peptide) liquid chromatography

FRD	fumarate reductase
GSH	glutathione (reduced form)
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-benzoquinones
[^{3}H]-5-azido-Q_0C_{10}	5-azido-2,3-dimethoxy[^{3}H]-6-decyl-1,4-benzoquinones
HPLC	high performance liquid chromatography
Ip	iron-sulfur protein
IPTG	isopropyl-β-D-thiogalactopyranoside
β-ME	β-mercaptoethanol
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	20mM sodium/sodium phosphate buffer, pH 7.3, containing 150mM NaCl
Pd-C	palladium on activated carbon
PMSF	phenylmethylsulfonylfloride
PVDF	Polyvinylidene difluoride
Q	ubiquinone
$Q^{\cdot}$	radical of ubiquinone

$QH^{\cdot}$	protonated form of ubiquinone radical
QH_2	ubiquinol (reduced form of ubiquinone)
Q_0C_{10}	2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone
QCR	quinol cytochrome *c* reductase
QFR	quinol fumarate reductase
QPs	ubiquinone-binding (membrane-anchoring) proteins in SQR
SCR	succinate cytochrome *c* reductase
SDH	succinate dehydrogenase
SDS	sodium dodecylsulfate
SQR	succinate-ubiquinone reductase
TBS	20 % Tris-Cl buffer, pH 7.4, 500 mM NaCl
TTBS	20 % Tris-Cl buffer, pH 7.4, 500 mM NaCl, 0.05 % Tween-20
TTFA	2-thenoyltrifluoroacetone
UV	ultra violet
X-gal	5-bromo-4-chloro-3-indolyl-b-galactopyranoside

INTRODUCTION

Living organisms require a continual input of free energy to maintain the living state. About 95% of the energy required is obtained from a coupling reaction known as oxidative phosphorylation which takes place in the inner mitochondrial membrane. Oxidative phosphorylation is carried out by the mitochondrial respiratory (electron transfer) chain and ATP synthase. The energy released from the oxidation of NADH or succinate by the mitochondrial respiratory chain is conserved as an electrochemical proton gradient ($\Delta\mu H^+$), which is then utilized by an ATP synthase complex to synthesize adenosine triphosphate (ATP). The electrochemical proton gradient, also known as protonmotive force, consists of two components, a proton concentration gradient (ΔpH) and a membrane electrical potential ($\Delta\psi$) (1).

The mitochondrial respiratory chain is composed of four electron transfer complexes: NADH-ubiquinone reductase (NQR), succinate-ubiquinone reductase (SQR), ubiquinol-cytochrome *c* reductase (QCR) and cytochrome *c* oxidase (CcO). They are commonly referred to as Complex I, II, III, and IV, respectively. Figure 1 shows electron and proton transfer paths in the mitochondrial respiratory chain. Succinate and NADH, which mainly are derived from the citric acid cycle, are oxidized to fumarate and NAD^+ by donating electrons to ubiquinone by SQR and NQR, respectively. The electrons on reduced ubiquinone (ubiquinol) are then transferred to cytochrome *c* by QCR. Reduced

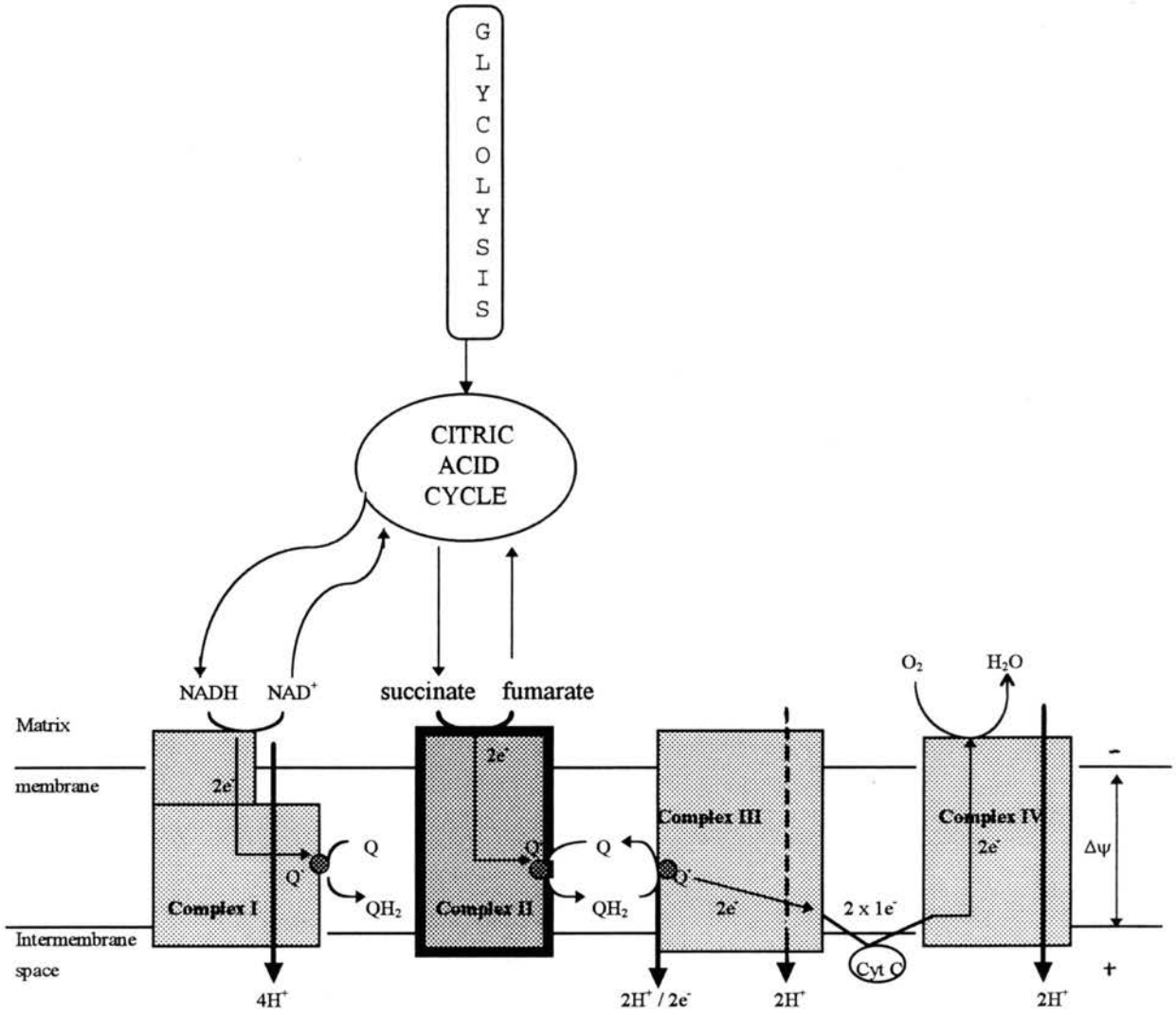


Figure 1. Electron and Proton Transfer Paths in The Mitochondrial Respiratory Chain.
(Modified from reference (48))

cytochrome *c* is finally oxidized by donating the electrons to molecular oxygen by CcO producing water. Electrons transferred through NQR, QCR, and CcO, are accompanied with translocation of protons across the mitochondrial inner membrane to generate a proton gradient for ATP synthesis (1, 2).

In recent years studies of mitochondrial electron transfer have focused on ubiquinone (Q). Q is a lipophilic molecule and abundant in the inner mitochondrial membrane. It has been considered as a mobile molecule shuttling electrons between SQR and QCR and between NQR and QCR. Recently, this free status of Q (Q-pool) (3, 4) has been challenged by an idea that Q has to be bound to protein in order to exert its electron transfer activity (4-6). Although evidence supporting these two ideas is accumulating, it is possible that both free Q and protein-bound Q are involved in the electron transfer chain. If the Q molecule which participates in electron transfer is strongly bound (high affinity) to its specific binding site, but is in redox equilibrium with free Q, then the ideas of Q-pool and Q-binding function are complementary.

Although the electron transfer catalyzed by SQR does not generate proton gradient for ATP synthesis, study of SQR has been vigorous because of its unique position in cell metablism and its catalysis of a Q-mediated electron transfer reaction. SQR serves as an electron entrance for succinate, which is produced by the citric acid cycle enzyme, into the respiratory chain for sequential electron transfer to produce ATP. A regulatory function of this enzyme in energy metabolism is also likely. Furthermore, SQR has a relatively simpler subunit structure than NQR and QCR, making it an ideal system for studying Q-mediated mitochondrial electron transfer reactions.

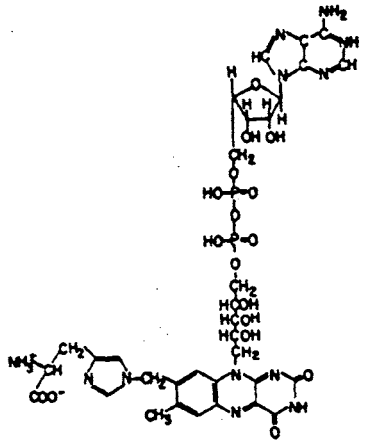
Bovine heart mitochondrial SQR was first isolated by Ziegler and Doeg (7). However, that isolation procedure was poorly designed; the yield was low with variable purity. A simple and reproducible procedure was introduced in 1982 by our group (8). This method involves treatment of succinate-cytochrome *c* reductase with Triton X-100 to dissociate SQR from QCR followed by calcium phosphate column chromatography at different pH's. Purified SQR contains five protein subunits with apparent molecular weights of 70, 28, 14, 11, and 9 kDa, estimated by high resolution SDS-PAGE (9). The complex has six prosthetic groups: FAD, three iron-sulfur clusters ([2Fe-2S], [3Fe-4S], and [4Fe-4S] clusters), cytochrome b_{560} heme, and Q (see Table I). The chemical structures of these prosthetic groups are shown in Figure 2.

SQR can be resolved into two reconstitutively active parts: a soluble succinate dehydrogenase (SDH) and a membrane-anchoring protein fraction. SDH has been purified and characterized in several laboratories (10, 11, 15, 16). Purified SDH possesses two types of enzymatic activities: redox dye activity and reconstitutive activity. The redox dye activity of SDH is its ability to transfer electrons from succinate to artificial electron acceptors, such as phenazine methosulfate and ferricyanide. The reconstitutive activity is its ability to reconstitute with QPs to form SQR. The succinate-low K_m ferricyanide activity of SDH correlates to its reconstitutive activity. Purified SDH contains two protein subunits with apparent molecular masses of 70 and 28 kDa (16), corresponding to the two larger subunits in SQR. The 70-kDa subunit contains a FAD and is called Fp; the 28 kDa subunit houses all three iron-sulfur clusters found in SQR and is named Ip. The amino acid sequences of Fp and IP subunits of SDH are available

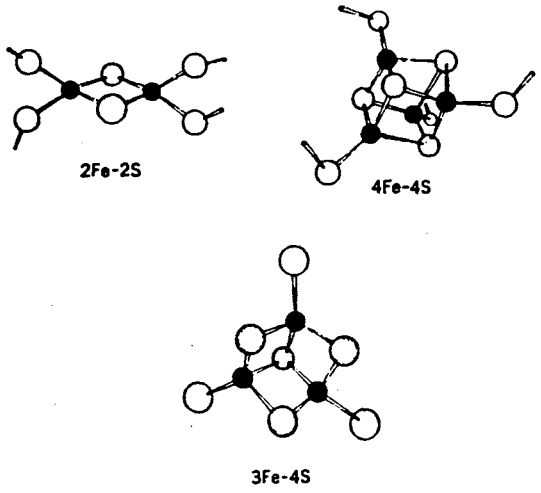
TABLE I

THE SUBUNIT COMPOSITION OF BOVINE HEART SUCCINATE-UBIQUINONE REDUCTASE AND ITS NH_2-TERMINAL AMINO ACID SEQUENCE

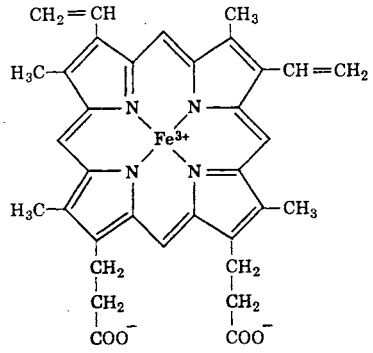
Molecular Mass (kDa)	Name	Prosthetic group (content in SQR)	Midpoint potential (mV)	NH_2-terminal Amino Acid	Ref.
70	Flavin protein (Fp)	His-FAD (5.8nmol/mg)	-79	SAKVSDAISA-	9, 8
27	Iron sulfur protein (Ip)	2Fe-2S 3Fe-4S 4Fe-4S (48 ngatom/mg)	0 +60 -260	AQTAA-	10
14	Q-binding protein (QPs1)	Heme (1.2nmol/mg)	-180	LGTTAKEEMER-	11,12
11	Q-binding protein (QPs2)			unclear	
9	Q-binding protein (QPs3)	Quinone (2nmol/mg)	+84	SDSKAASL-	12



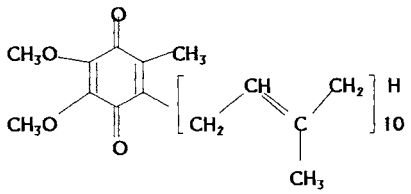
8-a-N(3)-histidyl FAD



Iron-sulfur Clusters



Ferroprotoporphyrin IX



Ubiquinone

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

through peptide (17) and nucleotide sequencing (18). Comparison of the available amino acid sequences of Fp and Ip subunits of SDH from all sources reveals a substantial degree of similarity (10, 11).

FAD is covalently linked to the Fp subunit in SQR via a 8α-N(3)-histidyl linkage (Fig. 2) (19, 20) and its midpoint potential is determined to be -79 mV which is higher than that of free FAD (-219 mV) (10, 11). Possibly the covalent linkage of FAD to protein raises the redox potential of FAD, allowing it to be reduced by succinate (11). Mutagenesis studies of *E. coli* fumarate reductase proved that succinate oxidation by FAD is only possible when FAD is covalently bound to protein (21). The FAD is also functionally, and probably, structurally located close to the substrate (succinate) binding site. A conserved tripeptide (His-Pro-Thr) in addition to conserved Arg, His, and Cys residues in the Fp was tentatively assigned to the active site by chemical modification and mutagenesis studies of *E. coli* fumarate reductase and *B. subtilis* SQR (10, 11). The histidine of this tripeptide is believed to play an important role in the protein donor/acceptor function by assisting the deprotonation of succinate (22).

The three iron sulfur clusters in the Ip subunit of SDH, irrespective of their origin, are named: S-1, a $[2Fe\text{-}2S]^{2+}$ cluster; S-2, a $[4Fe\text{-}4S]^{2+}$ cluster, and S-3, a $[3Fe\text{-}4S]^{2+}$ cluster (Fig. 2). All three clusters are ligated by cysteine residues of the protein (10, 11) and these cysteines of Ip are conserved in all species. The midpoint potentials of these three iron-sufur clusters in reconstitutively active SDH and SQR are determined as: S-1, 0 mV; S-2, -260 mV; and S-3, 60 mV (11, 23). Since S-2 center has extremely low potential and is not reducible by succinate, some reports have ruled out direct participation

of this iron-sulfur cluster in catalysis of SQR (10, 23). However, there is still a possibility for center S-2 being involved in SQR catalysis if the determined S-2 center potential is artificially low due to anticooperative interaction among the redox carriers in the sample (11). The reconstitution activity of SDH is directly proportional to the amount of S-3 center (24).

The reconstitutively active, membrane-anchoring protein fraction has been isolated and characterized in several laboratories (12-14). It is named as CII-3 and -4 (12), cytochrome b_{560} (13), and QPs (14). The term, QPs (Quinone-binding Protein in SQR), will be used for the membrane-anchoring protein fraction throughout this thesis because it is named by our group. All these preparations show two protein bands in the SDS-PAGE system of Weber and Osborn (25). However, the two-subunit QPs is further resolved into three protein subunits (26) by the high resolution SDS-PAGE system of Schägger *et al.* (9). The apparent molecular masses of these three subunits are 14, 11, and 9 kDa, corresponding to the three smaller subunits of SQR. They are named QPs1, QPs2, and QPs3, respectively.

The function of QPs is to provide membrane docking for SDH and the Q-binding site for SQR. Soluble SDH can catalyze electron transfer from succinate to redox dyes, but it cannot catalyze the 2-thenoyltrifluoroacetone (TTFA)-sensitive electron transfer from succinate to Q. Addition of QPs to soluble SDH converts SDH to the membrane-bound, TTFA-sensitive SQR with detectable ubisemiquinone radical formation, indicating that QPs provides membrane docking for SDH (12-14) and the Q-binding site for the complex (27, 28). It is not clear, at present, whether one, two, or three QPs protein

subunits are required for membrane docking and the Q-binding functions of QPs, because attempts to dissociate QPs into their individual subunits have, so far, been unsuccessful.

Isolated QPs contains 25 nmoles of cytochrome b_{560} per mg protein (29). Since cytochrome b_{560} in SQR is sub-stoichiometric to FAD and is not reducible by succinate, the direct participation of cytochrome b_{560} heme in catalytic function of SQR is ruled out. However, a close physical association of cytochrome b_{560} with SDH is evidenced by the following observations (29): (A) the dithionite reduced form of cytochrome b_{560} in isolated QPs has a symmetrical α-absorption peak, which upon reconstitution with SDH becomes slightly broadened and shows a shoulder at around 553 nm, identical to that of cytochrome b_{560} in SQR. (B) Dithionite reduced cytochrome b_{560} in QPs is reactive to carbon monoxide while cytochrome b_{560} in SQR is not reactive to carbon monoxide. Upon addition of SDH to QPs, the QPs becomes insensitive to carbon monoxide treatment. (C) The redox potential of cytochrome b_{560} in QPs is -144 mV, which is higher than that of cytochrome b_{560} in SQR (-185 mV). Upon addition of SDH, the redox potential of about 46% of the cytochrome b_{560} in QPs becomes identical to that of cytochrome b_{560} in SQR. (D) Cytochrome b_{560} in QPs shows two epr signals, g=3.07 and g=2.92, whereas cytochrome b_{560} in SQR exhibits only one epr signal at g=3.46. When QPs is reconstituted with SDH to form SQR, the g=3.46 epr signal reappears at the expense of the g=3.07 signal.

In addition to the structural role of cytochrome b_{560} in SQR, this cytochrome may, also, serve as a low potential electron entrance for fumarate reduction (29), under special conditions. Under anaerobic conditions dithionite or NADH reduced cytochrome b_{560} in

SQR or QPs in the presence of SDH is fully oxidized by fumarate, suggesting that cytochrome b_{560} serves as the electron entrance for fumarate reduction (29).

The ligand for the cytochrome b_{560} heme in SQR has been found as bis-histidine (30, 31). At present, it is not known whether both histidines come from QPs1, or one of them is in QPs2 or QPs3, even though QPs1 is believed to be cytochrome b_{560} in mitochondrial SQR. The latter possibility will be ruled out if a cytochrome b_{560} heme-containing QPs1 is obtained.

Isolated SQR contains 2 nmoles of tightly bound Q per mg protein. The preparation is 40-50% deficient in Q content. The Q deficiency is determined by the extent of activity increase in Q-stimulated DCPIP reduction by succinate in the presence of excess Q in the assay mixture. The midpoint potentials of the two half reactions ($Q/Q^{\cdot}$ and $Q^{\cdot}/QH_2$) of Q in SQR were estimated to be 40 and 128 mV, respectively (28). The existence of Q-binding protein in SQR was proven by the detection of a TTFA-sensitive ubisemiquinone radical (28). Unlike the ubisemiquinone radical detected in QCR, the ubisemiquinone radicals detected in SQR do not show power saturation (28). The ubisemiquinone radical formation requires the presence of QPs, indicating that QPs provides a Q-binding site in this complex. Identification of QPs subunits responsible for Q-binding and location of the Q-binding domain are part of this thesis project.

Study of Q:protein interaction in SQR with various Q-derivatives has generated vast information on the structural specificity of the Q-binding site in the complex. SQR requires Q with a five carbon alkyl side chain to exert maximum activity, while QCR needs Q with a 10 carbon chain length for maximum activity, indicating that the Q-binding sites

in these two complexes are different (32). The methoxy group at the 2- and 3- positions of the benzoquinone ring of the Q molecule is more important than the methyl group at the 5-position when Q is used as electron acceptor for SQR. Replacing the 5-methyl group with hydrogen causes a slight increase in activity (33). However, replacing one or both of 2- and 3-methoxy groups with a methyl completely abolishes electron-acceptor activity (33). By using [^{3}H]azido-Q derivatives to study Q:protein interaction in QCR, the Q-binding proteins in this complex have been identified. We intend to use azido-Q derivative to identify Q-binding protein in SQR.

Although the electron transfer path in SQR are not yet fully understood, the dual pathway model (Fig. 3) (34), proposed by Cammack, has been popular. This model hypothesizes reduction of Q at two different sites. At one site, the two low potential redox components of SQR, center S-2 (-260 mV) and cytochrome b_{560}, (-180 mV) are needed to reduce Q to QH· and mediate electron transfer between the low potential FAD/FADH· (-127 mV) and Q/QH· (40 mV) couples. At another site, centers S-1 (0 mV) and S-3 (60 mV) are involved to reduce QH· to QH_2 and mediate electron transfer between high potential FADH·/$FADH_2$ (31 mV) and QH·/QH_2 (128 mV) couples.

Electron transfer from succinate to FAD by a two-electron step is a thermodynamically unfavorable reaction, even if FAD is indeed located close to the active site, because the redox potential for the succinate/fumarate couple is +30 mV and for FAD/$FADH_2$ couple -79 mV. How can this thermodynamatically unfavorable electron transfer reaction take place ? There are indications that the relaxation energy associated with a conformational change may assist this electron transfer step. SQR was reported to

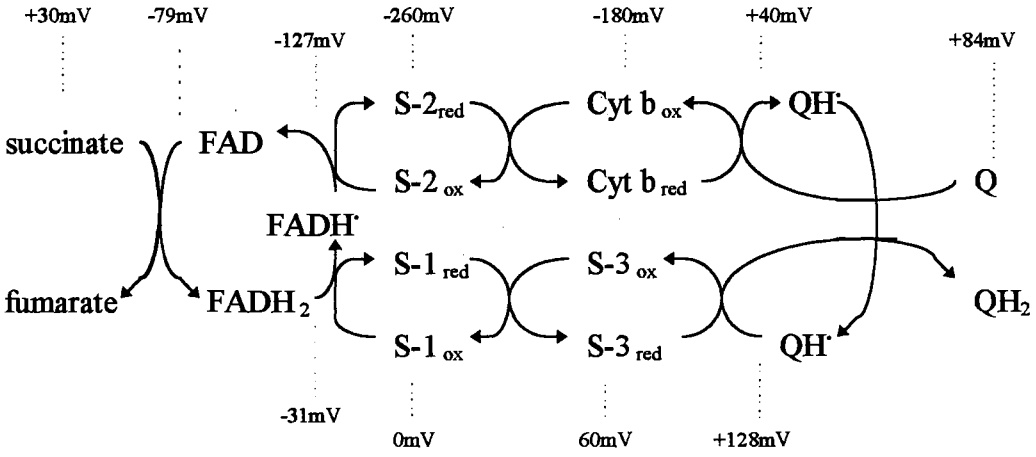


Figure 3. Dual Pathway Model for Electron Transfer through Succinate-Q Reductase. (Modified from reference (34))

have different substrate affinities for oxidized and reduced states, indicating that a conformational change is associated with the change in redox state of enzyme. The dissociation constant, K_d, for succinate is 16-fold higher for reduced SQR compared to oxidized enzyme. The K_d for fumarate is 4-fold higher for the the oxidized enzyme than for the reduced one (10).

FAD serves as a converter from n=2 to n=1 during oxidation of succinate. It accepts electrons from succinate in a two-electron step and donates electrons to iron-sulfur clusters in two sequential one-electron steps. Oxidation of $FADH_2$ by iron-sulfur clusters with two one-electron transfer reactions is supported by the detection of very stable flavosemiquinone radicals in SQR (10). $FADH_2$ donates its first electron to high potential iron-sulfur center S-1 to generate FADH· which then subsequently donates another electron to low potential iron-sufur center S-2 to generate oxidized FAD. Thus, dual electron transfer paths are generated for subsequent electron transfers from S-1 to S-3 and from S-2 to cytochrome b_{560} to reduce Q to QH· and QH· to QH_2, respectively.

The observation of Q· Q· interaction in SQR by EPR study (35) supports the presence of two Q-binding sites in SQR. Moreover, mutagenesis study of *E. coli* quinol-fumarate reductase identified several amino acids essential for enzymatic activity in the membrane anchor proteins (36). By mapping these residues on the proposed secondary structure, two quinone-binding sites were revealed, like the well characterized two quinone binding sites of the photosynthetic reaction center (36, 37). These results further support the speculation of the existence of two Q-binding sites in SQR.

Figure 4 shows the proposed topological arrangement of subunits and redox centers of SQR in the mitochondrial inner membrane. This arrangment is consistent with data obtained from biochemical and biophysical studies of SQR and accomodates the proposed dual-pathway model of electron flow in the complex. The Fp subunit of SDH is located at the matrix surface of the mitochondrial inner membrane, which can receive the substrate, succinate, from and send the fumarate back to the citric acid cycle (38-41). The Ip subunit of SDH serves as the link between QPs and the Fp subunit (39, 40). QPs subunits are mostly buried in the membrane.

Interaction of SDH with QPs is known to be through hydrophobic and ionic reactions (6) as SDH can be detached from QPs by either chaotropic reagents or alkaline pH treatment. The ionic interaction is further supported by chemical modification studies of QPs and SDH revealing that two carboxyl groups and a histidine residue in QPs (42, 43) and an amino group in SDH (44) are essential for this interaction.

The S-3 iron-sulfur cluster is located in the hydrophobic region of the Ip subunit of SDH, and QPs is generally believed to bind in the vicinity of the S-3 center (27). The Ip subunit of SDH is possibly associated with the inner membrane via a short NH_2-terminal membrane anchor from the observation that the NH_2-terminus of Ip consists of a stretch of nine uncharged residues (45). EPR studies suggest that FAD is 12-18 Å apart from S-1, and S-1 is 9-12 Å from S-2 and 10-20 Å from S-3 (10, 46).

The interaction of QPs with the Ip subunit of SDH is probably through the NH_2-terminal portion of QPs1, as the proposed secondary structure of QPs1, based on the deduced amino acid sequence (26), shows three transmembrane helices with its NH_2-

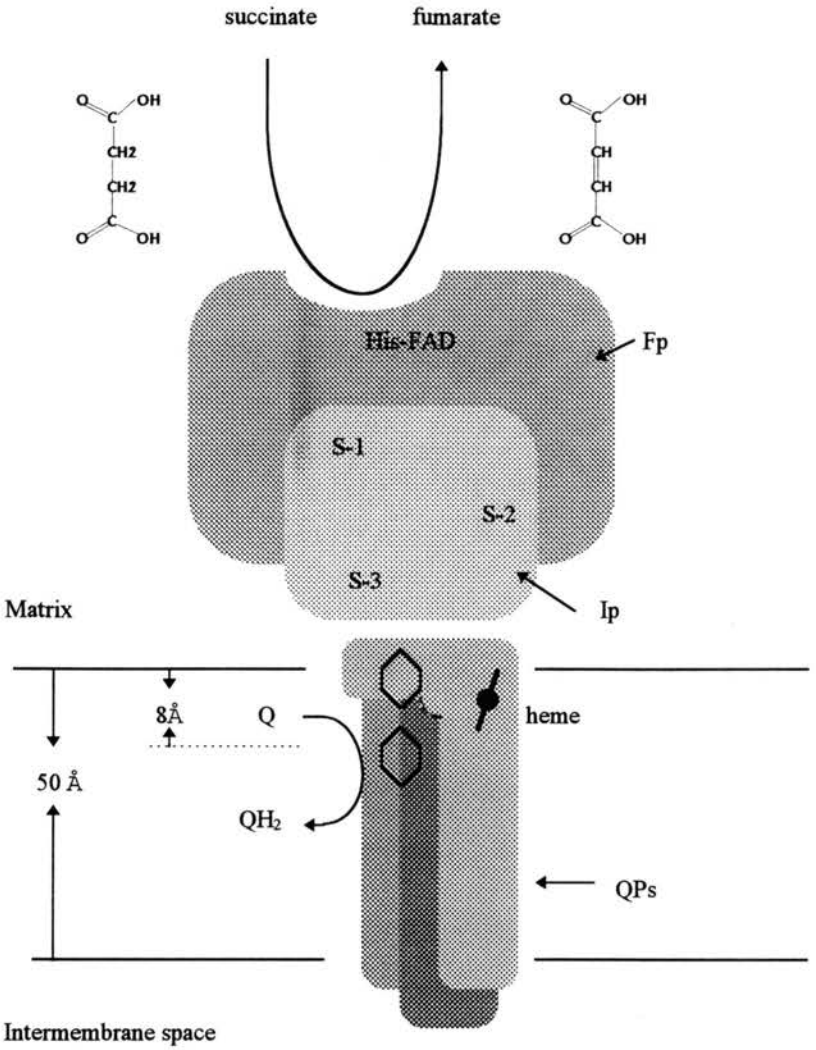


Figure 4. Topological Arrangement of Subunits and Redox Centers in Succinate-Q Reductase (Modified from reference (10)).

terminus on the matrix side. It is also possible that all three subunits of QPs are involved in interaction with the Ip subunit of SDH. At present, the topological arrangement of QPs subunits in the membrane is unknown. Study on this aspect is currently in progress in our laboratory.

The two ubisemiquinone radicals in SQR are about 8 Å apart as estimated by EPR spectral analysis. Their alignment is not face to face but edge to edge (47). The dipolar axis (quinone-quinone direction) is approximately perpendicular to the membrane plane in a trans-membrane fashion as a rigidly bound quinone pair (47). By examining the power saturation characteristics of S-3 and coupled signal, at least one of the Q's was found to be proximal to S-3 (35). Also, it is suggested that cytochrome b_{560} may be necessary for the binding and simultaneous stabilization of two semiquinones (27).

References

1. Nicholls, D. G., and Ferguson, S. J. (1992) Bioenergetics 2. Academic Press, New York.
2. Hatefi, Y. (1985) *Ann. Rev. Biochem.*, **54**, 1015-1069.
3. Lenaz, G, Fato, R., Battino, M., Mandrioli, E., and Castelli, G. P. (1987) in *Bioenergetics: Structure and Function of Energy Transducing Systems* (Ozawa, T. and Papa, S. eds.) pp 49-61, Japan Scientific Societies Pressss, Tokyo.
4. Kroger, A. and Klingenberg, M. (1973) *Eur. J. Biochem.*, **34**, 358-368.

5. Yu, C. A. and Yu, L. (1987) in *Bioenergetics: Structure and Function of Energy Transducing Systems* (Ozawa, T. and Papa, S. eds.) pp 81-99, Japan Scientific Societies Pressss, Tokyo.

6. Yu, C. A. and Yu, L. (1981) *Biochim. Biophys. Acta*, **639**, 99-128.

7. Ziegler, D. M. and Doeg, K. A. (1962) *Arch. Biochem. Biophys.*, **97**, 41-50.

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

9. Schägger, H., Link, T. A., Engel, W. D., and von Jagow, G. (1986) *Methods Enzymol.* **126**, 224-237.10. Hederstedt, L., and Ohnishi, T. (1992) in Molecular Mechanisms in Biogenetics (Ernster, L., ed.) pp. 163-198, Elsevier Science.

10. Hederstedt, L., and Ohnishi, T. (1992) in Molecular Mechanisms in Biogenetics (Ernster, L., ed.) pp. 163-198, Elsevier Science.

11. Ackrell, B. A. C., Johnson, M., Gunsalus, S. P., and Cecchini, G. (1992) in Chemistry and Biochemistry of Flavoenzymes (Muller, F., ed.) Vol III, pp 229-297, CRC Press, London.

12. Ackrell, B. A. C., Ball, M. B., and Kearney, E. B. (1980) *J. Biol. Chem.*, **255**, 2761-2769.

13. Hatefi, Y., and Galante, Y. M. (1980) *J. Biol. Chem.*, **255**, 5530-5537.

14. Yu, C. A., and Yu, L. (1980) *Biochemistry*, **19**, 3579-3585.

15. Keilin, D. and King, T. E. (1958) *Nature*, **181**, 1520-1522.

16. Davis, K. A. and Hatefi, Y. (1971) *Biochemistry* **10**, 2509-2516.

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

18. Birch-Machin, M. A., Farnsworth, L., Ackrell, B. A. C., Cochran, B., Jackson, S., Bindorff, L. A., Aitken, A., Diamond, A. G., and Turnbull, D. M. (1992) *J. Bio. Chem.*, **167**, 11553-11558.

19. Walker, W. H. and Singer, T. P. (1970) *J. Biol. Chem.*, **245**, 4224-4225.

20. Salach, J., Walker, W. H., Singer, T. P. (1972) *Eur. J. Biochem.*, **26**, 267-278.

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

22. Vik, S. B. and Hatefi, Y. (1981) *Pro. Natl. Acad. Sci. USA*, **78**, 6749-6753.

23. Ohnishi, T., Salerno, J. C., Winter, D. B., Lim, J., Yu, C. A., Yu, L. and King, T. E. (1976) *J. Bio. Chem*, **251**, 2094-2104.

24. King, T. E., Ohnishi, T., Winter, D. B. and Wu, J. T. (1976) in Iron and Copper Proteins (Yashunobu, K. T., Mower, H. F. and Hayaishi, O., eds.), pp. 182-227, Plenum Pub. Co., New York.

25. Weber, K. and Osborn, M. (1969) *J. Biol. Chem.*, **244**, 13599-13604.

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

27. Xu, Y., Salerno, J. C., Wei, Y. H., and King, T. E. (1981) *Biochem. Biophys. Res. Comm.*, **144**, 315-322.

28. Miki, T., Yu, L., and Yu, C. A. (1992) *Arch. Biochem. Biophys.* **293**, 61-66.

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

30. Peterson, J., Vibat, C., Gennis, R. B. (1994) *FEBS Letters*, **355**, 155-156.

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

32. Yu, C. A., and Yu, L. (1982) *Biochemistry*, **21**, 4096-4101.

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

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

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

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

37. Okamura, M. Y., and Feher, G. (1992) *Ann. Rev. Biochem.*, **61**, 861-896.

38. Klingenberg, M. (1970) *Eur. J. Biochem.*, **13**, 247-253.

39. Merli, A., Capaldi, R. A., Ackrell, B. A. C., and Kearney, E. B. (1979) *Biochemistry*, **18**, 1393-1400.

40. Girdlestone, J., Blisson, R., and Capaldi, R. A. (1981) *Biochemistry*, **20**, 152-156 .

41. Johnson, M. K., Kowal, A. T., Morningstar, J. E., Oliver, M. E., Whittaker, K., Gunsalus, R. P., Ackrell, B. A. C., and Cecchini, G. (1988) *J. Biol. Chem.*, **263**, 14732-14738.

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

43. Paudel, H. K., Yu, L., and Yu, C. A. (1991) *Biochim. Biophys. Acta*, **1056**, 159-165.

44. Yu, L., McCurley, J. P, and Yu, C. A. (1987) *Biochim. Biophys. Acta*, **893**, 75-82.

45. Clarkson, G. H. D., Neagle, J., and Lindsay, J. G. (1991) *Biochem. J.*, **273**, 719-724.

46. Ohnishi, T. (1987) *Current Topics in Bioenergetics*, **15**, 37-65.

47. Saleno, J. C., Harmon, H. J., Blum, H., Leigh, J. S., and Ohnishi, T. (1977) *FEBS Letters*, **82**, 179-182.

48. Hägerhäll, C. (1994) On the Structure and function of Succinate-Ubiquinone Oxidoreductase using *Bacillus subtilis* as a model system. Akademisk Avhandling, University of Lund, Lund.

CHAPTER I

THE AMINO ACID SEQUENCE OF QPs1

Structure-function study of SQR requires knowledge of the amino acid sequences of all the subunits in this complex. The amino acid sequences of Fp and Ip subunits of SDH are available by sequencing peptide (1) and nucleotide sequences (2), respectively, but the amino acid sequences of QPs subunits are mostly lacking. Recently, a cDNA encoding QPs1 has been obtained in our lab by immunologically screening the bovine heart cDNA library in λgt11 with antibody against QPs1 (3). Since purified QPs1 protein, obtained from preparative SDS-PAGE, had its NH_2-terminus blocked and was resistant to all proteolytic enzyme digestion, confirmation of this QPs1 cDNA clone was achieved by mass spectrometry. The protonated molecular fragments detected in the laser desorption time-of-flight mass spectrum of QPs1 correspond to the assigned fragments in the deduced amino acid sequence of QPs1.

In the QPs1 cDNA clone the coding sequence for mature QPs1 was assigned to start with ATG, 27 bp downstream from the 5' end, with a total of 396 bp encoding 132 amino acid residues. This assignment was based on the largest molecular mass fragment in the mass spectrum of QPs1, 14320.2 (m/e), and an apparent molecular weight of QPs1 determined by SDS-PAGE of 14,000 daltons. This assigment leaves room for revision because the insolubility of QPs1 may complicate QPs1 molecular weight detemination by

mass spectrometry and hydrophobic protein molecular masses determined by SDS-PAGE are usually not very accurate. Of course, the most direct way to assign the start of the coding sequence for QPs1 is to have a partial NH_2-terminal amino acid sequence of QPs1 available.

The failure of our previous attempts in obtaining the partial NH_2-terminal amino acid sequence of QPs1 was due to the blockage of the NH_2-terminus in isolated samples. It was reported (4) that proteins isolated by SDS-PAGE often have their NH_2-terminus blocked resulting from the interaction of the amino group with unreacted acrylamide solution in gels after polymerization. This kind of NH_2-terminus blockage of the protein can be prevented by using a residual acrylamide-free SDS-PAGE gel. This report motivated us to re-investigate the nature of NH_2-terminus blockage in isolated QPs1 and to obtain its partial NH_2-terminal amino acid sequence. Obtaining the complete nucleotide and amino acid sequences of QPs1 are essential for structure-function study of this protein, such as localization of Q-binding domain and the molecular genetic study of QPs1.

The uncertainty of the NH_2-terminus status of QPs1 raised the possibility of the QPs1 cDNA clone obtained in our laboratory being an incomplete clone which lacks an NH_2-terminal presequence. It is highly likely that nascent QPs1 is synthesized with a presequence, like the membrane-anchoring proteins of yeast SQR, to direct it to the inner mitochondrial membrane. Although information on the presequence is not important for structure-function studies of QPs1, it will help us understand the processing of QPs1 in the cell.

In this charpter I report isolation of a NH_2-teminus unblocked QPs1 by SDS-PAGE and the partial NH_2-terminal amino acid sequence of QPs1. I also present a revised amino acid sequence of QPs1 deduced from a revised nucleotide sequence. The presence of a presequence for QPs1 is confirmed by nucleotide sequencing of a longer QPs1 cDNA clone.

Experimental Procedures

Materials

DNase I (Type IV), RNase A, ampicillin, agarose, sodium thioglycolate, 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), bovine serum albumin, DCPIP, Triton X-100, sodium dodecylsulfate (SDS), N-[2-hydroxy-1,1-bis (hydroxymethyl) ethyl]glycine (Tricine), and sodium cholate were obtained from Sigma. Restriction endonucleases, T4 DNA ligase, were purchased from either Promega Corporation or Bethesda Research Laboratories, Inc. Polyvinylidene difluoride (PVDF) membranes were from Millipore. Other chemicals were obtained commercially in the highest purity available.

Bacterial Strains and Plasmids

E. coli JM109[*rec*A1, *endo*A1, *gyr*A96, *thi*$^-$, *hsd*R17, *sup*E44, *rel*A1 Δ(*lac, pro*)F'*tra*D36*pro*AB *lac*Iq, *lacZ* M15] was used as host for pGEM-3Zf(+) (Promega). *E. coli* strains were grown in LB medium (5). When necessary, ampicillin (100 μg/ml) was added.

DNA Manipulation and DNA Sequencing

Restriction enzyme digestion, large-scale isolation and minipreparation of plasmid DNA, isolation and purification of Lamda phage DNA were performed according to reported methods (5). The sequencing primers were synthesized and DNA sequencing was performed by the Recombinant DNA/Protein Resource Facility at Oklahoma State University.

Enzyme Preparations and Assays

Bovine heart SQR was prepared from SCR by the method previously reported (6). SQR containing fractions obtained from the second calcium phosphate-cellulose column were concentrated by 43% ammonium sulfate saturation and centrifugation at 48,000 x *g* for 20 min. The precipitate obtained was dissolved in 50 mM potassium/sodium phosphate, 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 Cary spectrophotometer (model 219) or Shimadzu UV-2101PC. The reaction mixture used for the Q-stimulated DCPIP reduction assay contains 38 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 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 $mM^{-1}cm^{-1}$.

Electroblotting of QPs1 onto a PVDF Membrane

SQR was treated with 1% β-mercaptoethanol and 1% SDS for 2 hrs at 37°C. 20-μl aliquots of the digested samples were applied to wells of an SDS-PAGE gel which had been pre-electrophoresed overnight at 45 V with an anode buffer containing 0.2 M Tris-Cl, pH 8.9, and 0.1 mM sodium thioglycolate and a cathode buffer containing 0.1 M Tris, 0.1 M Tricine, 0.1% SDS, and 0.1 mM sodium thioglycolate, pH 8.2. The proteins in SDS-PAGE gel were electroblotted to a PVDF membrane according to the procedure of LeGendre *et al.* (4) using CAPS transferring buffer (10 mM CAPS, 10% methanol, pH 11) for 15 min . After the transfer was complete, the membrane was rinsed several times with HPLC-grade H_2O before being stained with Coomassie blue R-250 (0.1% in 50% methanol) for 2 min to visualize the proteins bound to the membrane. The blue band corresponding to QPs1 was cut out and subjected to NH_2-terminal sequence analysis.

Amino Acid Sequence Determination

Amino acid sequence 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

Revised Amino Acid and Nucelotide Sequences of QPs1

The partial NH_2-terminal amino acid sequence of QPs, obtained by microsequencing of QPs1 protein blotted on a PVDF membrane, is: LGTTAKEEMER-. The success of this partial NH_2-terminal amino acid sequence determination relies on the isolation of a QPs1 protein having the NH_2-terminus unblocked during SDS-PAGE. The use of a pre-electrophoresed SDS-PAGE gel and inclusion of 0.1 mM sodium thioglycolate in the running buffer prevented blockage of the NH_2-terminus during electrophoresis. The blockage of protein NH_2-temini during SDS-PAGE is reported to be due to the covalent linkage of NH_2-terminus with free acrylamide remaining in the gel (4). The residual ammonium persulfate and TEMED in the gel may catalyze this reaction. Therefore, pre-electrophresing the gel overnight, can remove all the remaining free acrylamide, ammonium persulfate, and TEMED. The inclusion of sodium thioglycolate in the running buffer of SDS-PAGE can keep the NH_2-terminus reduced to prevent any possible reactions.

From the partial NH_2-teminal amino acid sequence of QPs1, it was obvious that our previously reported amino acid sequence for QPs1 deduced from nucleotide sequencing is 8 amino acid residues short. The coding sequence for mature QPs1 starts from 7 base pairs, instead of 31 base pairs, downstream from the 5' end of our isolated QPs1 cDNA clone (3). Figure 5 shows the revised amino acid and nucleotide sequences of QPs1. The molecular weight of mature QPs1 is 15,149. Apparently the cDNA clone

```
                                                                  TTG AGA CAT GTT
                                                  [ ( M   A   A   L   L ) L   R   H   V
GGC CGT CAC TGC CTC CGT GCC CAC CTT AGT CCT CAG CTC TGT ATC AGA AAT GCT GTT CCT
 G   R   H   C   L   R   A   H   L   S   P   Q   L   C   I   R   N   A   V   P ]

1                                               11
TTG GGA ACC ACA GCC AAA GAA GAG ATG GAG AGG TTC TGG AGT AAG AAC ACT ACT TTA AAC
 L   G   T   T   A   K   E   E   M   E   R   F   W   S   K   N   T   T   L   N
21                                              31
CGT CCT TTG TCT CCC CAT ATC AGC ATC TAC GGC TGG TCT CTT CCC ATG GCG ATG TCC ATT
 R   P   L   S   P   H   I   S   I   Y   G   W   S   L   P   M   A   M   S   I
41                                              51
TGC CAC CGT GGC ACT GGT ATT GCC TTG AGT GCA GGA GTC TCT CTT TTT GGC TTG TCA GCC
 C   H   R   G   T   G   I   A   L   S   A   G   V   S   L   F   G   L   S   A
61                                              71
CTC TTG GTC CCT GGG AGC TTT GAG TCT CAT TTG GAA TTT GTG AAG TCC CTG TGT TTG GGG
 L   L   V   P   G   S   F   E   S   H   L   E   F   V   K   S   L   C   L   G
81                                              91
CCA GCA CTG ATC CAC ACA GCC AAA TTT GCA CTT GTC TTC CCT CTC ATG TAT CAC ACC TGG
 P   A   L   I   H   T   A   K   F   A   L   V   F   P   L   M   Y   H   T   W
101                                             111
AAT GGG ATC CGA CAC TTG ATG TGG GAT CTA GGA AAA GGC CTG ACG ATT TCC CAG CTA CAC
 N   G   I   R   H   L   M   W   D   L   G   K   G   L   T   I   S   Q   L   H
121                                             131
CAG TCT GGA GTG GCT GTC CTG GTT CTT ACT GTG TTG TCC TCT GTA GGG CTG GCA GCC ATG
 Q   S   G   V   A   V   L   V   L   T   V   L   S   S   V   G   L   A   A   M
TGA
 *

AGAGCTGAAGTTCCCAGTGTCATCATCCTACAAATTATCACATTGACCTGTCTTCCTGTTTGTTACTCTTGTCTTTGGC
CAGGGCAAAGTTATTCTGATTTGTTTAGACCCTTCTTTGCTTGCAGATCCCCTTGGAGCTCAGCAGTAGAGTACCTTAC
AGAACCATAACGGTGGAAAAGGGTCCAGTTTTCTCCTTATTTATAAGGATGGAATGACTGCAAAAACTCCCTTTTCTTG
CCCCCGCGTTGCAGCCTACTCTGGGCTTGGGAGCCTTCTTTCTCTCTCCATGTTAGGCCTTGATTTATGCTGAGGGTCA
GCCTTTGGCTCCTTTTTTCCTGAGACAGTGGAAACAATGCCAGCTCTGTGGCCTCTGCCGTGGGGACTGGGAGTGGGGAT
CCGGGTGCCACTGCCTGTGGGTTGCTGGCTCAAAGGACAATTCTGTCCATTGGTCAGAGCCCAGGGTCTTTAGCGCCCA
CATAGTGTGACTGAAAGGAGAGGGGCCCGAATTC
```

Figure 5. Nucleotide Sequence of cDNA Encoding Bovine QPs1 and Its Deduced Amino Acid Sequence. ([ ], leader sequence; underlined, NH_2-terminus amino acid sequence determined by peptide sequencing; **bold,** added nucleotide and amino acid sequence in the longer cDNA clone; ( ), missing leader sequence of our clone; number, amino acid number of mature QPs1)

for QPs1 isolated in our laboratory is a partial clone; it contains a complete sequence for mature QPs1, but lacks some amino acid residues of the leader peptide of QPs1. In fact, the isolated QPs1 clone contains only two codons for amino acid residues, Proline (P) and Valine (V), from the COOH-terminal end of the leader peptide.

Presequence of QPs1

In order to study the presequence of QPs1, a complete cDNA clone for QPs1 which encodes mature QPs1 and presequence is required. A longer cDNA clone for QPs1 was available in our laboratory making it a likely candidate containing the complete presequence of QPs1. Therefore, the 5' end region of this cDNA insert was sequenced using a synthetic squencing primer, GCACGCGGAAGAAGGCACATGGCT, which matches with the position 981-1004 on the λgt11 sequence. The clone is 1003 bp long. This longer QPs1 cDNA clone does not have the methione start coding sequence (ATG) between the 5' end start of cDNA insert and the the NH_2-terminus coding sequence of mature QPs1 (shown in Fig. 5), indicating that this is also an incomplete cDNA clone for QPs1. Before proceeding to isolate a complete cDNA clone for QPs1 from the cDNA library from other vendors, the presequence of QPs1 was reported from Dr. Capaldi's laboratory (7). There are five codons missing from the presequence obtained from our laboratory.

Figure 6 compares the leader peptide of QPs1 with those of SQR subunits from other eukaryotic species. The reported presequences of mitochondrial SQR subunits have a typical mitochondrial signal sequence. They contain a preponderance of basic (Arg and

MLSLKKSALSKLTLLRNTRTFTSSALVR	S.cerevisiae SDH1 (10)
MLNVLLRRKAFCLVTKKGM	S.cerevisiae SDH2 (11)
MSAMMVKLGLNKSALLLKPSAFSRAAALSSSRRLLFNTARTNFLSTSPLK	S.cerevisiae SDH3 (12)
MMLPRSMKFMTGRRIFHTATVARAFQSTAKKS	S.cerevisiae SDH4 (13)
MSGVRGLSRLLSARRLALAKAWPTVLQTGTRGFHFTVDGNKRA	Human liver Fp (14)
-----WRTCLQASRG	Human liver Ip (15)
MSGVAAVSRLCARPALALTCTKWSAAWQTGTRSFHFTVDGNKRS	Bovine heart Fp (16)
(MAALL)LRHVGRHCLRAHLSPQLCIRNAVP	Bovine heart QPs1 (7, this chapter)

Figure 6. Comparison of Amino Acid Sequence of Presequences From Different Eukaryotic Sources. (Reference numbers are indicated.)

Lys) and hydroxylated (Ser and Thr) residues and lack acidic residues (Glu and Asp) (8). It was suggested that mitochondrial membrane proteins, which contain the consensus sequence for cleavage by the matrix processing protease in their presequence, follow the two-step cleavage processing for mitochondrial targeting, and those which contain argine at the -2 upstream position follow a single-step cleavage processing. The consensus sequence is RX(F)XX(S), where R is arginine; X is any amino acid; (F) is phenylalanine or other hydrophobic residues; and (S) is serine, threonine, or glycine. Since all four *S.cerevisiae* SQR peptides have the consensus sequences, these proteins may follow the two-step cleavage processing for mitochondrial targeting. Human liver mitochondrial Fp and Ip subunits of SDH, and bovine heart Fp have arginine at the -2 upstream position (9) and thus follow single-step cleavage. QPs1 is more likely to follow the two-step cleavage because its presequence has a similar consensus sequence (RAHLS) and no arginine at the -2 upstream position.

References

1. Yao, Y., Wakabayashi, S., Matsuda, S., Matsubara, H., Yu, L., & Yu, C. A. (1986) in Iron-Sulfur Protein Research (Matsubara, H., Katsube, T., and Wada, K., eds) pp. 240-244, Japan Scientific Societies Press, Tokyo.
2. 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.
3. Yu, L., Wei, Y.-Y., Usui, S. & Yu, C. A. (1992) *J. Biol. Chem.* **267**, 24508-24515.

4. LeGendre, N., Mansfield, M., Weiss, A., & Matsudaira, P. (1993) A Practical Guide to Protein and Peptide purification for Microsequencing, second edition, pp. 71-82. Academic Press.

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

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

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

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

9. Brand, U., Yu, L., Yu, C.A., and Trumpower, B. L. (1993) *J. Biol. Chem.*, **268**, 8387-8390.

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

11. Hirawake, H., Wang, H., Kuramochi, T., Kojima, S., and Kita, K. (1994) *J. Biochem.*, **116**, 221-227.

12. Kita, K., Oya, H., Gennis, R. B., Ackrell, B. A. C., and Kasahara, M. (1990) *Biochem. Biophys. Res. Comm.* **166**, 101-108.

13. Birch-Machin, M. A., Farnsworth, L., Ackrell, B. A. C., Cochran, B., Jackson, S., Bindorff, L. A., Aitken, A., Diamond, A. G., & Turnbull, D. M. (1992) *J. Biol. Chem.*, **167**, 11553-11558.

14. Johnson, K. S., Harrison, G. B. L., Lightowlers, M. W., O'Hoy, K. L., Cougle, W. G., Dempster, R. P., Lawrence, S. B., Vinton, J. G., Heath, D. D., and Rickard, M. D. (1989) *Nature*, **338**, 585-587.

15. Strandberg, L., and Enfors, S.-O. (1991) *Appl. Environ. Micro.*, **57**, 1667-1674.

16. Hartman, J., Daram, P., Frizzell, R., Rado, T., Benos, D. J., and Sorscher, E. J. (1992) *Biotech. Bioenerg.*, **39**, 828-832.

CHAPTER II

IDENTIFICATION OF THE UBIQUINONE-BINDING DOMAIN IN QPs1 OF SUCCINATE-UBIQUINONE REDUCTASE

The goal of this thesis work is to understand the Q-mediated electron transfer reaction in mitochondrial SQR. The active species of Q in electron transfer is proposed to be a protein-bound Q entity. The presence of Q binding protein(s) in SQR is evidenced by the detection of a TTFA-sensitive ubisemiquinone radical in the complex and isolation of QPs which converts soluble SDH to a TTFA-sensitive succinate-Q reductase. The isolated QPs contains three protein subunits, QPs1, QP2, and QPs3, but it is not known which of the subunit(s) is responsible for the Q-binding function of QPs.

The availability of azido-Q derivatives in our laboratory allows us to study the Q:protein interaction and to identify the Q-binding protein in SQR by the photoaffinity labeling approach. Furthermore, if QPs1 is identified as the Q-binding protein in the complex by the photoaffinity labeling technique, the availability of the amino acid sequence of QPs1 (Chapter I) will facilitate our identification of the Q binding domain in this subunit.

The successfulness in isolation of the azido-Q linked peptide(s) from an azido-Q labeled protein relies on the susceptibility of isolated protein toward proteolytic enzyme digestion and separation of proteolytic peptides by HPLC. In this charpter I report the

detailed conditions for photoaffinity labeling of Q-binding protein(s) in SQR with the azido-Q derivative, and the detailed isolation procedure for the Q-binding domain in QPs1.

Experimental Procedures

Materials

Bovine serum albumin, DCPIP, Triton X-100, sodium cholate, TTFA, and potassium deoxycholate were obtained from Sigma. Molecular weight standards and urea were from BioRad. n-Dodecyl-β-D-maltoside was from Anatrace. Silica gel 1B-F (2.5 x 7.5 cm) was from J. T. Baker. Insta-gel liquid scintillation cocktail was from Packard Instrument Co. Other chemicals were of the highest purity commercially available.

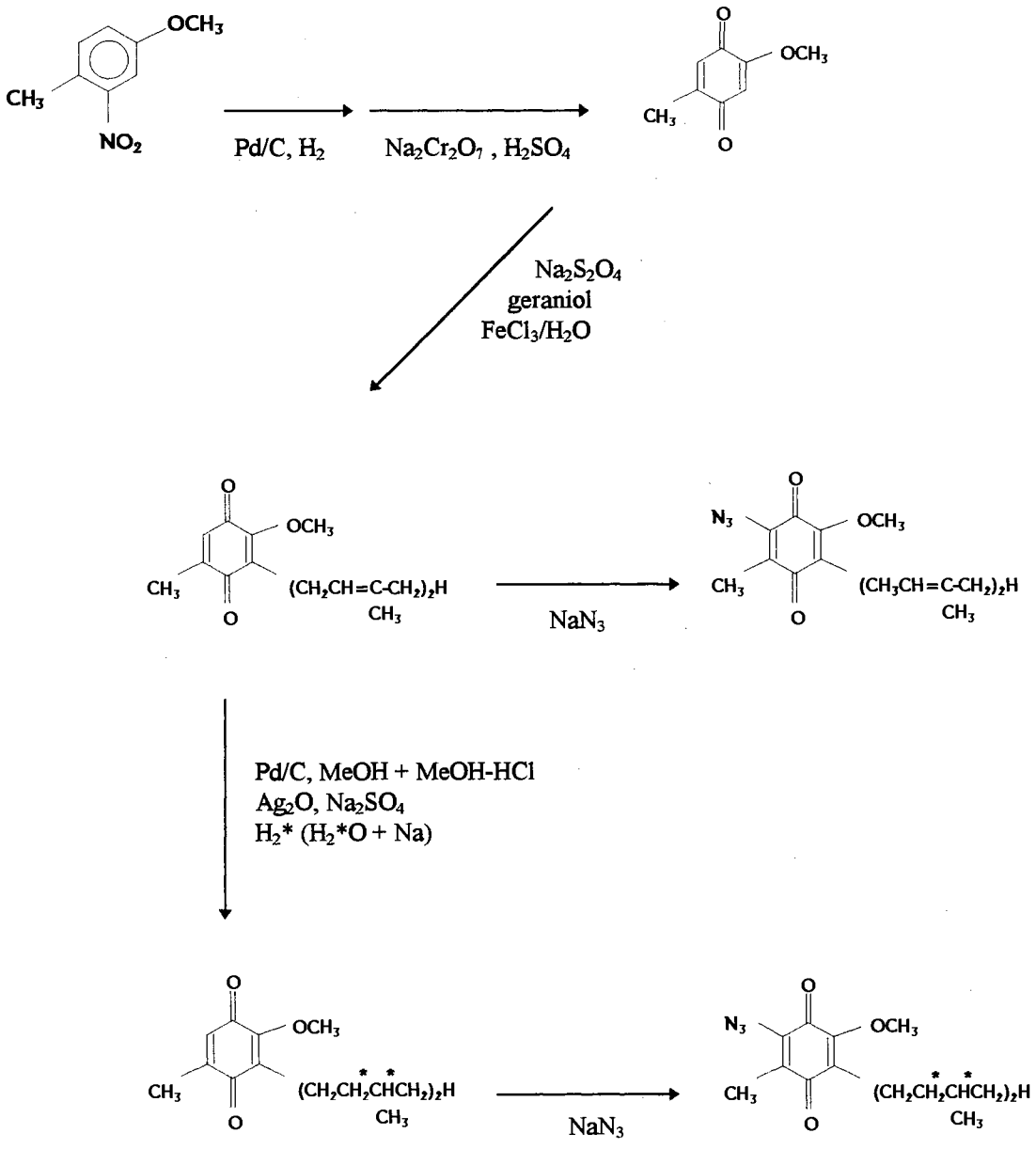
The ubiquinone derivatives, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (Q_0C_{10}), 3-azido-2-methoxy- and 3-azido-2-methoxy[^{3}H]-5-methyl-6-decyl-1,4-benzoquinones (3-azido-Q_0C_{10} and [^{3}H]-3-azido-Q_0C_{10}), and 5-azido-2,3-dimethoxy- and 5-azido-2,3-dimethoxy[^{3}H]-6-decyl-1,4-benzoquinones (5-azido- Q_0C_{10} and [^{3}H]-5-azido-Q_0C_{10}) were synthesized according to methods reported previously (1). Calcium phosphate was prepared according to Jenner (2) 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-Dimethyl[^{3}H]-Octyl)-1,4-Benzoquinones (Azido-Q and [^{3}H]Azido-Q)

Scheme I summarizes the procedure for synthesis of azido-Q and [^{3}H]azido-Q. Azido-Q and [^{3}H]azido-Q was synthesized basically according to the method reported (1, 3). Six-g of 4-methyl-3-nitroanisole (3) were mixed with 1g of 10% Pd-C in 95ml of methanol. After addition of 5ml 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 600ml of cold 0.5 M sulfuric acid. Then 250ml of a cold solution containing 27g sodium dichromate and 20 ml concentrated sulfuric 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 $CHCl_3$ 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 $CHCl_3$, 0.5 g of yellow crystals of 2-methyl-5-methoxy-1,4-benzoquinone were obtained.

Sixty-mg of 2-methoxy-6-methyl-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 $BF_3O(C_2H_5)_2$ over 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

Scheme I. Synthesis of 3-Azido-2-Methyl-5-Methoxy-6-Geranyl-1,4-Benzoquinone and 3-Azido-2-Methyl-5-Methoxy-6-(3,7-Dimethyl[^{3}H]-Octyl)-1,4-Benzoquinone.



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 in the thin-layer plate. 2-Methyl-5-methoxy-6-geranyl-1,4-benzoquinone was eluted with ether and upon removal of solvent, 9 mg of orange oil were 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): oxi., 263 nm; red., 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 (1), 25 mg of 2-methyl-5-methoxy-6-geranyl-1,4-benzoquinone was dissolved in 1ml of acetic acid (90%), and incubated at 75 °C with constant stirring. The subsequent operations were performed in the dark. Three additions of sodium azide solution (17 mg sodium azide in 0.05 ml of water each) were made over a period of 3 hours. After the additions of sodium azide, 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 the 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). UV_{max} (95% ethanol): oxi., 306 nm; red., 298 nm.

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

hydrogen 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 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 hrs 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 57,300 cpm/nmol, which was determined in a Beckman liquid scintillation counter.

Enzyme Preparations and Assays

Bovine heart SQR was prepared from SCR by the method previously reported (4) except that succinate was omitted from all buffers used in the purification procedure. SQR containing fractions obtained from the second calcium phosphate-cellulose column were concentrated by 43% ammonium sulfate saturation and centrifugation at 48,000 x *g* for 20 min. The precipitate obtained was dissolved in 50 mM potassiun/sodium phosphate, pH 7.8, containing 0.2 % sodium cholate and 10 % glycerol.

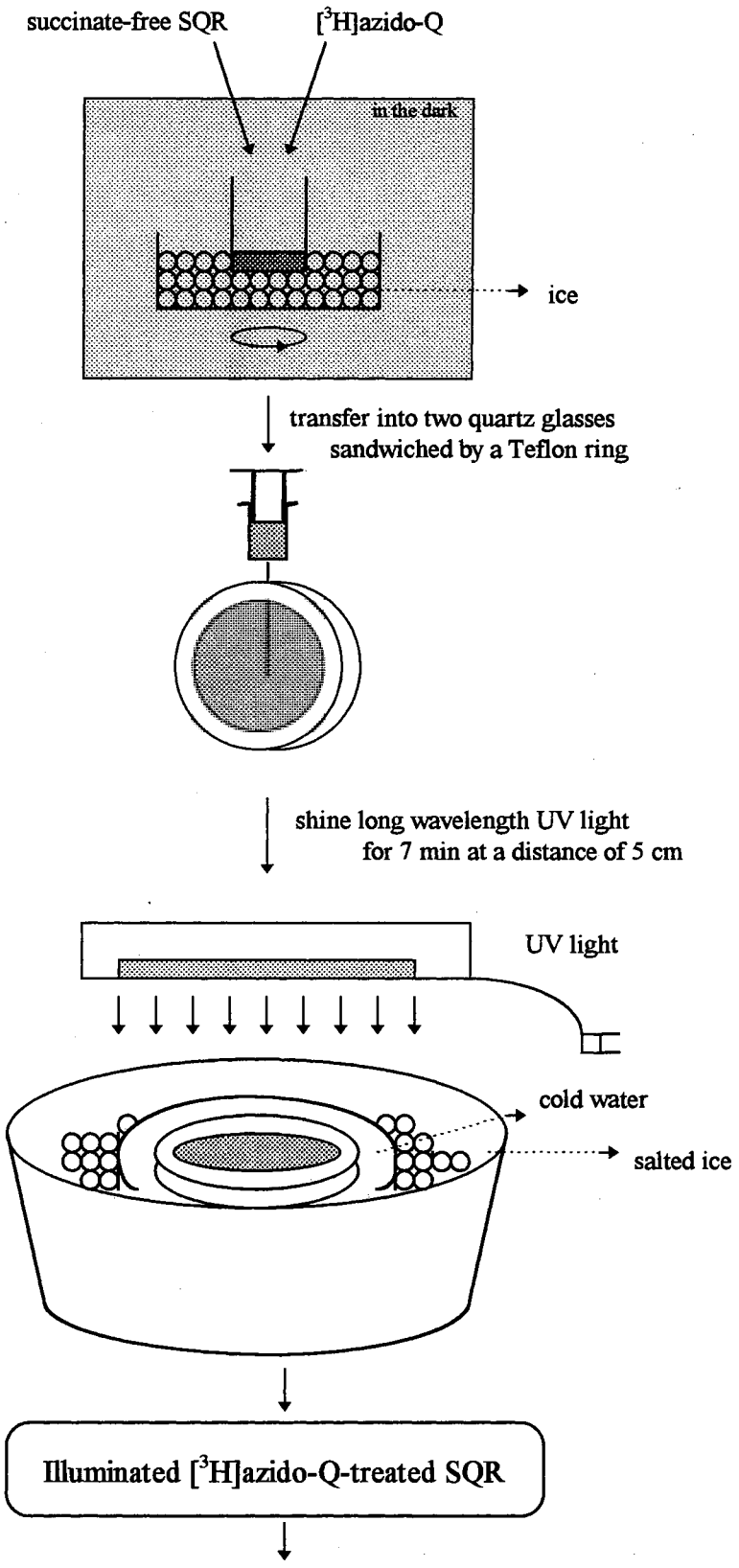
SQR activity was assayed for its ability to catalyze Q reduction or Q-stimulated DCPIP reduction by succinate. The assays were performed at room temperature in a Cary

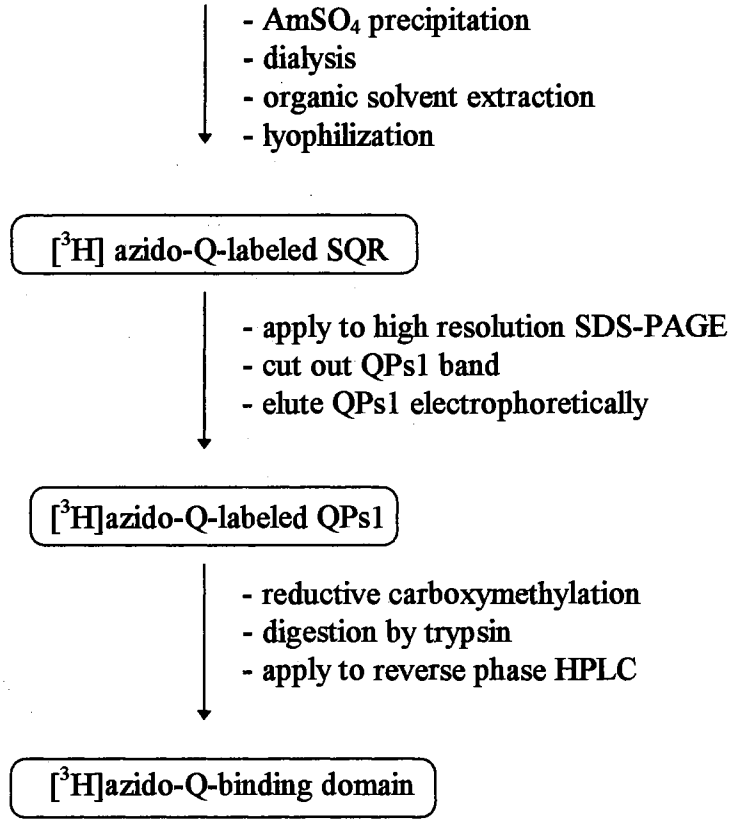
spectrophotometer (model 219) or Shimadzu UV-2101PC. The reaction mixture used for the Q reduction assay contains 100 μmol of sodium/potassium phosphate buffer, pH 7.4, 20 μmol of succinate, 10nmol of EDTA, 25 nmol of Q_0C_{10} and 0.01 % of *n*-dodecyl-β-maltoside in the total volume of 1 ml. The reduction of Q_0C_{10} was followed by measuring the absorption decrease at 275 nm, using a millimolar extinction coefficient of 12.25 $mM^{-1}cm^{-1}$. The reaction mixture used for the Q-stimulated DCPIP reduction assay contains 38 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 in the total volume of 1 ml. The reduction of DCPIP was followed by measuring the absorption decrease at 600nm, using a millimolar extinction coefficient of 21 $mM^{-1}cm^{-1}$.

Isolation of [^{3}H]Azido-Q labeled QPs1

A brief procedure from labeling of SQR with [^{3}H]azido-Q to isolation of Q-binding peptide is summarized in scheme II. 10ml of succinate-Q reductase, 1.5 mg/ml, in 50 mM sodium/potassium phosphate buffer, pH 7.8, containing 10 % glycerol and 0.2 % sodium cholate were incubated with 125 μl of [^{3}H]azido-Q (9.6 mM in 95 % ethanol) at 0 °C for 10 min in the dark. The specific radioactivity of azido-Q used was 1.6×10^4 in 95 % ethanol and 6.6×10^3 cpm/nmol in 50 mM potassium/sodium phosphate buffer, pH 7.8, containing 10 % glycerol and 0.2 % sodium cholate in the presence of SQR. This mixture was transferred to an illumination apparatus made from two quartz glasses sandwiched by a Teflon ring. The apparatus was immersed in cold water in a Petri dish surrounded with

Scheme II. Isolation of [^{3}H]-Azido-Q-Binding Peptide.




- $AmSO_4$ precipitation
- dialysis
- organic solvent extraction
- lyophilization
[^{3}H] azido-Q-labeled SQR
- apply to high resolution SDS-PAGE
- cut out QPs1 band
- elute QPs1 electrophoretically
[^{3}H]azido-Q-labeled QPs1
- reductive carboxymethylation
- digestion by trypsin
- apply to reverse phase HPLC
[^{3}H]azido-Q-binding domain

salted ice to maintain the temperature of the water at 0 °C. The sample was illuminated with long wavelength UV light for 7 min at a distance of 5 cm from the light source.

The illuminated [^{3}H]azido-Q-treated SQR was precipitated by 43 % ammonium sulfate saturation and centrifuged at 48,000 x *g* for 20 min. The precipitate was dissolved in 10 mM Tris-Cl buffer, pH 7.8, and dialyzed against double distilled water overnight with one change of water. The free Q or the phospholipid bound Q was extracted from the protein by organic solvent (1). The dialyzed sample (0.8ml) was mixed with 2 ml of methanol and 1 ml chloroform, and incubated at room temperature for 30 min with occasional shaking. After incubation, 1 ml of H_2O and 1 ml of chloroform were added and the mixture was kept at room temperature for another 10 min with occasional shaking. The chloroform layer was separated from the aqueous layer by centrifugation and discarded. The aqueous layer was extracted once more with 1ml chloroform and the chloroform layer was removed. The methanol in the aqueous layer was evaporated under a stream of nitrogen gas before the solution, which contained the photolyzed protein, was subjected to lyophilization. The lyophilized sample was suspended in 20 mM Tris-Cl, pH 8.0 containing 1 % SDS. β-mercaptoethanol was added to a final concentration of 1 % and the solution was incubated at 37 °C for 2 h before it was subjected to preparative SDS-PAGE. The SDS-PAGE gel was prepared according to Schägger *et al.* (5) except 8 M urea was used in the separating gel instead of 13 % glycerol. The SDS-β-mercaptoethanol treated sample was loaded onto 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 run at 15 V for 2 h and then

at 35 V for another 17 h with a Mini-protean II cell of Bio-Rad. The protein bands were visualized by fluorescence under UV. The SDS-PAGE pattern of the fluorescamine-treated sample was identical to that of the untreated sample as established by Coomassie Blue staining. The QPs1 protein band was excised from the SDS-PAGE gel and the protein was eluted with an electroeluter from Bio-Rad.

Reductive Carboxymethylation of Azido-Q-Labeled QPs1

The QPs1 protein solution was subjected to reductive carboxymethylation by a modification of the method reported previously (6). The electrophoretically eluted QPs1 protein solution was concentrated to a protein concentration of about 3 mg/ml by membrane filtration using Centricon-10 and precipitated with 50 % cold acetone (-20°C). The protein precipitate was suspended in 1 ml of cold water and centrifuged at 100,000 x *g* for 1 hr to remove the trace of acetone remaining in the protein. The precipitate thus obtained was homogenized in 0.1 M Tris, 6 M guanidine HCl, 1 mM EDTA, pH 8.3. Dithiothreitol was added to the suspension to a final concentration of 2 mM. The solution was made anaerobic by passing argon through it and then incubated at 37 °C for 1 h. Iodoacetic acid, neutralized with NaOH, was added to give a final concentration of 5 mM. Argon was flushed over the surface of the reaction mixture, the vessel was sealed, and alkylation was allowed to go on in the dark at 37 °C for 1 h. Complete alkylation was achieved by an additional treatment with 1 mM dithiothreitol and incubation with 2 mM iodoacetate under the same conditions. The alkylated protein solution was treated with β-mercaptoethanol to a final concentration of 1 % and dialyzed

against 50 mM ammonium bicarbonate, pH 8.5 overnight at 4 °C with one change of buffer.

Trypsin Digestion of the Carboxymethylated Azido-Q-Labeled QPs1

The carboxymethylated QPs1 protein was collected from the dialyzed sample by centrifugation at 100,000 x *g* for 30 min. The collected precipitates were suspended in 0.1 M ammonium bicarbonate-0.1 mM $CaCl_2$ to a final protein concentration of 1mg/ml and digested with trypsin at 37 °C for 2 hr using a trypsin:QPs1 ratio of 1:50 (w/w). After a 2 hr incubation, a second addition of trypsin was made (1:100) and the incubation continued at 37 °C for 22 h.

Isolation of Ubiquinone-Binding Peptides

One hundred-microliter aliquots of the trypsin-digested QPs1 were separated by high performance liquid chromatography (HPLC) on a Synchropak RP-8 column (0.46 x 25 cm) using a gradient formed from 0.1 % trifluoroacetic acid and 90 % acetonitrile containing 0.1% trifluoroacetic acid with a flow rate of 1 ml/min. One-milliliter fractions were collected. The absorbance at 214 nm and the radioactivity of each fraction were measured.

Amino Acid Sequence Determination

Amino acid sequence 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

Preparation of SQR

Succinate, the substrate for SDH, has been routinely included in the preparation of SQR to stabilize the enzyme complex. However, the succinate-containing SQR preparation is not suitable for studying Q:protein interactions using the azido-Q derivative because in the presence of succinate, the azido-Q derivative is slowly reduced by SQR to aminoquinol, thus losing its photoaffinity labeling ability. Although the rate of conversion of azido-Q to aminoquinol by SQR in the presence of succinate is very slow, the reaction is complete within 20 min at 0 °C in the dark when the molar ratio of azido-Q to SQR in the system is less than 50. The conversion of azido-Q to aminoquinol was evident from the change of absorption characteristics and of mobility on TLC. Upon reduction, a purple spot with the R_F value of 0.57 (aminoquinol) was observed with concurrent disappearance of a yellow spot with the R_F value of 0.90 (azido-Q), on a TLC plate developed with a hexane:ether (2.5 :1) mixture. When a succinate-free SQR was incubated with 10-fold molar excess of azido-Q, no purple spot was observed on the TLC plate after 20 min incubation at 0 °C in the dark.

The succinate-free, SQR was prepared by replacing Tris-succinate buffer with Tris-Cl buffer during the separation of SQR from QCR in Triton X-100 treated succinate-cytochrome c reductase. Succinate was omitted from the subsequent buffers used in calcium phosphate column chromatography.

It has been shown that the interaction of azido-Q derivatives with the cytochrome *b*-*c*1 complexes from various sources (1, 7, 8) requires prior removal of endogenous Q from the complex. The reason for this is that the binding affinity of azido-Q derivatives to the Q-binding proteins (sites) is weaker than that of endogenous Q_{10}. The succinate-Q reductase, prepared according to the procedure described (4), is 40-50 % deficient in Q content. The Q deficiency in SQR preparations is measured by the extent of activity increase of Q-stimulated DCPIP reduction by succinate in the presence of Q_0C_{10} (25 μM) in the assay mixture. Attempts to further remove Q from SQR preparations by prolonged washing of the 2nd calcium phosphate: cellulose column or by repeating the ammonium sulfate fractionation in the presence of 0.5 % sodium cholate, were unsuccessful. Therefore, a partially Q-deficient, succinate-free SQR was used in this study.

Properties of Azido-Q derivatives

Three azido-Q derivatives, 3-azido-Q_0C_{10}, 5-azido-Q_0C_{10}, and azido-Q (Fig. 7), were synthesized and tested for their suitability for identifying Q-binding sites in succinate-Q reductase. All these derivatives exhibit partial electron acceptor activity for succinate-Q reductase. The maximal activity was 35, 23, and 8 % (Fig. 8) for 5-azido- Q_0C_{10}, 3-azido-Q_0C_{10}, and azido-Q, respectively, compared with Q_0C_{10}.

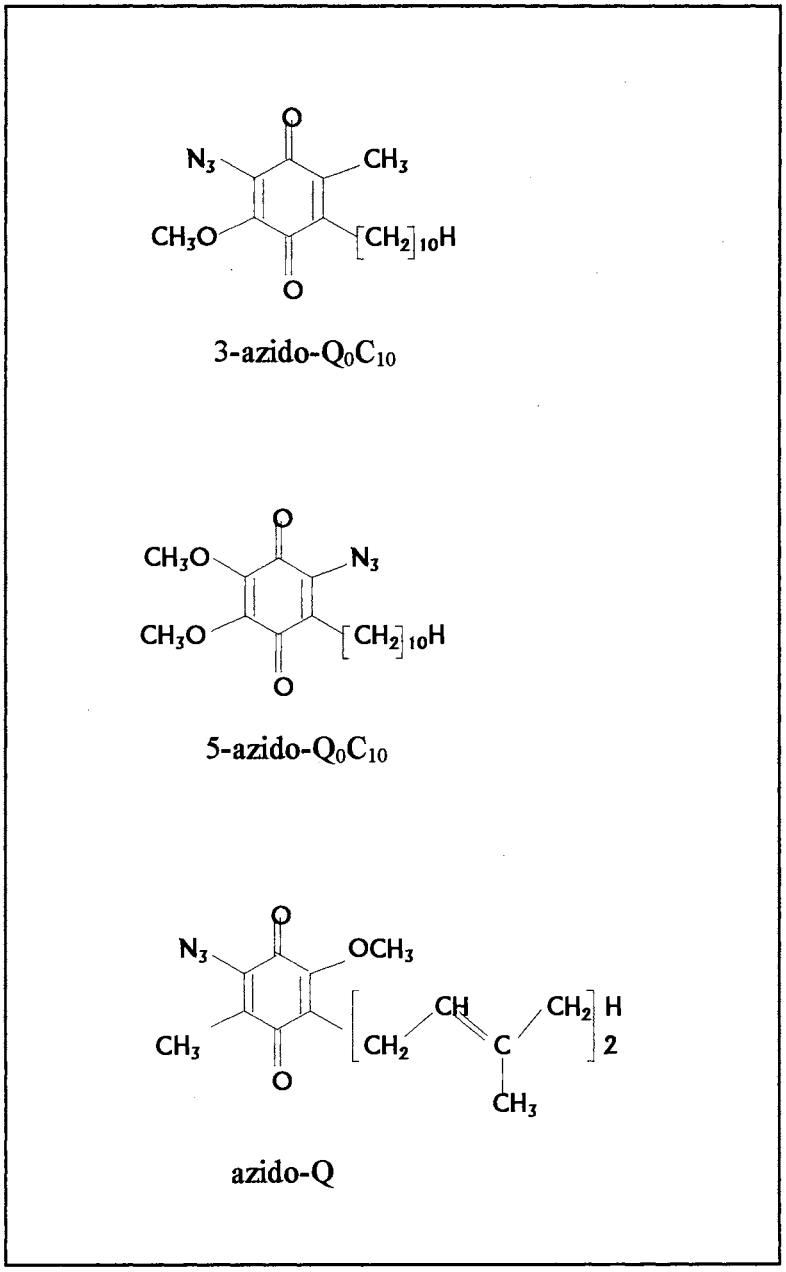


Figure 7. Structures of Azido-Quinone Derivatives.

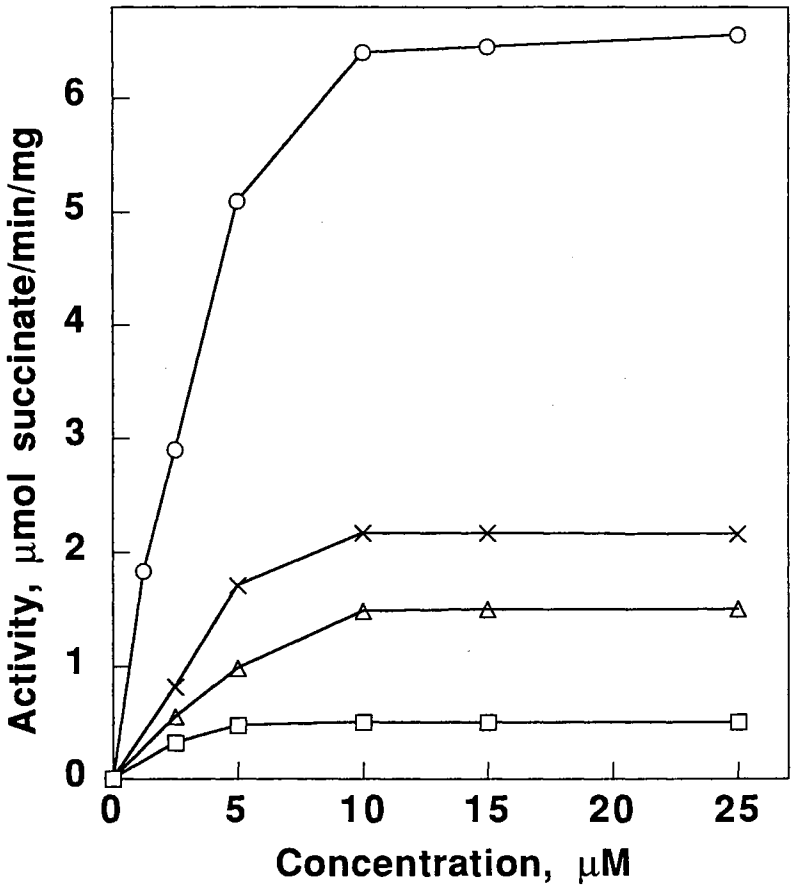


Figure 8. Titration of Succinate-Q Reductase Activity with Q Derivatives. 1 ml assay mixture conatins 100μmol of sodium/potassium phosphate buffer, pH 7.4, 20 μmol of succinate, 10 nmol of EDTA, 0.01 % of n-dodecyl-β-maltoside, and indicated amounts of Q_0C_{10}(○), 5-azido-Q_0C_{10} (x), 3-azido-Q_0C_{10} (Δ), and azido-Q (□). The reaction was started with the addition of succinate-Q reductase. The reduction of Q derivatives was followed by the absorbtion decrease at 275 nm, 315 nm, 289 nm, and 304 nm for of Q_0C_{10}, 5-azido-Q_0C_{10}, 3-azido-Q_0C_{10}, and azido-Q, respectively. A millimolar extinction coefficient of 12.25 was used for the oxidized minus reduced form of Q-derivatives.

In order to use these azido-Q derivatives to identify the Q-binding proteins and to facilitate the study of the Q:protein interaction, it is necessary to synthesize them in a radioactive form. [^{3}H]-3-azido-Q_0C_{10} and [^{3}H]-5-azido-Q_0C_{10} were prepared by methylation of the hydroxyl group(s) on 3-H-2-hydroxy-5-methyl- and 5-H-2,3-dihydroxy-6-decyl-1,4-benzoquinones, respectively, with C[^{3}H]$_3$I before being subjected to nucleophilic substitution with NaN_3. And [^{3}H]-azido-Q was obtained by hydrogenation of geranyl group on 3-H-2-methyl-5-hydroxy-6-geranyl-1,4-benzoquinones with tritium before azidolization. These [^{3}H]azido-Q derivatives have the same electron acceptor activity for SQR as the unlabeled compounds (Table II). However, when SQR was incubated with 40 molar excess of these [^{3}H]azido-Q derivatives for 10 min at 0 °C in the dark prior to illumination with a long wavelength UV light for 7 min, only the [^{3}H]azido-Q treated sample showed inactivation and radioactivity uptake by protein (Table II), indicating that this [^{3}H]azido-Q derivative is suitable for studying the Q:protein interaction in SQR. The failure to detect 3-azido- Q_0C_{10} and 5-azido- Q_0C_{10} uptake by SQR during illumination is most likely due to intramolecular cyclization between the generated nitrene and its neighboring methoxy or methylene group on the Q molecule, during illumination. This [^{3}H]azido-Q has been successfully used to identify the Q-binding site in cytochrome *b*-*c*1 complexes from several sources (1, 7, 8).

Effect of Azido-Q Concentration on SQR Activity After Illumination

When SQR was incubated with various concentrations of [^{3}H]azido-Q and illuminated, the activity decreased as the concentration of azido-Q increased. Maximum

TABLE II

ELECTRON ACCEPTOR AND PHOTOAFFINITY LABELING ACTVITIES OF [^{3}H]AZIDO-Q DERIVATIVES FOR SUCCINATE-Q REDUCTASE

[^{3}H]-Azido-Q derivatives	Electron acceptor activity for SQR[a]	SQR treated with [^{3}H]Azido-Q derivatives [b]		
		Activity		Radioactivity uptake by protein upon illumination
		-hv	+hv	
	μmol succ./min/mg	μmol succ./min/mg		cpm/mg
Q_0C_{10}	7.10			
3-N_3-2-C[^{3}H]$_3$O-5-CH_3-6-decyl-1,4-benzoquinone	1.58	7.10	7.08	0
5-N_3-2,3-di C[^{3}H]$_3$O-6-decyl-1,4-benzoquinone	2.53	7.05	7.01	0
3-N_3-2-CH_3-5-CH_3 O-6-(3,7-di C[^{3}H]$_3$-octyl)-1,4-benzoquinone	0.62	7.08	4.21	5.73×10^4

[a] 25 μM of indicated Q-derivatives were included in the assay mixture for succinate-Q reductase (SQR).

[b] 200-μl aliquots of succinate-Q reductase, 1.5 mg/ml, in 50 mM phosphate buffer, pH 7.8, containing 0.2% sodium cholate and 10% glycerol, were mixed with 5 μl of [^{3}H]azido-Q derivatives, 13.2 mM in 95% ethanol. These [^{3}H]-azido-Q-treated succinate-Q reductase preparations were incubated in the dark for 10 min prior to illumination. Aliquots were withdrawn from [^{3}H]azido-Q treated samples, before and after illumination, for enzymatic and radioactivity uptake assays. The activity and radioactivity uptake were performed as described in "Experimental Procedures". The radioactivities for 3-N_3-2-C[^{3}H]$_3$O-6-decyl-1,4-benzoquinone, 5-N_3-2,3-di-C[^{3}H]$_3$O-5-CH_3-6-decyl-1,4-benzoquinone, and 3-N_3-2-CH_3-5-CH_3O-6-(3,7-di C[^{3}H]$_3$-octyl)-1,4-benzoquinone were 5.9×10^3, 1.3×10^2, and 6.6×10^3cpm/nmol, respectively, in 50 mM phosphate buffer, pH 7.8, containing 0.2% sodium cholate and 10% glycerol in the presence of succinate-Q reductase.

inactivation of approximately 35 % was obtained when 40 mol of azido-Q/mol of FAD was used (Fig. 9). Inactivation was not due to inhibition of SQR by photolyzed products of azido-Q because, when azido-Q was photolyzed in the absence of reductase and then added to SQR, no inhibition was observed. The maximum inactivation of 35%, which is in close agreement with the 40-50 % deficiency of Q in the preparation, suggests that the affinity of endogenous Q for its binding site is much stronger than that of the azido-Q derivative. Since SQR activity is assayed in the presence of excess Q_0C_{10} (25 μM), the extent of inactivation of the azido-Q-treated SQR, after illumination, is a measure of the fraction of Q-binding sites covalently linked to azido-Q.

If azido-Q can occupy 35 % of the Q-binding sites in the reductase, one would expect to see a decrease in the activity of the azido-Q-treated sample before illumination, because this derivative has less than 10 % of the electron-transfer efficiency of Q_0C_{10}. The failure to observe a decrease in activity prior to illumination may be due to the fact that the concentration of Q_0C_{10} in the assay mixture is several orders of magnitude higher than that of azido-Q. Thus Q_0C_{10} can easily displace azido-Q from the binding sites, and the inferior electron-transfer activity of azido-Q is not expressed. After illumination, the covalently linked azido-Q cannot be displaced by Q_0C_{10} and inhibition occurs.

Correlation Between Azido-Q Incorporation and Inactivation of SQR

Figure 10 shows the effect of illumination time on the inactivation of, and azido-Q incorporation into, SQR. When azido-Q-treated SQR was illuminated at 0°C for various lengths of time, activity decreased as illumination time was increased; maximum

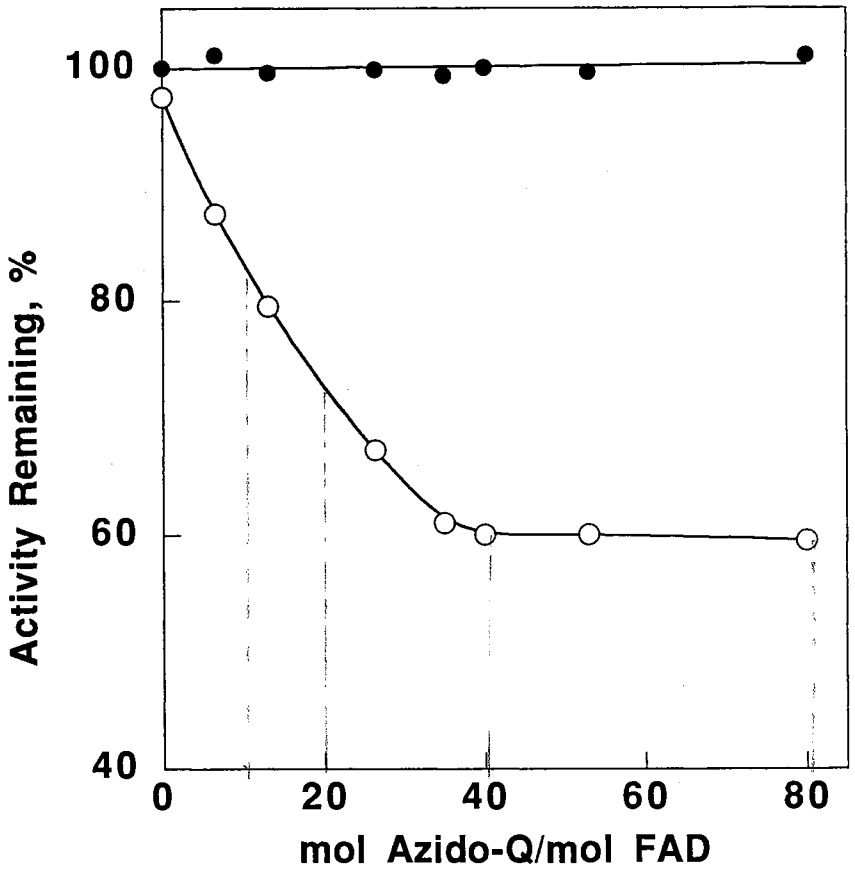


Figure 9. Effect of Azido-Q Concentration on Succinate-Q Reductase Activity After Illumination. Aliquots (0.2 ml) of succinate-Q reductase, 1 mg/ml (5.5 μM FAD), in 50 mM phosphate buffer, pH 7.8, containing 10% glycerol and 0.2% sodium cholate were mixed with 5 μl of an alcoholic solution of azido-Q derivative (concentrations indicated), in the dark. After incubation at 0 °C for 10 min, the samples were illuminated for 7 min at 0 °C. Succinate-Q reductase activity was assayed before(●)and after (○) illumination. 100% activity is succinate-Q reductase without treatment with azido-Q and without illumination.

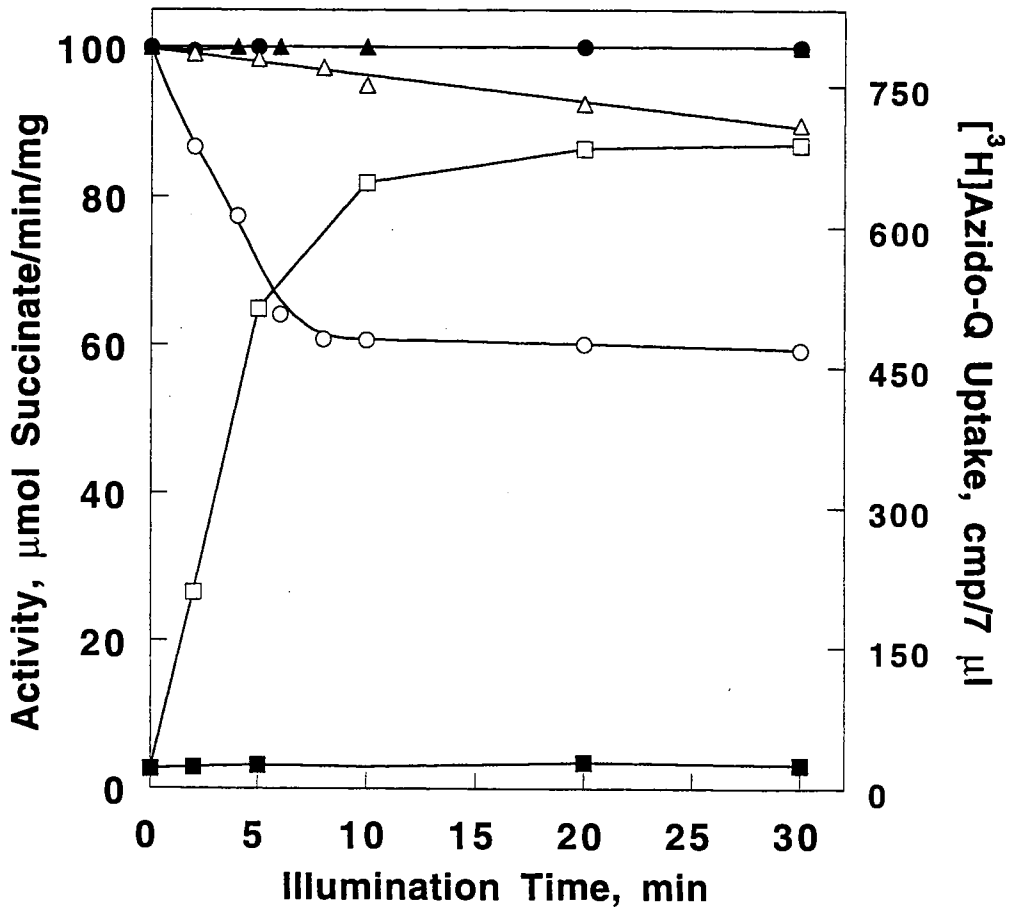


Figure 10. Effect of Illumination Time on Azido-Q Uptake and Inactivation of SQR. Aliquots (0.4 ml) of SQR, 1.5 mg/ml, in 50 mM phosphate buffer, pH 7.8, containing 10 % glycerol and 0.2 % sodium cholate were mixed with 10 μl of ethanol (control) and 10 μl of [^{3}H]azido-Q (13.5 mM in 95 % ethanol) in the dark. After incubating for 10 min at 0oC in the dark, samples treated with [^{3}H]azido-Q (●,○) and controls (▲, Δ) were divided into two equal portions: one was placed in the quartz cuvette and wrapped with aluminium foil (● , Δ) and the other was placed in the quartz cuvette (○, Δ) with 0.2-cm light path. All samples were put in the same photolysis chamber and illuminated with long-wavelength UV light. The temperature in the photolysis chamber was maintained at 0 °C. At indicated time intervals, 2-μl aliquots were withdrawn and assayed for SQR activity. To determine the radioactivity incorporation of [^{3}H]azido-Q into the protein, aliquots (7-μl) of [^{3}H]azido-Q treated sample, with (□) and without (■) illumination, were spotted on Whatman No.3 paper in the dark. When all samples were spotted and dried, the paper was developed with chloroform/methanol (2:1, v/v). Proteins at their original spots were cut out and placed in scintillation vials containing 5 ml of Insta-Gel. Radioactivity was determined with a Packard Model 1900 CA Tri-Carb liquid scintillation analyzer.

inactivation (35%) was reached in 7 min. The amount of azido-Q uptake by protein paralleled the extent of inactivation until the maximum was reached, indicating that inactivation results from binding of azido-Q to the ubiquinone binding site. Although illuminating for longer than 7 min caused no further decrease in activity, azido-Q uptake continued, but at a much slower rate, suggesting that this slower incorporation is due to nonspecific binding of azido-Q to proteins. The photoinactivation rate of succinate-Q reductase was affected by the protein, alcohol, and detergent concentrations in the system. Interaction with azido-Q derivative was most effective (*i.e.*, showed the greatest degree of inactivation after illumination) when the reaction system contained SQR, 1-1.5 mg/ml, sodium cholate, 0.2%, and alcohol, <3%.

Radioactivity Distribution of Azido-Q among Subunits of SQR

Since the uptake of the azido-Q derivative by SQR during illumination correlated with the enzymatic inactivation, it is reasonable to assume that the azido-Q derivative was bound specifically to the Q-binding site(s). Thus, the distribution of the covalently bound azido-Q among the subunits of SQR after SDS-PAGE should indicate the specific Q-binding polypeptide in this enzyme complex. Figure 11 shows the distribution of ^{3}H radioactivity among subunits of SQR. Prior to SDS-PAGE the reductase was extracted with organic solvent to remove most of the non-protein-bound azido-Q, such as free azido-Q or detergent-azido-Q or lipid-azido-Q adducts. The SQR contained 0.16 mol of azido-Q/ mol cytochrome b_{560}. Radioactivity was found predominately in QPs1, suggesting that this protein provides the Q-binding site in this segment of the electron-

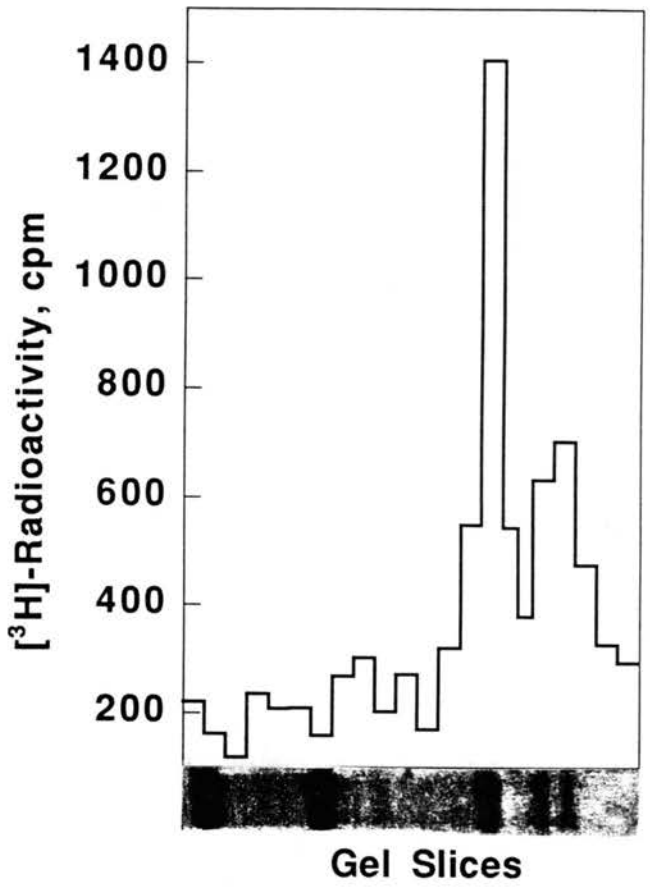


Figure 11. Distribution of Radioactivity Among Subunits of Succinate-Q Reductase. 0.2 ml of succinate-Q reductase, 1.5 mg/ml, in 50 mM phosphate buffer, pH 7.8, containing 10% glycerol and 0.2% sodium cholate were mixed with 10 μl of [^{3}H]azido-Q (13.5 mM in 95% ethanol) in the dark. After incubation at 0 °C for 10 min, the sample was illuminated for 7 min at 0 °C. The illuminated sample was extracted with a mixture of chloroform/methanol (2:1) and lyophilized. The lyophilized sample was dissolved in 20 mM Tris-Cl buffer, pH 8.0, containing 1% SDS and 1% β–mercaptoethanol and incubated at 37 °C for 2 hrs. The digested sample was treated with fluorescamine and applied to SDS-PAGE gel. The protein bands were visualized by fluorescence under UV and sliced. The portion containing no protein was also sliced to the same size as that of protein bands. The gel slices were smashed and mixed with 5 ml of Insta-Gel, and the radioactivity was determined.

transfer chain. Some radioactivity was found in QPs2 and QPs3; about 45% and 50% of that observed in QPs1. No radioactivity was found in the subunits of succinate dehydrogenase. Since the amount of radioactive labeling in QPs1 was proportional to the extent of inactivation of the reductase, participation of QPs1 in Q-binding is established.

Although lesser amounts of radioactivity were found in QPs2 and QPs3, involvement of these two subunits in formation of the Q-binding site of SQR cannot be ruled out. The smaller azido-Q uptake by QPs2 and QPs3, compared to QPs1, may result from portions of the Q-binding sites being contributed by these two subunits, or from the preferable occupation of these two subunits by the endogenous Q present in the partially Q-deficient SQR used in this investigation. The role of QPs2 and QPs3 in Q-binding needs further investigation. The availability of a reconstitutively active, Q-depleted SQR, and of the amino acid sequences of QPs2 and QPs3, would greatly help in the assessment of the involvement of QPs2 and QPs3 in Q-binding of SQR.

Preparation and Properties of Reductive Carboxymethylated, Azido-Q-labeled QPs1

In order to facilitate the identification of the Q-binding domain in QPs1 through isolation and sequencing of an azido-Q-linked peptide, it is absolutely necessary to have isolated azido-Q-labeled QPs1 free from contamination with unbound azido-Q and completely susceptible to proteolytic enzyme digestion. The [^{3}H]azido-Q-labeled-QPs1 was isolated from illuminated, [^{3}H]azido-Q-treated SQR by a procedure involving ammonium sulfate fractionation, dialysis, organic solvent extraction, lyophilization, preparative SDS-PAGE, electrophoretic elution, and cold acetone precipitation. Three

steps in the isolation procedure: organic solvent extraction, SDS-PAGE, and 50% cold acetone precipitation, are used to remove non-protein-bound azido-Q, some of which occurs as adducts with detergent or phospholipid. The electrophoretically eluted QPs1 shows only one band in SDS-PAGE (see lane 1 of Fig. 12). The SDS, free azido-Q, and detergent-azido-Q adducts present in eluted QPs1 preparation were removed by the acetone precipitation step. About 35% of the QPs1 protein present in SQR was recovered in the final purification step, assuming that the molecular mass of succinate-Q reductase is 136 kDa and that it contains 1 mol of QPs1 per mol of reductase. This relatively low yield of QPs1 is probably due to a very thin slicing of the QPs1 band from SDS-PAGE gel to avoid contamination with QPs2 protein.

The isolated azido-Q labeled QPs1 was highly aggregated and resistant to proteolytic digestion. Inclusion of 0.1% SDS and 2 M urea in the digestion mixture did not increase proteolytic efficiency. Modification of isolated azido-Q-labeled QPs1 protein by reductive carboxymethylation rendered the protein susceptible to trypsin and chymotrypsin digestion. The reductive carboxymethylation step was carried out in 50 mM Tris-Cl buffer, pH 8.3, containing 6 M guanidinium chloride. Guanidinium chloride (6 M) was found to be more effective than 50% N, N-dimethylformamide (6) in denaturation and in reduction of QPs1 with dithiothreitol. The modified isolated QPs1 contained 0.16 mol of azido-Q/ mol cytochrome b_{560}.

Isolation and Characterization of Ubiquinone-binding Peptides of QPs1

Figure 13 shows the distribution of radioactivity among the tryptic peptides of

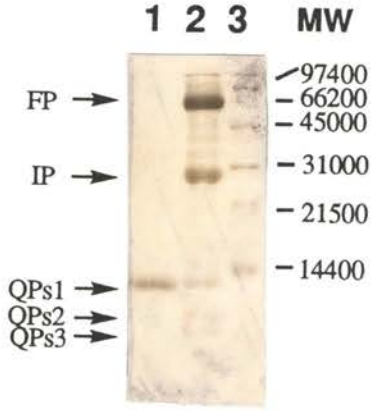


Figure 12. SDS-PAGE of Purified [^{3}H]Azido-Q-Labeled QPs1. *Lane* 1, electrophoretically eluted QPs1; *lane* 2, succinate-Q reductase, and *lane* 3, molecular weight standards (phosphorylase *b*, 97400; bovine serum albumin, 66200; ovalbumin, 45000; carbonic anhydrase, 31000; soybean trypsin inhibitor, 21500; and lysozyme, 14400).

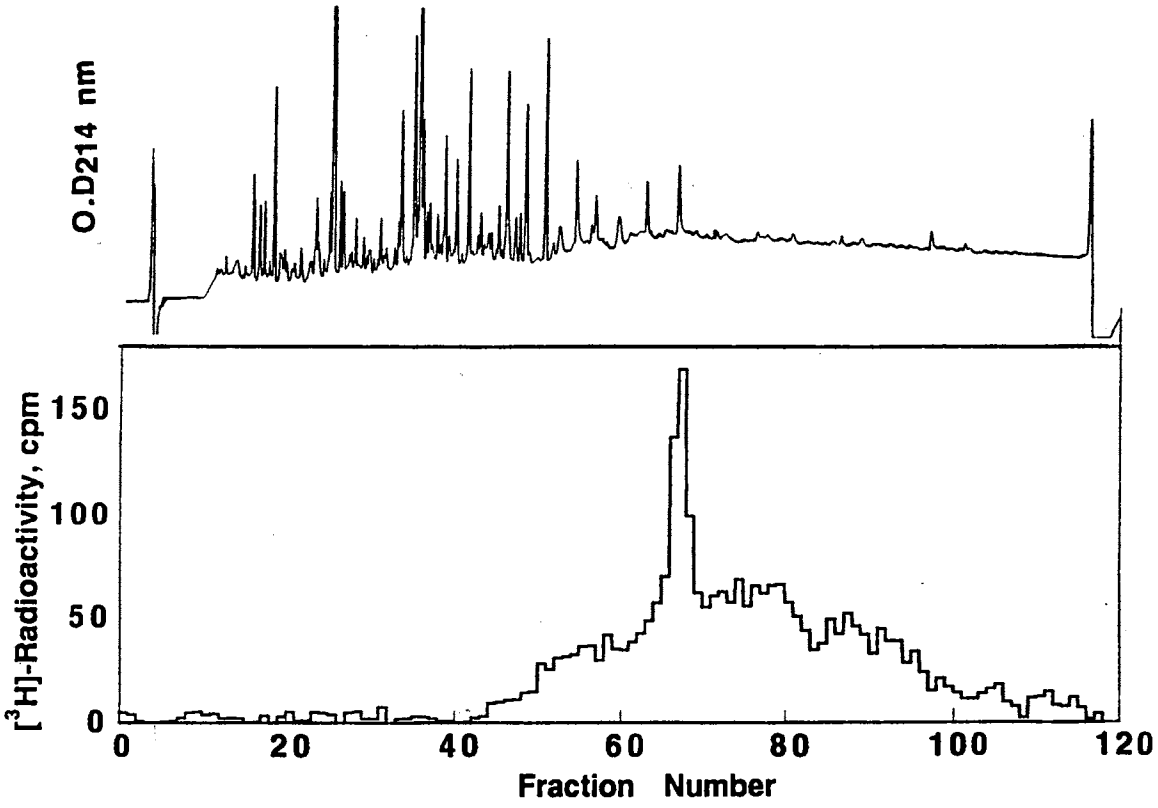


Figure 13. ^{3}H Radioactivity Distribution on HPLC Chromatogram of Trypsin-Digested, [^{3}H]Azido-Q-labeled QPs1 Protein. The [^{3}H]azido-Q-labeled QPs1 (1 mg/ml, 7.8 x 10^{4} cpm/mg) was digested with trypsin, and 100-μl aliquots of digested solution were subjected to HPLC separation as described under "Experimental Procedures." One hundred-μl aliquots were withdrawn from each tube for radioactivity determination.

QPs1 separated by HPLC. When carboxymethylated [^{3}H]azido-Q-labeled QPs1 protein was digested with trypsin followed by HPLC separation using a Synchropak RP-8 column eluted with a gradient formed from 0.1% trifluoroacetic acid and 90% acetonitrile in 0.1% trifluoroacetic acid, the majority of the radioactivity was found in a fraction with retention time of 66.9 min (P-67). Although the presence of 0.1% trifluoroacetic acid in the buffer system greatly increased the peptide resolution of QPs1 on HPLC, these acid conditions cause a partial release of radioactivity during chromatography (6). Radioactivity recovery from the HPLC separation of trypsin-digested QPs1 is about 62%.

The partial NH_2-terminal amino acid sequence of P-67 was determined to be GLTISQL-, which corresponds to amino acid residues 113-119 in the revised amino acid sequence of QPs1 (Fig. 14). Although the isolated azido-Q linked peptide (P-67) was from a tryptic peptide containing amino acid residues 113-140 of QPs1, the Q-binding domain, using the proposed structure of QPs1 (Fig. 14), is most likely located in the sequence connecting putative transmembrane helices 2 and 3. This region of the peptide is thought tp be extruded from the surface of the M side of the inner membrane. Threonine 115 is probably the amino acid covalently linked to azido-Q upon illumination. This deduction is based on finding that the intensity of the threonine peak is much less than that of the other amino acid peaks during sequencing of p-67. Neighboring amino acid residues, such as His^{105}, Trp^{108} and Asp^{109}, probably participate in the formation of Q-binding site. More detailed information on Q-binding must await the availability of the three-dimensional structure of SQR. The information obtained from this study, however, can serve as a basis for future mutagenesis studies to identify the essential amino acid residues involved in Q-binding.

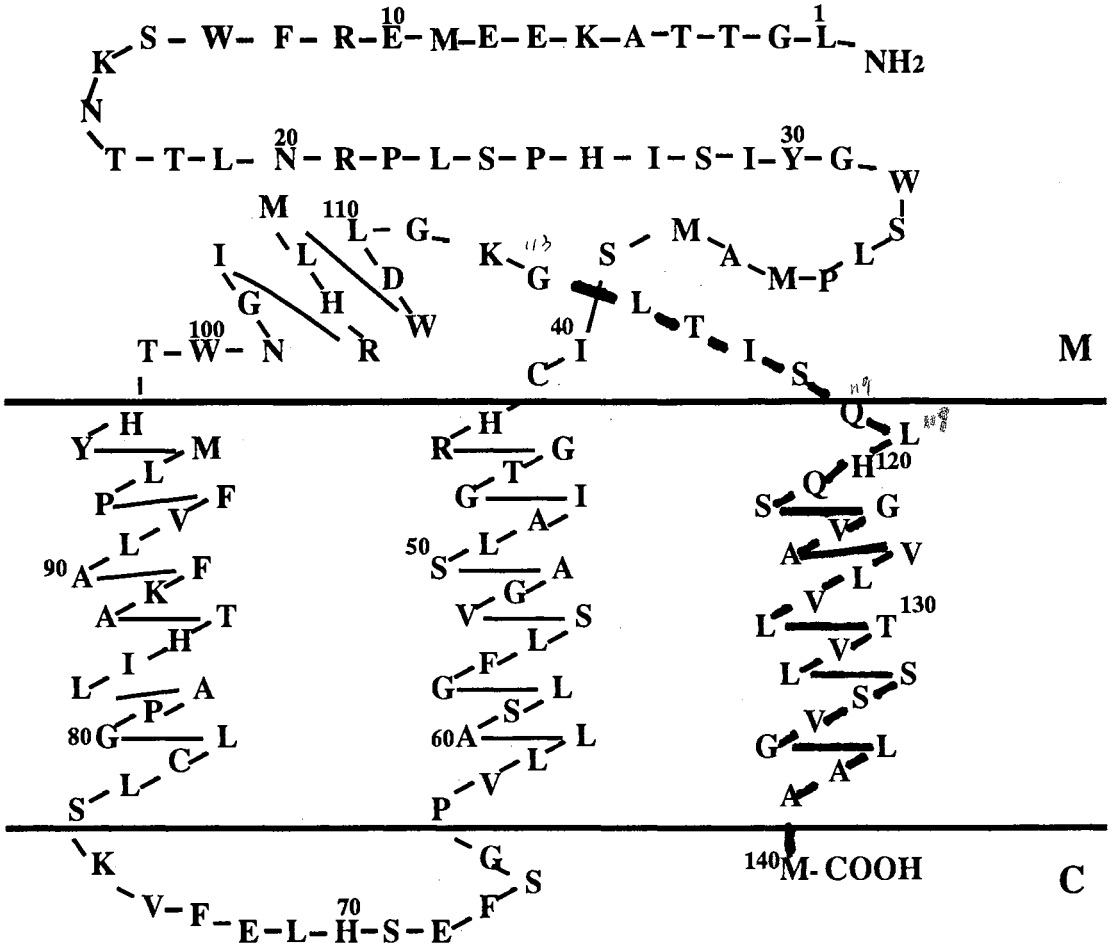


Fig. 14. Proposed Structure of QPs1. The amino acid sequence of QPs1 was revised by the addition of 8 amino acid residues, LGTTAKEE, to the NH_2-terminal end of our previously reported sequence (3). Bold line indicates the tryptic peptide isolated from P-67.

References

1. Yu, L., Yang, F-D., and Yu, C. A. (1985) *J. Biol. Chem.*, **260**, 963-973.
2. Jenner, E. L. (June 5, 1973) U.S. Patent 3737516.
3. Gu, L.-Q., Yu, L., and Yu, C. A. (1990) *Biochim. Biophys. Acta*, **1015**, 482-492.
4. Yu, L., and Yu, C. A. (1982) *J. Biol. Chem.*, **257**, 2016-2021.
5. Schägger, H., Link, T. A., Engel, W. D., and von Jagow, G. (1986) *Methods Enzymol.* **126**, 224-237.
6. He, D.-Y., Yu, L., and Yu, C. A. (1994) *J. Biol. Chem.*, **269**, 2292-2298.
7. Yu, L. and Yu, C. A. (1987) *Biochemistry*, **26**, 3658-3664.
8. Doyle, M. P., Li, L-B., Yu, L., and Yu, C. A. (1989) *J. Biol. Chem.*, **264**, 1387-1392.
9. Yu, L., Wei, Y-Y., Usui, S., and Yu, C. A. (1992) *J. Biol. Chem.*, **267**, 24508-24515.
10. Okamura, M. Y., and Feher, G. (1992) *Ann. Rev. Biochem.*, **61**, 861-896.

CHAPTER III

EXPRESSION OF QPs1 PROTEIN OF SUCCINATE-UBIQUINONE REDUCTASE

The Q-binding domain in QPs1 was identified by previous study to be in the sequence connecting transmembrane helices 2 and 3. To better understand the molecular structure of the Q-binding site requires knowledge of amino acid residues involved in Q-binding by this putative Q-binding domain. This can be achieved by an approach using expressed mutated QPs1 protein to reconstitute, *in vitro*, a QPs1-lacking complex to form SQR. In order to employ this *in vitro* reconstitution approach, the reconstitutively active QPs1-lacking SQR and an over-expressed, functionally active QPs1 are needed.

Recently, the small molecular weight Q-binding proteins, QPc (1) of bovine heart mitochondrial QCR and subunit IV (2) of the *Rhodobacter sphaeroides* cytochrome bc_1 complex, have been successfully expressed in *Escherichia coli* as glutathione S-transferase (GST) fusion proteins using the pGEX expression system. These reports, together with the availability of QPs1 cDNA in our laboratory, encouraged us to obtain functionally active QPs1 by expressing QPs1 in *E. coli* cells using the pGEX system. The use of pGEX expression system allowed one-step purification of recombinant fusion protein by a glutathione agarose gel, thus greatly simplifying the purification procedure.

In this chapter, I report the construction of a QPs1 expression vector, pGEX/QPs1, development of optimal conditions for expression of QPs1, solubilization of

recombinant GST-QPs1 protein from inclusion bodies, purification and characterization of recombinant GST-QPs1 fusion protein obtained from cell extracts and from solubilized inclusion bodies, reconstitution of cytochrome b_{560} from recombinant GST-QPs1 fusion protein and heme, and isolation of the heme-reconstituted recombinant QPs1.

Experimental Procedures

Materials

Restriction endonucleases, T4 DNA ligase, T4 DNA polymerase and T4 polynucleotide kinase were obtained from either Promega Corporation or GIBCO BRL. DNase I (Type IV), RNase A, IPTG, DMSO, gelatin, ampicillin, tetracycline, and X-gal were from Sigma. Nitrocellulose membrane was from MSI. Agarose, acrylamide, bis-acrylamide, BCIP, NBT, and goat anti-rabbit IgG alkaline phosphatase were from Bio-Rad.

δ-Aminolevulinic acid, ferrous sulfate, hemin chloride, sorbitol, betaine, glutathione (reduced form), glutathione-agarose beads, thrombin, and phenylmethylsulfonylfloride (PMSF) were from Sigma. LB agar, LB broth base, Select Peptone 140, and Select Yeast Extract were from GIBCO BRL. Oligonucleotides were synthesized by the Recombinant DNA/Protein Resource Facility at Oklahoma State University. Antibodies against QPs1, which were generated in rabbits and purified by the method previously reported (3), were available in our laboratory. Other chemicals were obtained commercially in the highest purity available.

Bacterial Strains and Plasmids

E. coli JM109[*rec*A1, *endo*A1, *gyr*A96, *thi*$^-$, *hsd*R17, *sup*E44, *rel*A1 Δ(*lac, pro*)F'*tra*D36, *pro*AB, *lac*Iq, *lacZ* M15] was used as host for pGEM-3Zf(+) (Promega), pSelect (Promega), and pGEX-2T (Pharmacia) plasmids. *E. coli* DH5α[F$^-$ 80d, *lacZ*ΔM15 Δ(lacZYA *arg*F)U169, *end*A1, *hsd*R17(r_k^- m_k^+), *deo*R, *thi*$^-$, *sup*E44, λ$^-$,*gyr*A96, *rel*A1] was used as final host for pGEX-2TH/QPs1. *E. coli* strains were grown in LB medium (4). When necessary, ampicillin (100 μg/ml) or tetracycline (15 μg/ml) were added.

DNA Manipulation and DNA Sequencing

Restriction enzyme digestion, large-scale isolation and minipreparation of plasmid DNA, and isolation and purification of Lamda phage DNA were performed according to reported methods (4). Site-directed mutagenesis was performed using the Altered Site *in vitro* Mutagenesis system from Promega (5). The sequencing primers were synthesized and DNA sequencing was performed by the Recombinant DNA/Protein Resource Facility at Oklahoma State University.

Isolation of Recombinant GST-QPs1 Protein from Crude Soluble Extract

Isolation of recombinant GST-QPs1 fusion protein from crude soluble extract of *E. coli* DH5α/pGEX/QPs1 cell culture was performed essentially according to the method described previously (1). Ten-milliliter of an overnight grown culture of *E. coli* DH5α/pGEX/QPs1 was used to inoculate 100-ml of bacterial growth medium (see Results

and Discussion) and incubated at 37 °C with vigorous shaking until the OD_{660nm} reached 0.9. The culture was cooled to about 27 °C and IPTG was added to a final concentration of 1mM to induce synthesis of the GST-QPs1 fusion protein. Cells were grown at 27 °C for 3 hours before being harvested by centrifugation at 8,000 x *g* for 15 min. Ten-gram of cell paste (which was obtained from 3 liter of the cell culture) were suspended in 20ml of 20mM sodium/pottasium phosphate buffer, pH 7.3, containing 150mM NaCl (PBS). PMSF was added to the cells to a final concentration of 1mM. The stock PMSF solution was 100mM in absolute alcohol. The cells were broken by sonicating (Heat Systems-Ultrasonics Inc., model W-220F) for 10 sec three times with 1 minute intervals. Triton X-100 was added to the broken cell suspension to a final concentration of 1% (w/v). The suspension was then stirred for one hour at 0°C and centrifuged at 16,000 x *g* for 20 min. The supernatant was taken and mixed with an equal volume of glutathione-agarose gel equilibrated with PBS. The gel mixture was gently shaken for two hours at 0°C and centrifuged at 5,000 x *g* for 10 min to collect the beads. After washing five times with 200ml of 50mM Tris-Cl buffer, pH 7.5, the GST-QPs1 fusion protein was eluted from the column with 50mM Tris-HCl, pH 8.0, containing 5mM glutathione and 0.25M sucrose. Sample was dialyzed against 50 mM Tris-Cl, pH 8.0, containing 0.25M sucrose. The dialyzed sample was concentrated to 10mg/ml with Centricon-10, mixed with glycerol to a final concentration of 10%, and frozen at -80°C until use. Protein concentration was determined by the Lowry method (6) using bovine serum albumin as a standard.

Enzyme Preparations and Assays

The cytochrome b_{560} content was determined by pyridine hemochromogen spectra using the millimolar extinction coefficient of 34.6 for reduced absorbance at 557 nm minus 600 nm (7) or by difference spectra using the millimolar extinction coefficient of 20.5 for reduced minus oxidized absorbance at 560 nm minus 575 nm (8).

SDH was prepared and assayed according to the reported method (9). The reconstitutive activity of QPs1 was assayed by its ability to give SQR activity upon mixing with SDH. Experimentally, GST-QPs1 fusion protein samples were mixed with SDH and incubated for 10 min at 0°C. One-ml assay mixture contained 100μmol of sodium/potassium phosphate buffer, pH 7.4, 50 nmol of DCPIP, 20 μmol of succinate, 10 nmol of EDTA, 16 nmol of Q_2 and 0.1mg of Triton X-100. The reduction of DCPIP was followed by an absorption decrease at 600 nm using a millimolar extinction coefficient of 21 $mMl^{-1}cm^{-1}$. Absorption spectra and activity assays were performed at room temperature in a Shimadzu UV-2101PC spectrometer.

SDS-PAGE and Western Blot Analysis

Analytical SDS-PAGE was performed in a Bio-Rad Mini-Protein dual slab cell using the gel system of Laemmli (10) with 10% or 12% of total monomer. The proteins on the SDS-PAGE gels were electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked with 20 % Tris-Cl buffer, pH 7.4, 500 mM NaCl (TBS) containing 3 % gelatin for one hour at room temperature and washed with TBS and TBS containing 0.05 % Tween-20 (TTBS) for 5 min, respectively. The anti-QPs1 antibodies

(15 μg/ml) in TTBS containing 1 % gelatine were 8then incubated with the membrane at room temperature overnight. After incubating with the primary antibodies, the blots were washed twice with TTBS for 5 min and then incubated with goat anti-rabbit IgG alkaline phosphatase conjugate (1: 2500 in TTBS containing 1 % gelatin) for one hour. After washing the membrane with TTBS and TBS for 5 min, respectively, the proteins were detected by showing alkaline phosphatase activity with BCIP and NBT as substrates. Prestained protein molecular weight standard was used to estimate the size of proteins on the gel and the membrane. The prestained protein molecular weight standard contained phosphorylase *b* (112,000), bovine serum albumin (84,000), ovalbumin (53,200), carbonic anhydrase (34,900), soybean trypsin inhibitor (28,700), and lysozyme (20,500).

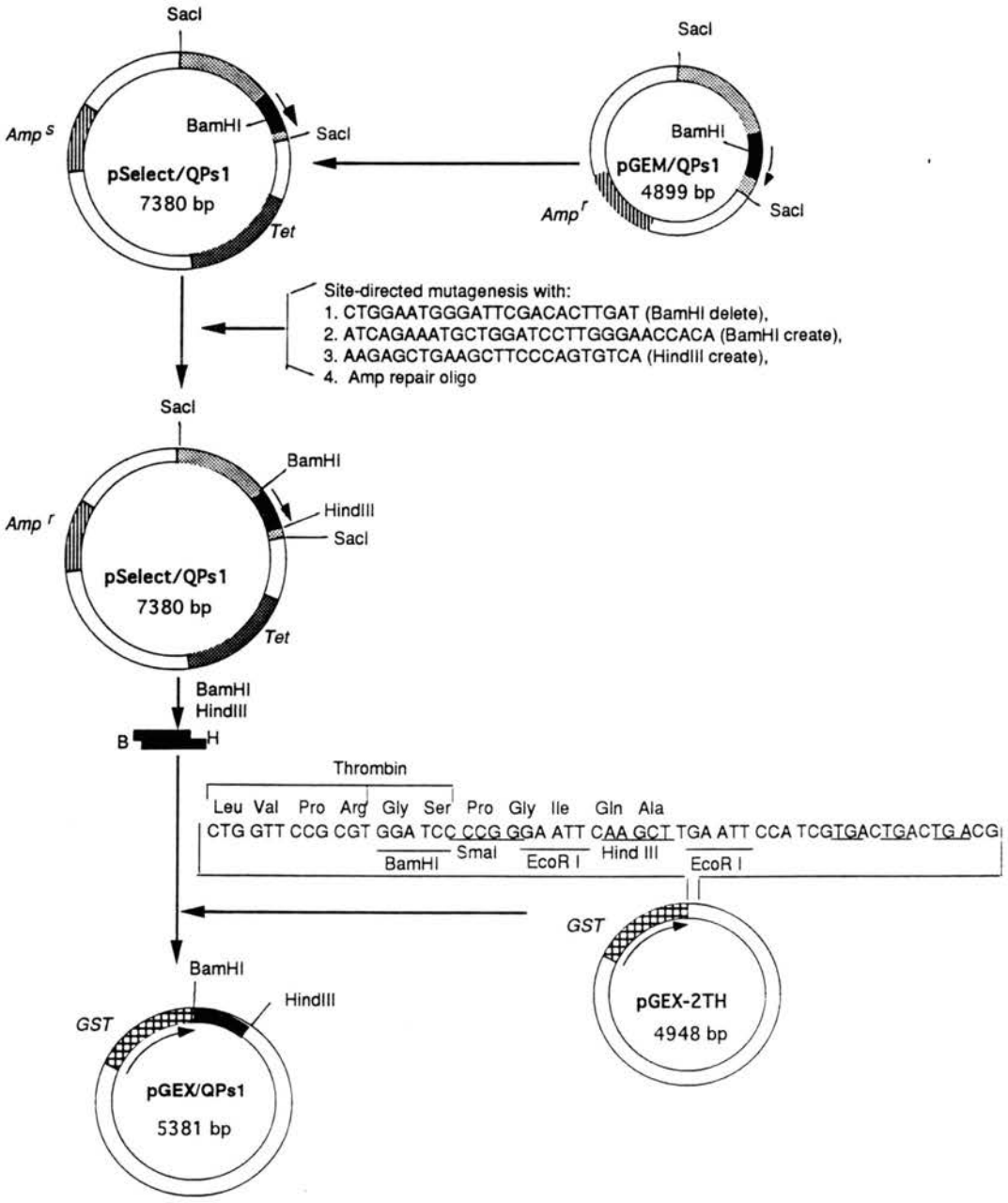
Results and Discussion

Construction of the Expression Vector for QPs1

Scheme III summarizes the protocol used for construction of the QPs1 expression vector, pGEX/QPs1. A new pGEX vector derivative, pGEX-2TH (1) which contained unique *BamH*I and *Hind*III sites (constucted by Dr. Usui), was used in this investigation. An in-frame fusion of the QPs1 gene with the glutathione-S-transferase gene in pGEX-2TH plasmid was achieved by generating a *BamH*I-*Hind*III fragment encoding mature QPs1 and subsequently ligating it into the *BamH*I and *Hind*III sites of the pGEX-2TH plasmid.

In order to obtain a *BamH*I-*Hind*III cDNA fragment encoding mature QPs1, an internal *BamH*I site in the sequence needed to be deleted before a *BamH*I site

Scheme III. Construction of the QPs1 Expression Vector, pGEX/QPs1.



(GGATCC) immediately upstream from the NH_2-terminus coding sequence (TTG) and a *Hind*III site (AAGCTT) 7-bp down stream from the stop codon (TGA) were created. This construction was achieved by using site-directed mutagenesis techniques.

A 1.7-Kbp SacI-fragment from pGEM/QPs1 was cloned into pSelect plasmid to generate pSelect/QPs1. The single stranded pSelect/QPs1 was used as a template for mutagenesis. The mutant oligonucleotide, CTGGAATGGGATTCGACACTTGAT, along with an ampicillin repair oligonucleotide was included in the mutagenesis system to delete the interal *BamH* I site in QPs1 sequence. Again, a *Sac*I fragment containing *BamH*I-deleted QPs1 sequence from pSelect $^{amp+}$ /$QPs1_{\Delta BamHI}$ was cloned into pSelect plasmid to generate pSelect $^{amp-}$ /$QPs1_{\Delta BamHI}$. The other two mutant oligonucleotides, ATCAGAAATGCTGGATCCTTGGGAACCACA (*BamH*I recognition), and AAGAGCTGAAGCTTCCCAGTGTCA (*Hind*III recognition), along with an ampicillin repair oligonucleotide were included in the mutagenesis system. The resulting plasmid, pSelect/$QPs1_{SBHS}$, was digested with *BamH*I and *Hind*III to produce a 433-bp fragment containing the QPs1 structural gene. This 433-bp *BamH*I-*Hind*III fragment was cloned into pGEX-2TH to generate pGEX/QPs1. pGEX/QPs1 was transformed into *E. coli* JM109 or *E. coli* DH5α. Transformants producing the recombinant GST-QPs1 fusion protein were identified by Western blot with antibodies against QPs1.

Effect of Induction Temperature, Media, and Time Length on Production of Recombinant QPs1 in *E. coli* DH5α

E. coli DH5α cells carrying pGEX/QPs1 produced the GST-QPs1 protein more efficiently than did *E. coli* JM109 cells containing this same plasmid. When overnight grown *E. coli* DH5α or JM109 cells carrying pGEX/QPs1 was inoculated into growth medium, respectively, DH5α cells required 2.5 hrs and JM109 cells required 3.5 hrs to reach the midpoint of their logarithmic growth phase. Furthermore, the level of expression of the fusion protein from *E. coli* DH5α cells was higher than that from *E. coli* JM109 cells when estimated by SDS-PAGE of whole lysate. Thus, QPs1 protein was expressed in *E. coli* DH5α cells.

When *E. coli* DH5α cells containing pGEX/QPs1 were treated with IPTG to induce systhesis of the GST-QPs1 fusion protein at 37°C for 3 hours, about 8% of the total cellular protein produced was recombinant GST-QPs1 fusion protein (see lane 1 of Fig. 15 (a)), indicating a high level of expression. The level of expression was estimated by comparing the color intensity of the 41-kDa protein band (GST-QPs1 fusion protein), which reacted with antibdies against QPs1, with that of total cellular protein bands in SDS-PAGE. However, when the cells were broken by sonication, only 5% (0.4% of total cellular protein) of the fusion protein produced was recovered in the crude extract. Addition of Triton X-100 (1%) to the broken cell culture did not improve the recovery of the fusion protein in the crude extracts, indicating that the majority of recombinant fusion protein were synthesized in insoluble aggregates (inclusion bodies) when cells were grown at 37°C.

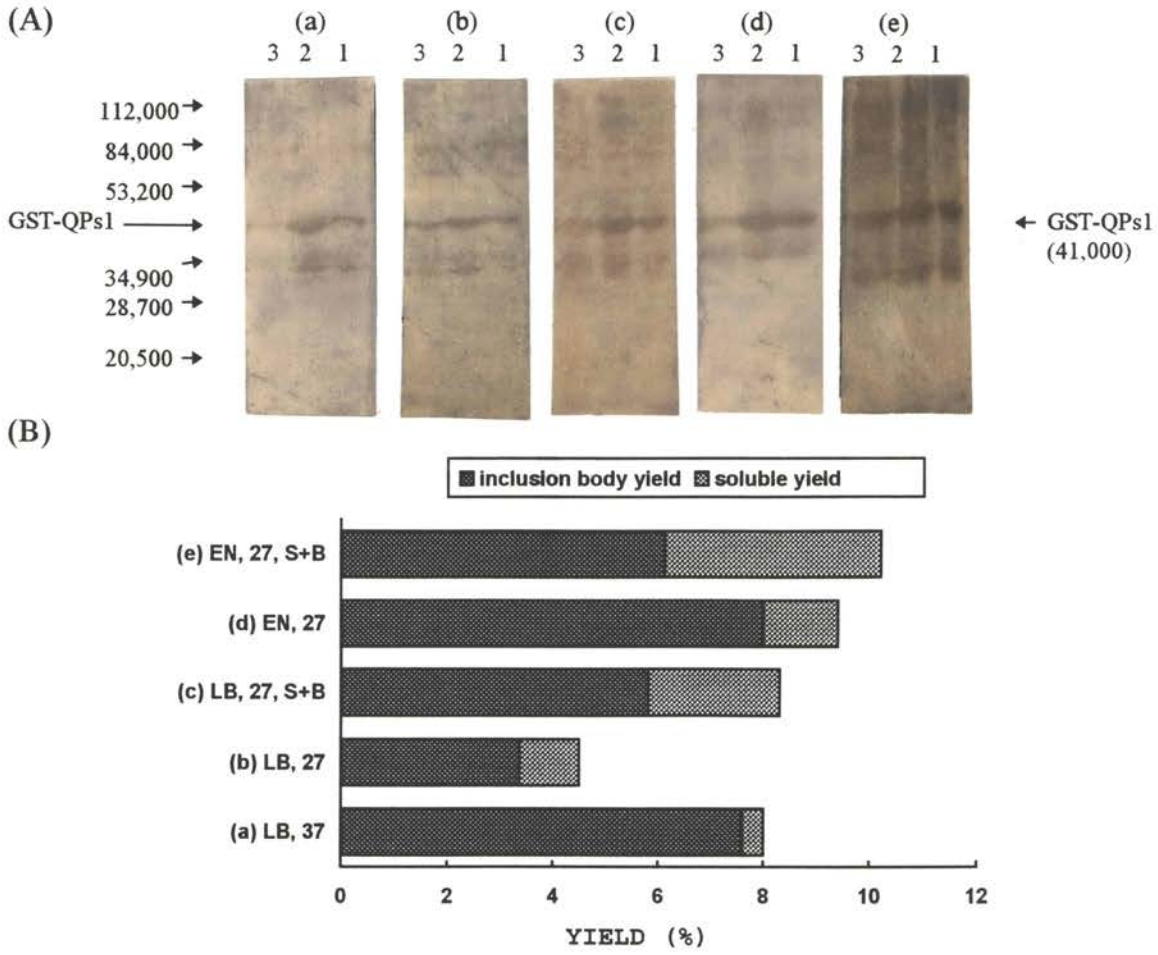


Figure 15. Total, Soluble and Inclusion Body Yield of GST-QPs1 Fusion Protein Expressed on Various Growth Condition. 1 ml of cell culture 3 hrs after IPTG induction was removed and centrifuged, and the precipitate was suspended in 1 ml of PBS (for total yield). 250 µl of Triton X-100 treated sonicated cell suspension were removed and centrifuged, and the precipitate was suspended in 1 ml of PBS for inclusion body yield. For soluble yield, the supernatant after centrifugation was used. And 30μl of 1% SDS and β-mercaptoethanol treated sample was applied to SDS-PAGE. (A), Western blots of the SDS-PAGE: (a) LB broth medium, at 37°C, (b), LB broth medium, at 27°C, (c), LB broth medium containing 440 mM sorbitol and 2.5 mM betaine, at 27°C, (d) enriched medium, at 27°C, and (e) enriched medium containing 440 mM sorbitol and 2.5 mM betaine, at 27°C. Lane1 is total yield, lane 2 is inclusion body yield, and lane 3 is soluble yield. (B), Total yield of GST-QPs1 fusion protein was estimated from the gel of SDS-PAGE by scanning it with densitometry. The yield from each fraction was obtained by comparing the color intensity of 41,000 Da protein band (GST-QPs1 fusion protein), which reacted with antibodies against QPs1-15 kDa, with that of other cellular protein bands.

The insolubility of recombinant GST-QPs1 fusion protein reduces its affinity for glutathionine agarose and thus limits the usefulness of the one-step purification scheme of the pGEX vector system. Only when the recombinant protein is soluble (or detergent soluble) and possesses a properly folded GST-active site, binding of the fusion protein to glutathione agarose is possible (11). There are two ways to achieve this goal: one is to change growth conditions to make cells produce active soluble recombinant protein; and the other is to develop methods to convert insoluble denatured recombinant GST-fusion protein into a soluble active form (1).

Changes in cellular environments are known to increase the yield of active recombinant protein either by encouraging the cells to adopt the active conformation or by increasing the stability of recombinant protein. Variation of media, induction conditions, and length of induction are factors that affect the cellular environments. We systematically examined the effect of these factors on the yield of soluble recombinant GST-QPs1 protein. Figure 15 compares the yield of GST-QPs1 fusion proteins produced in *E. coli* DH5α cells under five different growth conditions: (i) LB broth medium at 37°C; (ii) LB broth medium at 27°C; (iii) LB broth medium containing 2.5 mM betaine and 440 mM sorbitol at 27°C; (iv) enriched medium (2% Select peptone 140, 0.2% Na_2HPO_4, 0.1% KH_2PO_4, 0.8% NaCl, 1.5% Select yeast extract, and 0.2% glucose) at 27°C; (v) enriched medium containing 2.5 mM betaine and 440 mM sorbitol at 27°C.

Induction at 27°C improved the soluble yield over induction at 37°C, by 4-fold, but the total amount of recombinant GST-QPs1 produced by the *E. coli* culture induced at 27°C was only 60% of that produced by the culture induced at 37°C. This result was

compatible with previous reports of increased soluble yield of recombinant polypeptide in *E. coli* using low induction temperature (1, 11-15).

Although lowering the induction temperature increased the yield of soluble active GST-QPs1 fusion protein, about 75% of the recombinant fusion protein still remained in inclusion bodies. To further increase the soluble yield, a method involving the use of osmotic stress to facilitate the uptake of the "compatible solute" glycyl betaine (1, 13) was adopted. When *E. coli* DH5α/pGEX-QPs1 cells were grown at 27°C on LB medium containing 2.5 mM betaine and 440mM sorbitol, about 30% of the GST-QPs1 produced (accounts for 8.3% of the total cellular protein) was in soluble form. Although the % distribution (30%) of produced GST-QPs1 fusion protein in the soluble fraction for cells grown in the presence of betaine and sorbitol was only 5% higher than that for cells grown in the absence of these compounds (25%) at 27°C, the total yield of GST-QPs1 fusion protein increased from 5.3% to 8.3%. This suggests that inclusion of betaine and sorbitol in the growth medium not only promotes production of active soluble fusion protein, but also stimulates production of the fusion protein at low temperature. This result is similar to the report of increased the yield of active soluble GST-QPc by including betaine and sorbitol in the growth medium (1). Although the resason for the increased yield of active soluble recombinant protein is unknown, it has been suggested that increasing internal concentrations of compatible osmolytes, such as betaine which are believed to be excluded from the immediate domains of proteins, causes a thermodynamically unfavorable "preferential hydration" and thus minimization of solvent-protein contact and stabilization of protein structure (1, 13).

It has been reported that the recombinant protein can be induced in *E. coli* with the increased yield of the soluble form by using an enriched medium (14). When *E. coli* DH5α/pGEX-QPs1 cells were grown at 27°C on enriched growth medium such as peptone-phosphate medium (2% Select peptone 140, 0.2% Na_2HPO_4, 0.1% KH_2PO_4, 0.8% NaCl, 1.5% Select yeast extract, and 0.2% glucose), about 9.4% of the total cellular protein were GST-QPs1 fusion protein. About 15% of the fusion protein produced was in an active soluble form. Although the cells grown on enriched medium produced more fusion protein than the cells grown on LB medium, the percent distribution of produced GST-QPs1 in soluble fraction was lower than that for the cells grown on LB medium containing betaine and sorbitol.

Since betaine and sorbitol increased the soluble yield of recombinant GST-QPs1 for cells grown in LB medium, it was of interest to see whether these compounds could increase the soluble yield of recombinant fusion protein for cells grown in enriched medium. When only 2.5 mM betaine or 400 mM sorbitol was included into LB medium system, the soluble yield was increased to about 25% for each. However, addition of both 2.5 mM betaine and 400 mM sorbitol to enriched medium increased the soluble yield to 40% (Fig. 15 (e)). About 10.2 % of the total cellular protein was GST-QPs1 fusion protein. Therefore, enriched medium containing both 2.5 mM betaine and 400 mM sorbitol was the best condition for the production of the soluble recombinant GST-QPs1 fusion protein.

It should be mentioned that addition of Triton X-100 to the broken cell cultures obtained from all the above growth conditions did not improve the soluble yield of

recombinant protein, indicating that all the recombinant proteins in insoluble fraction after sonication existed as an inclusion body aggregate instead of as membrane bound form. However, Triton X-100 was included in our routine isolation procedure for recombinant GST-QPs1 fusion protein to reduce cellular protein contamination during glutathione-agarose gel affinity column chromatography (15).

Production of recombinant GST-QPs1 fusion protein was found to be dependent on IPTG-induction time (see Fig.16). The yield of the fusion protein increased, as the induction growth time was increased, to almost maximum yield which was obtained when cells were hearvested three hours after IPTG induction. When cells were grown for more than 3 hrs, the total yield did not increase much and the degradative products appeared more, suggesting that the recombinant protein was unstable and susceptible to protease digestion. In summary, the optimal condition for the production of soluble recombinant GST-QPs1 fusion protein is as follows: induction of recombinant GST-QPs1 fusion protein by IPTG at 27°C for three hours in *E. coli* DH5α cells containing pGEX/QPs1 grown on enriched medium containing 2.5 mM betaine and 440 mM sorbitol.

Solubilization of Recombinant GST-QPs1 Fusion Protein From Inclusion Body Precipitates

Although lowering the induction temperature and introducing enriched medium containing 2.5 mM betaine and 440 mM sorbitol increased the yield of soluble active GST-QPs1 fusion protein, about 60 % of the recombinant fusion protein produced still remained in inclusion body complexes. The production of the sensitive protein, which is

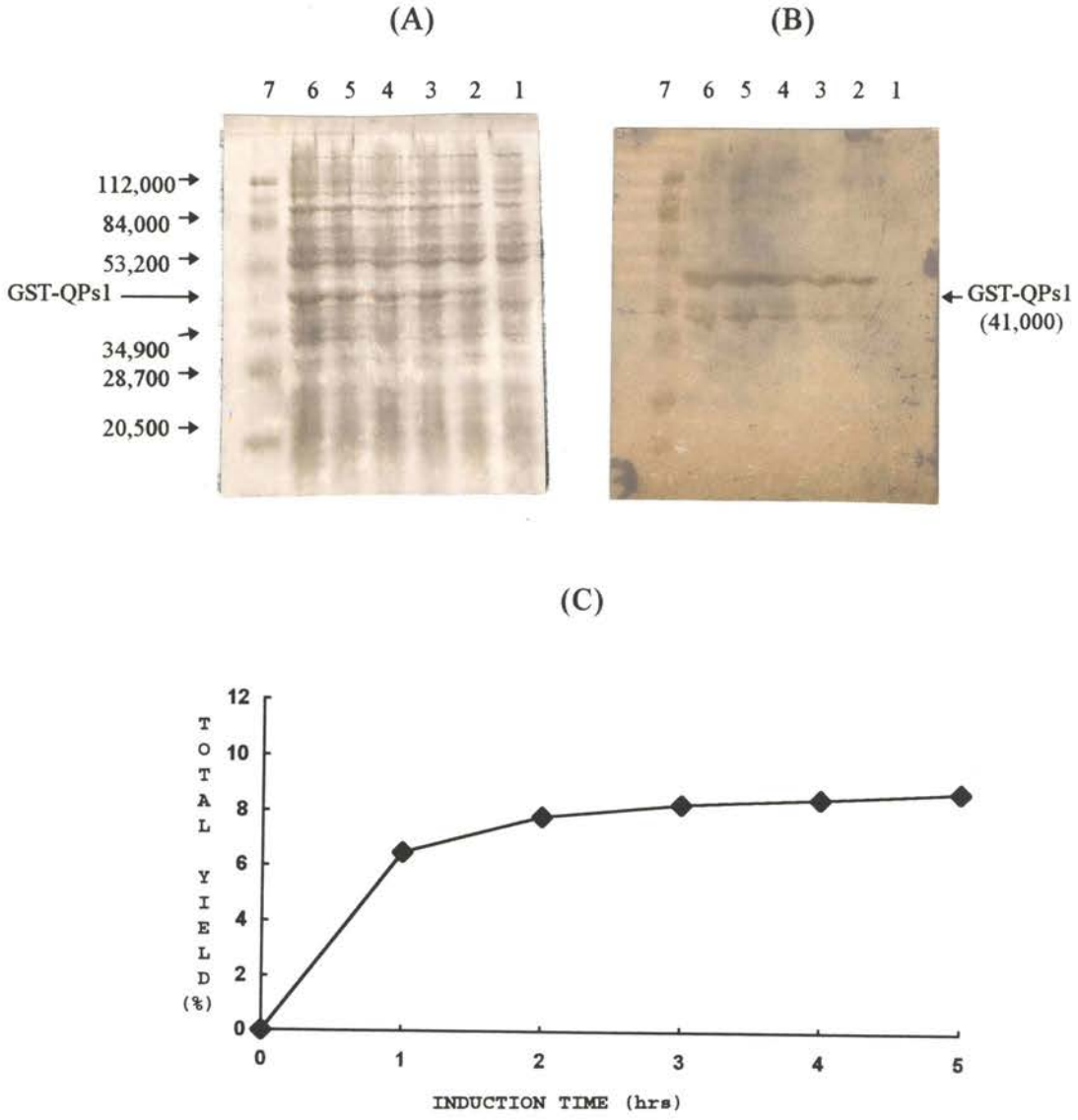


Figure 16. Effect of Induction Time by IPTG on Expression of GST-QPs1 Fusion Protein on LB Medium Containing 2.5 mM Betaine and 440 mM Sorbitol. 1 ml of cell culture was removed, centrifuged, and suspended in 500μl of PBS at 1, 2, 3, 4, and 5 hrs after inducing by IPTG. 30μl of suspension was subjected to SDS-PAGE. Total yield of GST-QPs1 fusion protein was estimated from the gel of SDS-PAGE by scanning it with densitometry. (A), SDS-PAGE of total cellular proteins before (lane 1), and 1 hr (lane 2), 2 hrs (lane 3), 3 hrs (lane 4), 4 hrs (lane 5), and 5 hrs (lane 6) after the IPTG induction. The prestained molecular weight standard (lane 7) was applied. (B), Western blot of (A). (C), The yield (%) of the GST-QPs1 fusion protein against total cellular proteins at each induction length was 0, 6.5%. 7.8%, 8.3%, 8.5%, and 8.75%, respectively.

degraded by proteases when expressed in microorganisms, in the form of inclusion bodies may offer a possibility to stabilize the protein (12). However, recovery of active recombinant protein from the inclusion body complexes has long been regarded as a formidable task due to the denaturation of the protein and the heterogeneous nature of inclusion bodies (1). Although general techniques for obtaining active recombinant protein from inclusion bodies have not been developed, some success has been reported (11, 13). In these cases, recombinant protein aggregates were solubilized with a high concentration of urea or other chaotropic reagents, and dialyzed to allow proper refolding of the protein structure (11, 13).

Solubilization of recombinant GST-QPs1 fusion protein from inclusion body precipitates was achieved by the urea-dialysis method. The inclusion body precipitates were suspended with 100 mM Tris-Cl, pH 7.4, containing 8 M urea to give the final concentration of urea of about 5 to 5.5 M. DTT was added to the urea-treated suspension to give a final concentration of 1 mM. After stirring for 30 min at room temperature, the mixture was centrifuged at 16,000 x *g* for 20 min. About 60% of the fusion protein in inclusion body complexes was recovered in the soluble fraction. The urea-solubilized inclusion body complexes were dialyzed against 1-liter of 50 mM Tris-Cl buffer, pH 7.4, containing 0.2% sodium cholate overnight with four changes of buffers. The dialyzed solution was centrifuged at 16,000 x *g* for 20 min to remove insoluble precipitates formed. About 20% of the recombinant GST-QPs1 fusion protein in urea-solubilized inclusion body fraction remained in soluble form after dialysis. The inclusion of 0.2% sodium cholate in the dialysis buffer slightly increased the yield of soluble GST-QPs1 fusion

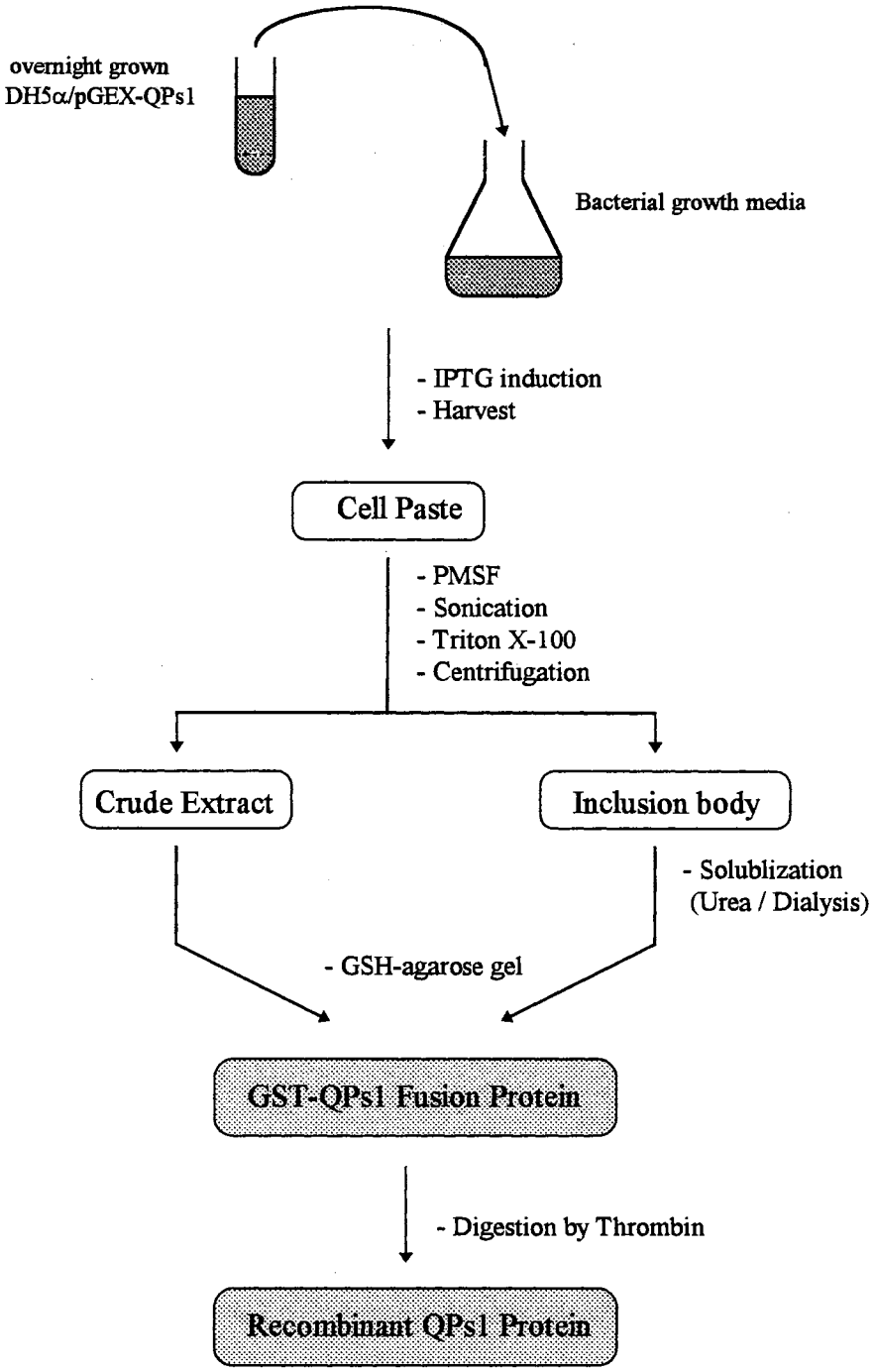
protein upon dialysis of the urea-solubilized inclusion body fraction. Thus, the recovery of soluble recombinant GST-QPs1 from inclusion body by the urea-dialysis method was about 12%.

Isolation and Properties of Purified Recombinant QPs1

Scheme IV outlines the protocol for isolation of recombinant QPs1 from *E. coli* DH5α/pGEX/QPs1 cell culture. Pure GST-QPs1 fusion protein was obtained from both crude soluble extract and solubilized inclusion body fraction by glutathione agarose gel chromatography (see Experimental Procedures), indicating that GST-QPs1 fusion protein in both two fractions possessed a properly folded GSH active site which was capable of binding to glutathione agarose.

The recombinant QPs1 was released from purified GST-QPs1 fusion protein by thrombin cleavage. When purified GST-QPs1 was incubated with thrombin (0.005 unit/μg of protein) at room temperature, QPs1 was progressively released from GST, with about 90 % being cleaved in a 1-hour incubation (Fig. 17). However, the cleavage of QPs1 from GST by thrombin never reached completion, even after prolonged incubation with increased amount of thrombin. The reason for this is unknown. The released GST, uncleaved GST-QPs1 fusion protein, and thrombin in thrombin-treated mixture were removed by Superose-12 FPLC column chromatography. The purified recombinant QPs1 was recovered in the column's void volume, indicating that the protein was in the highly aggregated form. The aggregation of QPs1 in aqueous solution results from the presence of three transmembrane helices in the protein (3). Although inclusion of 0.01% of

Scheme IV. The Protocol for Isolation of the Recombinant QPs1 Protein from *E. coli* DH5α Cells Containing pGEX/QPs1.



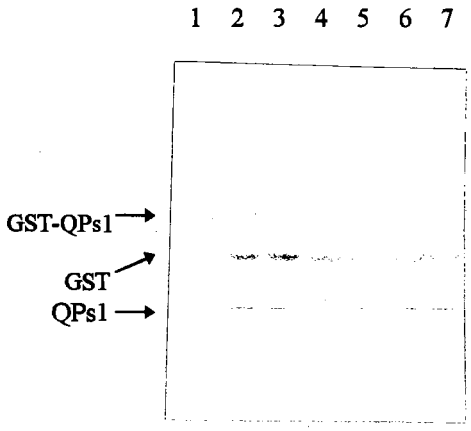


Figure 17. SDS-PAGE of the Isolated GST-QPs1 Before and After Cleavage By Thrombin. 20 μl of the fusion protein (7.9 mg/ml) was diluted in thrombin digestion buffer (20 mM Tris-Cl, 150 mM NaCl, 25 mM $CaCl_2$, pH 8.0) to a protein concentration of 0.5 mg/ml and thrombin (0.005 unit/ μg protein). n-Dodecyl maltoside (15 %) was included to a final concentration of 0.01%. Thrombin (0.005 unit / μg protein) was added to the sample solution. Before (line 1), 10, 30, 60, 120, 240 and 360 minutes (line 2 to 7, respectively) after incubation with thrombin, 20 μl of digested solution was withdrawn, treated with sample buffer containing 1 % SDS and 1 % β-ME, and subjected to SDS-PAGE.

dodecylmaltoside in the thrombin digestion mixture and in the buffer system during subsequent column chromatography did not prevent QPs1 from aggregation upon removal of GST, the recombinant QPs1 thus obtained was in detergent-dispersed form. The apparent molecular weight of recombinant QPs1 in 50 mM Tris-Cl buffer, pH 8.0, containing 150 mM sodium chloride and 0.01% dodecylmaltoside was over one million. When the QPs1 containing fraction recovered from Superose-12 column was subjected to SDS-PAGE, a band corresponding to the third subunit (QPs1) of purified SQR was observed.

Reconstitution of the Cytochrome b_{560}-Containing GST-QPs1 Fusion Protein by Heme

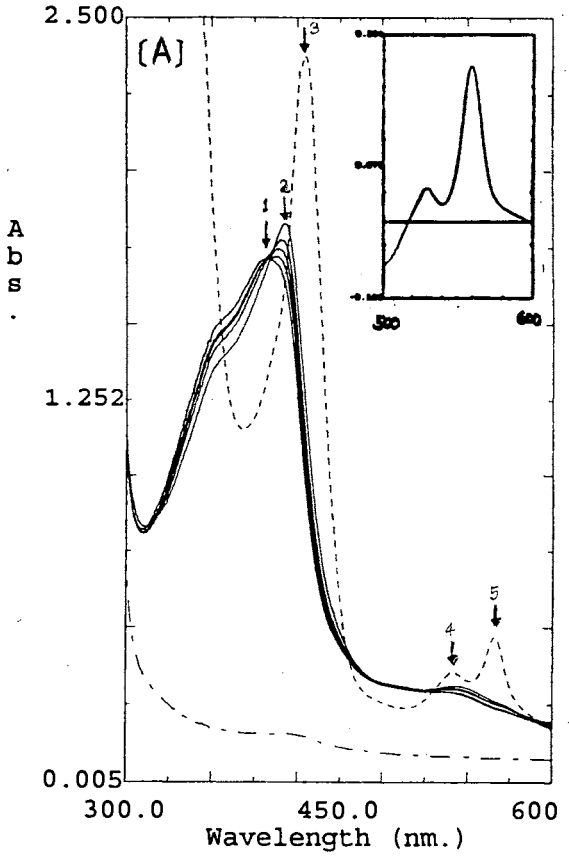
Although QPs1 is believed to be cytochrome b_{560}, with a bis-histidine ligand (16), isolated recombinant QPs1, obtained either from the crude soluble fraction or from inclusion body complexes, contained little cytochrome b_{560} heme. The possibility exists that lack of cytochrome b_{560} heme in recombinant QPs1 results from the inability of *E. coli* DH5α/pGEX-QPs1 cells to produce enough heme to be ligated into the QPs1 moiety in expressed GST-QPs1 fusion protein. In other words, the QPs1 in expressed GST-QPs1 fusion protein has the correct protein configuration for heme ligation and the two histidine residues of the ligand for cytochrome b_{560} are from QPs1 protein. One way to test this possibility is to reconstitute cytochrome b_{560} from recombinant QPs1 by heme. Although reconstituted b_{560}-containing QPs1 could be prepared by addition of heme to purified recombinant QPs1 or to recombinant GST-QPs1 followed by thrombin cleavage, the latter was chosen because GST-QPs1 fusion protein was soluble in aqueous solution and

exhibited as monomer form whereas isolated recombinant QPs1 was in a highly aggregated form.

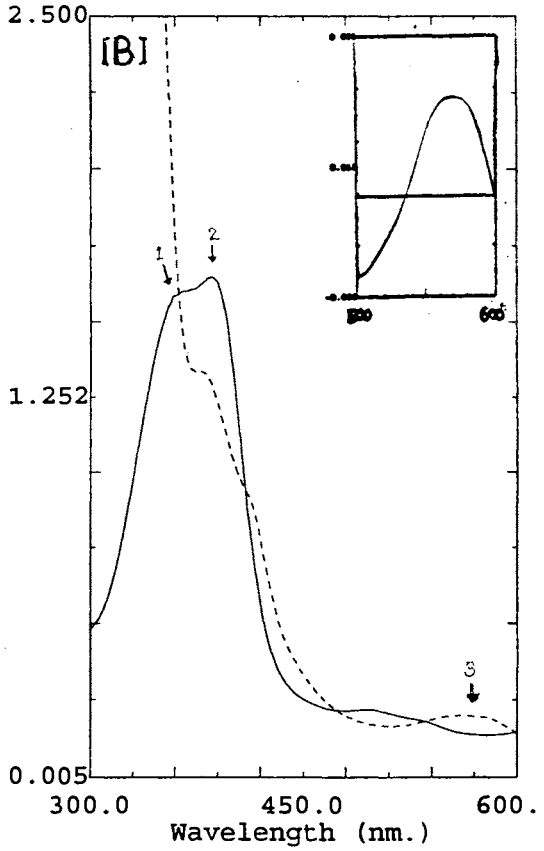
Figure 18A shows the restoration of cytochrome b_{560} to GST-QPs1 at the expense of added heme. When hemin chloride in DMSO was added to the fusion protein in 50 mM Tris-Cl buffer, pH 8.0, containing 0.25M sucrose, at 0.24 mole of heme per mole of fusion protein, the maximum absorption peak of oxidized form of the heme was progressively shifted from 398nm to 411 nm with increasing absorption intensity as incubation time increased. It took two hours to complete the spectral red shift and reach maximum increase in absorption intensity. When the reaction was complete, dithionite was added to reduce the sample. The reduced sample showed a symmetrical α-absorption at 559.5 nm, a broad β-absorption peak at 530 nm, and Soret absorption at 425 nm. These absorption characteristics were identical to those of cytochrome b_{560} in isolated reconstitutively active QPs preparation. They differed completely from reduced free heme spectra (see Fig. 18B). These results indicated that cytochrome b_{560} was reconstituted from recombinant GST-QPs1 fusion protein.

Restoration of cytochrome b_{560} to GST-QPs1 was heme concentration dependent. When a constant amount of isolated recombinant GST-QPs1 was incubated with various concentrations of hemin chloride, the peak height at 560 nm increased as the concentration of heme increased (Fig. 19A). Maximum increase of the peak height at 560 nm was obtained when 0.5 mole of heme per mole of fusion protein was used (see Fig. 19B). However, the amount of heme being ligated into the GST-QPs1 protein to form cytochrome b_{560} was less than 0.5 mole of heme /mol protein because the difference

Figure 18. Restoration of Cytochrome b_{560} from Isolated Recombinant GST-QPs1 Fusion Protein and Heme. (A), 3μl of hemin chloride (6.18 mM) in DMSO was added to 1ml of isolated GST-QPs1 fusion protein (3.1 mg/ml), and incubated at room temperature until no more change in oxidized Soret absorption peak (———). The isolated GST-QPs1 was in 50mM Tris-Cl buffer, pH 8.0, containing 0.25 M sucrose. Total incubation time was about 2hrs. The molar ratio of the added heme to the fusion protein was 0.24: 1 and the concentration of DMSO in the mixture was 0.3 %. When the reaction complete, the sample was reduced by dithionite (------). Heme concentration was determined by pyridine hemochromogen spectrum for free heme and difference spectra for heme in the protein. Oxidized form of the fusion protein is shown, too (_ - _ - _). (B), Absorption spectra of oxidized and reduced form of free heme. The same amount of hemin-chloride as (A) was added to 1 ml of the sample buffer. The inset in each panel shows difference spectrum of dithionite reduced minus oxidized form at α absorption region.

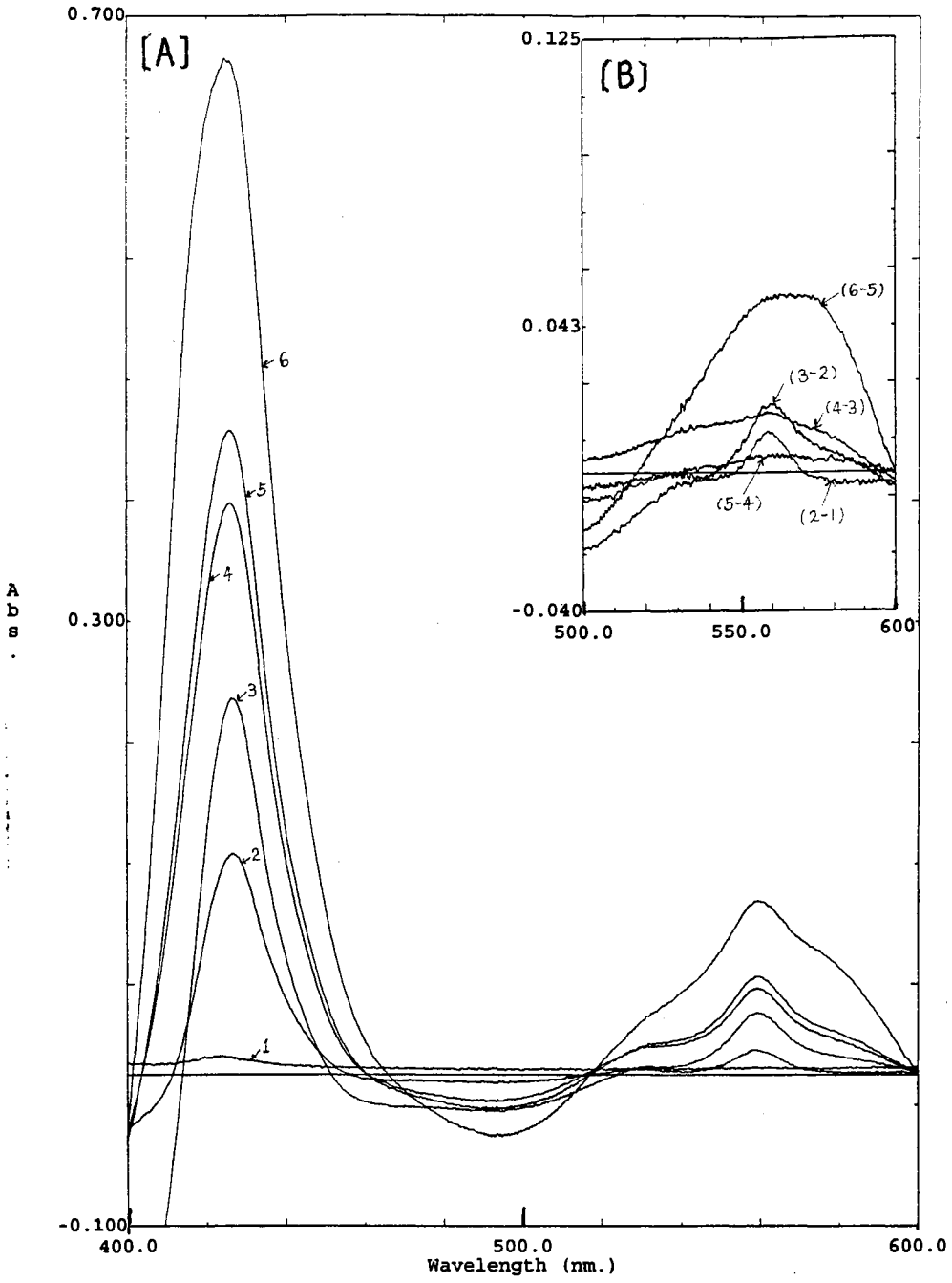


1, 398nm; **2**, 411nm; **3**, 425nm; **4**, 530nm; **5**, 559.5nm



1, 360nm; **2**, 386nm; **3**, 560-580nm

Figure 19. Difference Spectra of Reconstituted Cytochrome b_{560} From Isolated Recombinant GST-QPs1 and Various Amount of Heme. (A), Difference spectra of dithionite reduced minus oxidized reconstituted cytochrome b_{560} were obtained when 1ml of isolated GST-QPs1 (0.3 mg/ml) was incubated for 2 hour at room temperature with various amount of hemin chloride in DMSO. The molar ratios of heme to the fusion protein were 0 (line 1), 0.25 (line 2), 0.5 (line 3), 0.75 (line 4), 1 (line 5), and 2 (line 6) mole of heme / mole protein, respectively. The concentration of DMSO in the sample was kept lower than 0.3%. (B) Difference spectra of the two neighboring spectra of (A) in α absorption region. The numbers shown in (B) indicates those of spectra in (A).


[A]
[B]
0.700
0.300
-0.100
0.125
0.043
-0.040
500.0
550.0
600
400.0
500.0
600.0
Wavelength (nm.)
Abs.
1
2
3
4
5
6
(2-1)
(3-2)
(4-3)
(5-4)
(6-5)

spectra between dithionite reduced, 0.5 mole heme added GST-QPs1 mixture and 0.25 mole heme added GST-QPs1 mixture showed a small broad shoulder peak at 575 nm (see Fig. 19B) corresponding to reduced free heme α-absorption peak (see Fig. 18B). The amount of cytochrome b_{560} reconstitution may directly correlate to the amount of intact GST-QPs1 present in the isolated fusion protein preparation. The recombinant fusion protein purified from soluble cell extract was often contaminated with a 68 KDa-molecular mass protein and some degradative products of GST-QPs1 fusion protein , as shown in lane 5 of Figure 20C . About 40.5% of total protein in this GST-QPs1 preparation was intact GST-QPs1 fusion protein. This was estimated by comparing the color intensity of the 41-kDa protein band (GST-QPs1 fusion protein), which reacted with antibodies against QPs1, with that of total protein bands in SDS-PAGE of fusion protein preparation. Assuming that only intact GST-QPs1 fusion protein is capable of ligating with heme to form cytochrome b_{560}, then this reconstituted sample must contain about 0.38 mol b_{560} heme per mol of fusion protein.

It was reported (8) that reduced cytochrome b_{560} in SQR had α-absorption at 560.5 nm with a shoulder at 553 nm and was completely not reactive toward carbon monoxide (CO), whereas reduced cytochrome b_{560} in QPs had a symmetrical α-absorption at 560 nm and was reactive to CO. Addition of reconstitutively active SDH to QPs converts cytochrome b_{560} from CO reactive to CO non-reactive and causes the reappearance of the 553 nm α shoulder peak. Figure 20 compares absorption spectra and CO reactivity of the reconstituted cytochrome b_{560} in recombinant GST-QPs1 fusion protein in the presence and absence of SDH. As shown in Figure 21A, reduced

Figure 20. FPLC Chromatogram with A_{278} and A_{560} Obtained From Isolation of Heme-Reconstituted Recombinant QPs1 from GST-QPs1 Fusion Protein By Thrombin. 2 ml (6.2 mg) of heme-reconstituted recombinant GST-QPs1 (0.13 mole of heme / mole protein) was diluted with the same volume of thrombin digestion buffer (20 mM Tris-Cl, pH 8.0, containing 150mM NaCl, 25mM $CaCl_2$) and n-dodecylmaltoside (15 %) was added to a final concentration of 0.01 %. The diluted sample was incubated for 1hr at room temperature with thrombin (0.005 unit/μg protein). The thrombin treated sample was concentrated to 0.5 ml and applied to the Superose 12 FPLC column equilibrated with 50 mM Tris-Cl buffer, pH 8.0, containing 150 mM sodium chloride and 0.01% n-dodecyl maltoside. The fraction was collected with 4 min/ tube at a flow rate of 0.5 ml/min. The protein concentration was detected at 278nm (A) and the cytochrome b_{560} concentration was profiled (B). Twenty five-μl of each from fraction 4 to 7 (lane 1-4), isolated GST-QPs1 (lane 5), SQR (lane 6), and prestained molecular weight standard (lane 7) were subjected to SDS-PAGE (C) and Western Blot (D).

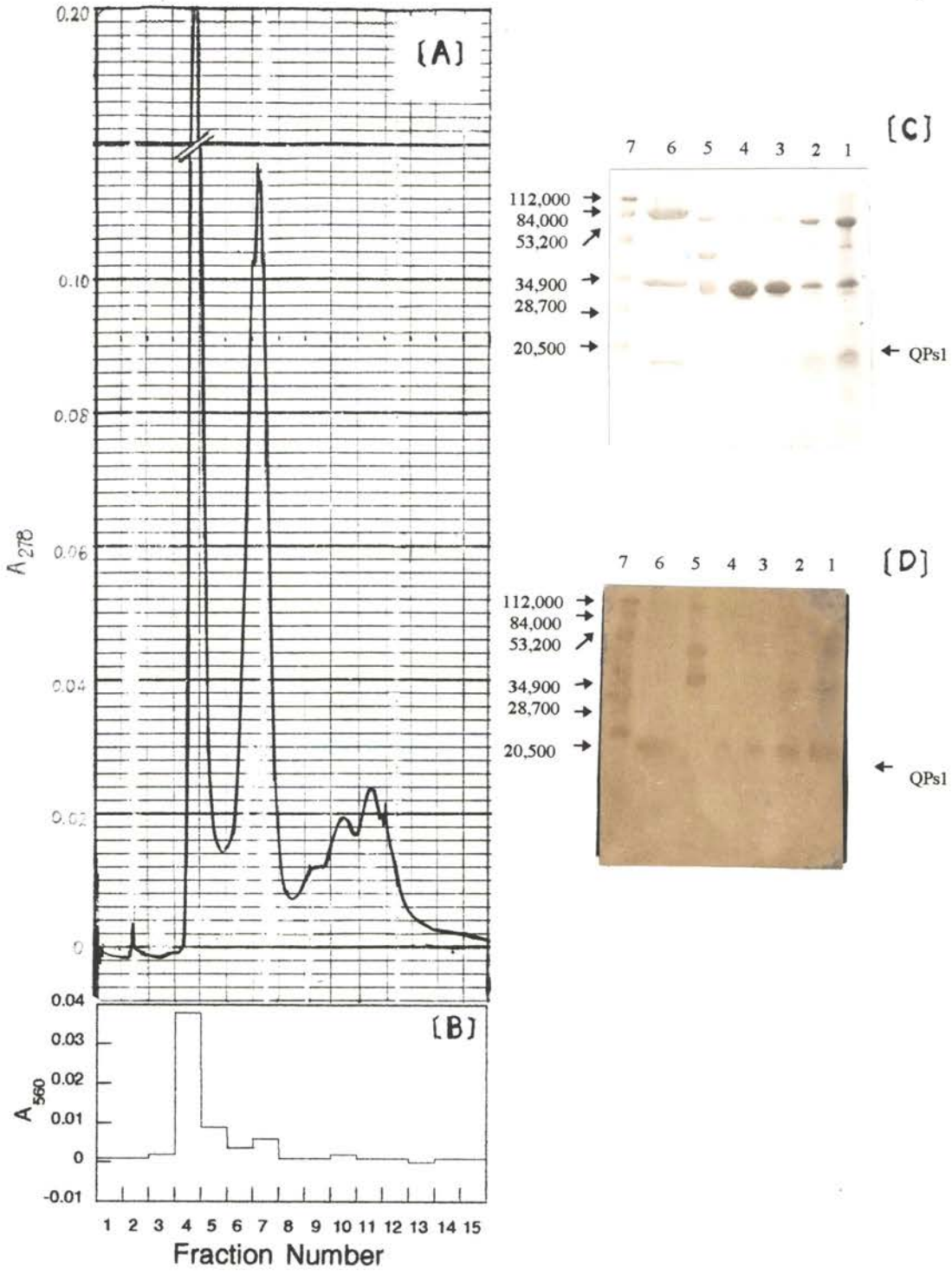
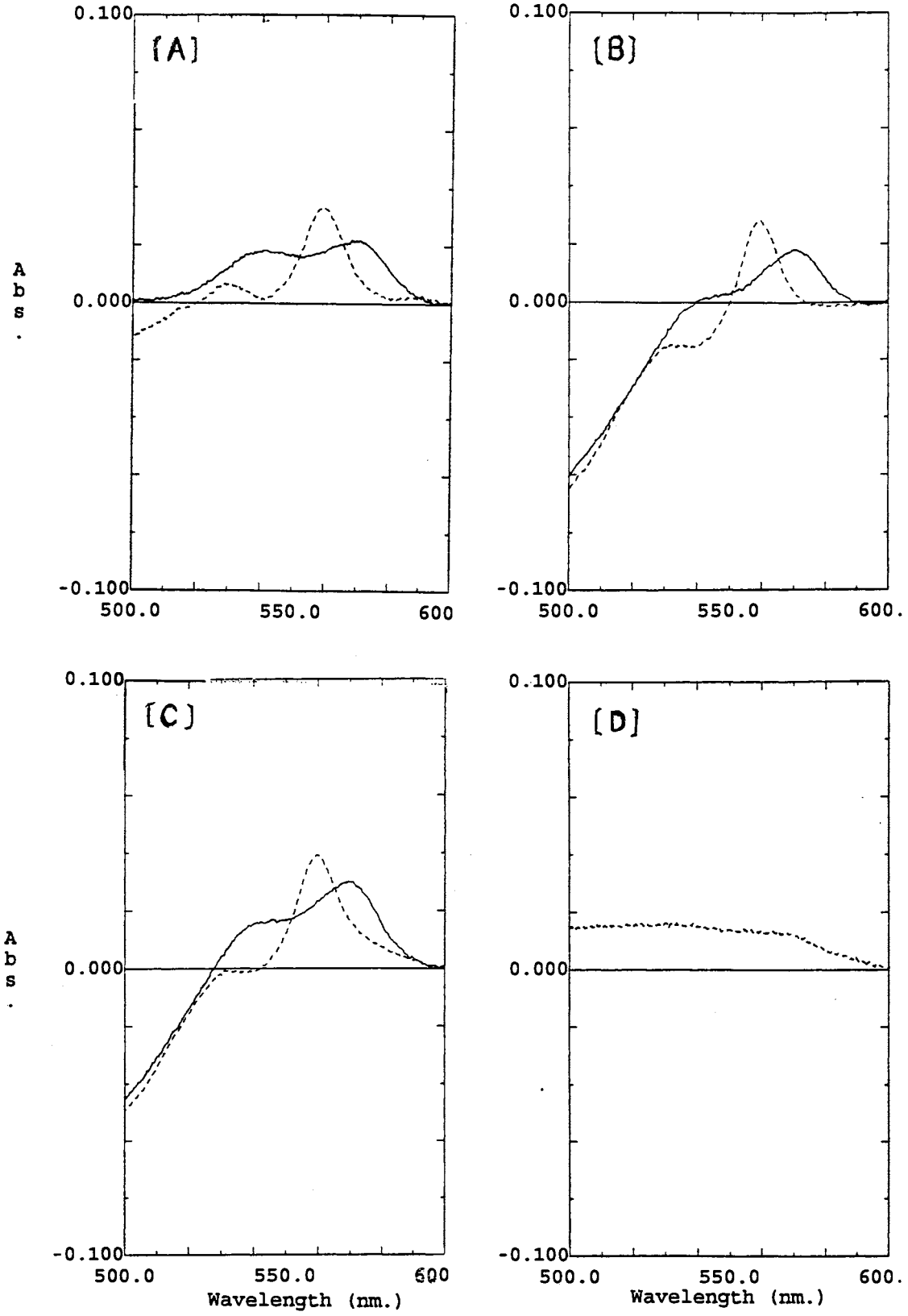
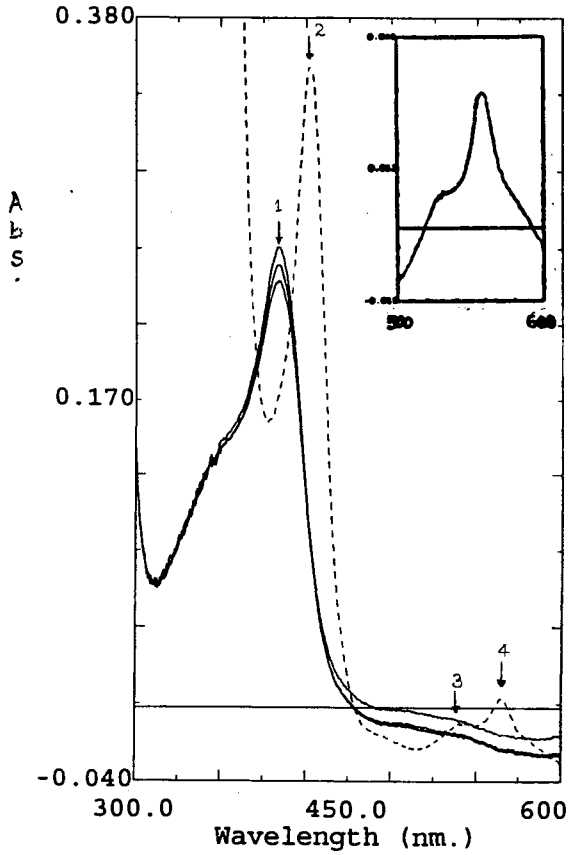

[A]
0.20
0.10
0.08
0.06
0.04
0.02
0
A_{278}
[B]
0.04
0.03
0.02
0.01
0
-0.01
A_{560}
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Fraction Number
[C]
7 6 5 4 3 2 1
112,000
84,000
53,200
34,900
28,700
20,500
QPs1
[D]
7 6 5 4 3 2 1
112,000
84,000
53,200
34,900
28,700
20,500
QPs1

Figure 21. Absorption Spectra and CO Reactivity of Cytochrome b_{560} in Heme-Reconstituted Recombinant GST-QPs1 in Presence or Absence of SDH. For spectral analysis, a divided cell, which is composed of two compartments, and in which the two samples can be mixed by gentle shaking, was used. One compartment contains 1ml of GST-QPs1 (1.5 mg/ml) containing 0.02% n-dodecyl maltoside and the other one contains 1ml of 0.1 M sodium/potassium phosphate buffer, pH 7.8, 20 mM sodium succinate, reconstitutively active SDH (3.5 mg/ml), or reconstitutively inactive SDH (3.5 mg/ml), respectively. For reference cell, the two solutions were kept separated and for sample cell, the two solutions were mixed, incubated for 30 min on ice, reduced by dithionite, and treated with CO. Absorption spectra of cytochrome after reduction (-------) and treatment with CO (_____) were obtained against the reference cell which had the same composition in each compartment as that of the sample cell. The reconstitutively active SDH was dealed undered anaerobic condition (under argon) and the reconstitutively inactive SDH was prepared by incubating the reconstitutively active SDH under aerobic condition for 2 hrs on ice. Absorption property and CO reactivity of cytochrome b560 in heme-reconstituted recombinant GST-QPs1 only (A) were compared with those in presence of reconstitutively inactive SDH (B) or active SDH (C). (D), Difference spectrum of CO reactivity in (C) minus that in (B).


[A]
[B]
[C]
[D]
0.100
0.000
-0.100
Abs.
500.0
550.0
600
600.
Wavelength (nm.)

reconstituted cytochrome b_{560} in recombinant GST-QPs1 fusion protein had a symmetrical α-absorption peak at 560 nm without the shoulder peak at 533 nm and totally reactive to carbon monoxide. Addition of reconstitutively active SDH to the heme-reconstituted fusion protein did not convert reconstituted cytochrome b_{560} from CO reactive to CO non-reactive (see Fig. 21B). As expected, no restoration of succinate-Q reductase was observed by this reconstitution. The failure to observe the interaction between SDH and reconstituted cytochrome b_{560} in GST-QPs1, through the detection of absorption spectral change and CO reactivity, may be due to the presence of GST in the NH_2-terminus of QPs1 or to the requirement of QPs2 or QPs3 for this interaction. Although cytochrome b_{560} spectral characteristics were restored to GST-QPs1 fusion protein upon addition of heme, it was not known whether the two histidines for liganding of cytochrome b_{560} were both from GST, or QPs1, or one from GST and one from QPs1 moiety of the fusion protein. GST contains 6 histidines and QPs1 has 7 histidines.

It was reported that BSA had binding affinity toward heme (17, 18). We observed a *b*-type cytochrome spectrum when heme was added to BSA (see Fig. 22). When heme in DMSO was added to bovine serum albumin (BSA) in 50 mM Tris-Cl buffer, pH 8.0, containing 0.25 M sucrose, a red shift of the soret absorption from 386 nm to 400 nm was observed. The absorption intensity at 400 nm decreased as incubation time increased and dithionite-reduced sample showed α absorption peak at 558 nm, β-absorption at 531, and Soret absorption at 422 nm. It should be noted that BSA contains 16 histidine residues.



1, 400nm; **2**, 421.8nm;
3, 531.2nm; **4**, 558nm

Figure 22. Absorption Spectra During Incubation of BSA with Heme and After Reduction By Dithionite. 1ml of BSA (2 mg/ml), in 50mM Tris-Cl buffer, pH 8.0, containing 0.25 M sucrose, was treated with hemin chloride (0.25 mole of heme / mole protein). The mixture was incubated at room temperature until no further change in oxidized Soret absorption peak (———) was observed. Total incubation time was about 2 hrs. At the end of incubation the sample was reduced by dithionite (------). The inset in each panel shows the difference spectrum of dithionite reduced minus oxidized form at α absorption region.

Isolation of b_{560} heme-containing QPs1

The observation of reconstitution of a *b*-type cytochrome in a solution of BSA to which heme has been added raised the possibility that the observed cytochrome b_{560} spectra in a solution of GST-QPs1 fusion protein to which heme has been added might result from heme ligation through histidine residues in the GST moiety of the GST-QPs1 fusion protein. On the other hand, the recent report of the bis-histidine ligand of cytochrome b_{556} of *E. coli* SQR being contributed by histidines from each sdh C and sdh D gene products (19) increased the possibility of involving both GST and QPs1 moieties in b_{560} heme ligation observed in cytochrome b_{560}-restored GST-QPs1 fusion protein. To determine the origin of histidine residues involved in heme ligation in cytochrome b_{560} reconstituted GST-QPs1 fusion protein, the reconstituted fusion protein was treated with thrombin and subjected to Superose-12 FPLC column chromatography separation of proteins. Figure 20A shows Superose-12 FPLC column profile of thrombin-cleaved, heme-reconstituted GST-QPs1 fusion protein. To avoid the complication resulting from the presence of free heme in reconstituted samples, 0.25 mole of heme was added per mole of recombinant GST-QPs1 fusion protein during reconstitution. The majority of reconstituted cytochrome b_{560} was found in a fraction eluted at the void volume of the column (see Fig. 20B). SDS-PAGE analysis of column fractions (Fig. 20C) confirmed that this b_{560} heme containing fraction was the QPs1 containing fraction. The QPs1 containing column fraction showed absorption characteristics (Fig. 23) identical to that observed in heme-reconstituted GST-QPs1 fusion protein, indicating that QPs1 is

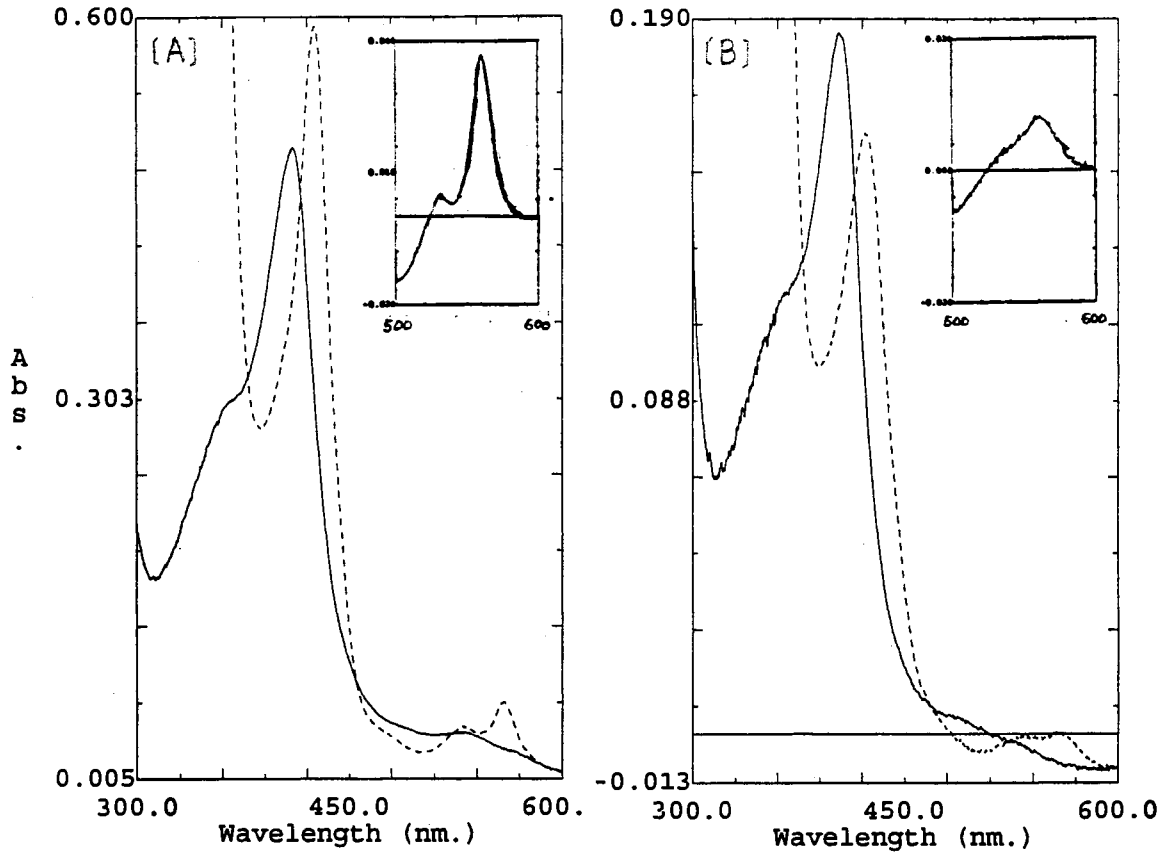


Figure 23. Absorption Spectra of cytochrome b_{560} in the Two Fractions Eluted from FPLC Chromatography. Absorption spectra of oxidized (———) and reduced (------) cytochrome b_{560} of isolated fraction 4 (A) and 7 (B) were detected. The inset shows difference spectrum of dithionite reduced minus oxidized cytochrome b_{560}.

responsible for heme ligation of cytochrome b_{560}. Since QPs1 contains seven histidine residues, which are the two hitidines involved in heme ligation is currently under investigation in our laboratory by using the site-directed mutagenesis approach.

References

1. Yu, L., Deng, K.-P., and Yu, C-A. (1995) *J. Biol. Chem.*, **270**, 25634-25638.
2. Chen, Y.-R., Yu, C. A., and Yu, L. (1996) *J. Biol. Chem.*, **271**, 2057-2062.
3. Yu, L.,Wei, Y.-Y., Usui, S. & Yu C. A. (1992) *J. Biol. Chem.* **267**, 24508-24515.
4. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Appendix 1, Cold Spring Harbor laboratory, Cold Spring Barbor, NY.
5. Altered Sites TM in Vitro Mutagenesis Systems, Technical Mannual, Promega Corporation.
6. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265-275.
7. Paul, K. G., Theorell, H., and Akesson, A. (1953) *Acta Chem. Scand.*, **7**, 1248-.
8. Yu, L., Xu, J-X., Haley, P. E., and Yu, C. A. (1987) *J. Biol. Chem.* **262**, 1137-1143.
9. Yu, C. A., and Yu, L. (1980) *Biochim. Biophys. Acta.* **591**, 409-420.
10. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
11. Hartman, J., Daram, P., Frizzell, R., Rado, T., Benos, D. J., and Sorscher, E. J. (1992) *Biotechnology & Bioengineering*, **39**, 828-832.

12. Strandberg, L., and Enfors, S.-O. (1991) *Appl. Environ. Micro.*, **57**, 1667-1674.

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

14. Moore, J. T., Uppal, A. U., Maley, F., and Maley, G. F. (1993) *Protein Expression and Purification*, **4**, 160-163.

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

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

17. Fagard, R., and London, I. M. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 866-870.

18. Datta, A., Haro, D., Sierra, J. M., and Ochoa, S. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 1148-1152.

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

CHAPTER IV

CLONING AND SEQUENCING OF cDNA ENCODING QPs2 OF SUCCINATE-UBIQUINONE REDUCTASE

While participation of QPs1 in Q-binding of SQR is established, the involvement of QPs2 and QPs3 in providing the Q-binding site is rather uncertain. When a Q-deficient, succinate-free SQR was photoaffinity labeled with [^{3}H]azido-Q derivatives, some of the radioisotope labels were detected in QPs2 and QPs3 (Chapter II) although the majority was found in QPs1. The radioactivities QPs2 and QPs3 were about 45% and 50% of that observed in QPs1. This 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 endogeneous Q present in the partially Q-deficient SQR. One way to test the Q-binding role of QPs2 and QPs3 is to attempt to isolate a [^{3}H]azido-Q linked peptide from [^{3}H]azido-Q labeled QPs2 or QPs3 obtained from [^{3}H]azido-Q labeled, Q-depleted SQR. However, identification of Q-bidning domains in QPs2 and QPs3 requires knowledge of the amino acid sequences of both subunits. It is best to obtain these two protein sequences by nucleotide sequencing because peptide sequencing of these proteins is experimentally difficult due to their resistance to proteolytic enzyme digestion.

In this chapter, I report the isolation of pure QPs2, production and purification of antibodies against QPs2, immunological screening of a bovine heart cDNA expression library in λgt11 to obtain a cDNA clone for QPs2, and nucleotide sequencing of five possible cDNA clones obtained for QPs2.

Experimental Procedures

Materials

Bovine serum albumin, DCPIP, Triton X-100, and sodium cholate were obtained from Sigma. Goat anti-rabbit IgG-alkaline phosphatase conjugate, protein A-horseradish peroxidase conjugate, SDS-PAGE molecular weight standards, alkaline phosphatase substrate kit, and DEAE Affi-Gel blue were purchased from Bio-Rad. Phenyl sepharose CL-4B and sephacryl S-300 SF were obtained from Pharmacia. Calcium phosphate was prepared according to Jenner (1), and mixed at a 3:1 ratio with cellulose powder prior to use in column chromatography.

Restriction endonucleases were obtained from either Promega Corporation or Bethesda Research Laboratories, Inc. Beef heart cDNA expression library in λgt11 was from Clontech. T4 DNA ligase were from Promega. DNase I, Type IV, RNase A, IPTG, X-gal, gelatin, BCIP, and NBT were from Sigma. TA cloning kits were from Invitrogen and Erase-a-Base System was from Promega. LB agar and LB broth base were from GIBCO. Nitrocellulose disks were from Schleicher & Schuell. PVDF membranes were from Millipore. Other chemicals were obtained commercially in the highest purity available.

Bacterial Strains, Phages, and Plasmids

E. coli Y1090r⁻[Δ*lac*U169Δ(lon, araD)139 *str*A *sup*F(trpC:Tn10)] was used as host for plaque screening and propagation of λgt11 recombinations. *E. coli* JM109[*rec*A1, *endo*A1, *gyr*A96, *thi*⁻, *hsd*R17, *sup*E44, *rel*A1 Δ(*lac*, *pro*)F'*tra*D36*pro*AB *lac*Iq, *lac*Z M15] was used as host for pGEM-3Zf (+) and pGEM-5Zf(+) plasmids. These plasmids, which were used for subcloning and sequencing, were obtained from Promega. *E. coli* strains were grown in LB medium (2). When necessary, $MgSO_4$ (10mM), maltose (0.2%), and ampicillin (100 μg/ml) were added.

Isolation of Pure QPs2 Protein

Submitochondrial particle (3), SCR (4), and SQR (5) were prepared according to methods reported previously. QPs was prepared from SQR by a Phenyl sepharose CL-4B (Pharmacia) column chromatography with different buffer systems. SQR obtained from the second calcium phosphate-cellulose column was diluted to 2 mg/ml with 50 mM Tris-Cl buffer, pH 7.4, containing 0.2 % sodium cholate, and incubated for 30 minutes at room temperature with stirring. The mixture was applied to a Phenyl sepharose CL-4B column equilibrated with 50 mM Tris-Cl buffer, pH 7.4, containing 0.2 % sodium cholate. The column was washed stepwise with three column volumes of each washing buffer. The washing buffers were 50mM Tris-Cl buffer, pH 7.4, containing 0.2 %, 1 %, 2 % sodium cholate, and 2 % sodium cholate with 4M urea. The crude QPs was eluted with 50 mM Tris-Cl, pH 7.4, containing 0.2 % SDS. Crude QPs was treated with 1 % SDS and 1 %

β-mercaptoethanol and subjected to high resolution SDS-PAGE. The QPs2 containing band was excised from the gel. Pure QPs2 was obtained by electrophoretic elution of the QPs2 containing gel slices. The purity of eluted QPs2 was confirmed by SDS-PAGE.

Production and Purification of Antibodies Against QPs2

Pure QPs2 was concentrated to about 3 mg/ml with Centricon-10, and precipitated by 50% acetone at 0°C. This treatment removed most of SDS and other water-soluble contaminants. The precipitate was washed once with H_2O, flushed with argon to remove the remaining acetone, and suspended in H_2O. The mixture was centrifuged for 1 hour at 150,000 x *g* to remove any water-soluble impurities and the trace acetone remaining in the solution. The precipitate thus obtained was suspended in H_2O to a protein concentration of 2 mg/ml. QPs2 suspension (0.5 ml) was emulsified with an equal volume of Freund's complete adjuvant and subcutaneously injected into a rabbit subcutaneously. Boosters of QPs2 (0.5mg) were given weekly for one month, blood was collected by cardiac puncture, and serum was obtained after clotting.

Antibodies against QPs2 were purified at 0-4 °C as described (7). 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 50 mM NaCl, and dialyzed against this same buffer. The dialyzed solution containing IgG was applied to a DEAE Affi-Gel blue column (5 times the serum volume) equilibrated with 20 mM Tris-HCl, pH 8.0, containing 50 mM NaCl. The effluent containing IgG was collected and concentrated by ultrafiltration with

an Amicon PM-30 membrane. IgG was further purified on a Sephacryl S-300 SF column equilibrated with phosphate buffer saline (PBS) to remove the contaminating IgM. IgG fraction was concentrated by ultrafiltration with Amicon PM-30 membrane to give a protein concentration of around 5 mg/ml. Sodium azide was added to give a final concentration of 0.02 %. Samples were frozen at -80°C until use.

DNA Manipulation

Restriction enzyme digestion, large-scale isolation and minipreparation of plasmid DNA, isolation and purification of lamda phage DNA were performed according to reported methods (2)

Immunological Screening of a Bovine Heart cDNA Library with Antibodies Against QPs2

The beef heart cDNA expression library in λgt11 was immunologically screened with antibodies against QPs2, using *E. coli* Y1090r$^-$ as host, essentially according to the method described previously (8, 9). Antibodies against QPs2 (30 μg/ml) were preincubated with *E. coli* lysate (a final protein concentration of 1mg/ml) for 1 hour at room temperature. This *E. coli* lysate treated anti-QPs2 antibodies were used as the primary antibody and Goat anti-rabbit IgG alkaline phosphatase conjugate was the secondary antibody. BCIP and NBT were used for as substrates to detect alkaline phosphatase activity.

DNA Sequencing

DNA sequencing was performed in our lab by the dideoxy method of Sanger (10) or at the Recombinant DNA/Protein Resource Facility at Oklahoma State University. The sequencing primers used were the T7, SP6 promoter primer, M13 forward, and M13 reverse which were obtained from Promega or synthesized by the Recombinat DNA/Protein Resource Facility at Oklahoma State University. The cDNA fragments generated from restriction enzyme digestion of QPs2 cDNA were subcloned into pGEM 3Zf(+) or pGEM-5Zf(+) plasmid (from Promega) and sequenced.

Electroblotting of QPs2 Protein onto a PVDF Membrane for Microsequencing

SQR was treated with 1% β-mercaptoethanol and 1% SDS for 2 hrs at 37 °C. Twenty-μl aliquots of the SDS and β-ME treated samples were applied to wells of an SDS-PAGE gel which had been pre-electrophoresed for overnight at 45 V with an anode buffer containing 0.2 M Tris-Cl, pH 8.9, and 0.1 mM sodium thioglycolate and a cathode buffer containing 0.1 M Tris, 0.1 M Tricine, 0.1% SDS, and 0.1 mM sodium thioglycolate, pH 8.2. The proteins in SDS-PAGE gel were electroblotted to a PVDF membrane according to the procedure of LeGendre *et al.* (6) with CAPS transfer buffer (10 mM CAPS, 10 % methanol, pH 11) for 15 min . After the transfer was complete, the membrane was rinsed several times with HPLC-grade H_2O and stained with Coomassie blue R-250 (0.1 % in 50 % methanol) for 2 min to visualize the proteins bound to the membrane. The blue band corresponding to QPs2 protein was cut out and subjected to NH_2-terminal sequence analysis.

Isolation of QPs2 protein for Amino Acid Sequencing

The conditions for SDS-PAGE of SQR were the same as those described in microsequencing. The QPs2 band in SDS-PAGE gel was excised and the protein was electrophoretically eluted from the gel slices. The eluted QPs2 was concentrated by membrane filtration with Centricon-10. The sample was washed several times with H_2O and concentrated to about 2 mg/ml to remove all the small molecular weight impurities which have absorbance at 278 nm. The concentrated QPs2 was precipitated with 50 % cold acetone (-20 °C) to remove SDS in the sample. The sample was air-dried to remove the residual acetone and subjected to peptide sequencing.

Amino Acid Sequence Determination

Amino acid sequence 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

Immunological Specificity of Antibodies against QPs2

When the immunological specificity of antibodies against QPs2 was characterized by Western blotting using QCR, SCR, SQR, *E. coli* SQR, *E. coli* lysate, and isolated QPs2 as antigens, no binding was found with SDH moiety of SQR, *E. coli* SQR, QPs1, and QCR. However, anti-QPs2 antibodies reacted with *E. coli* lysate and QPs3, in

addition to QPs2, suggesting that anti-QPs2 antibodies contained other IgGs which can react with *E. coli* cellular proteins and QPs3. The reason for the cross-reactivity of anti-QPs2 antibodies with QPs3 could be that the preparation of QPs2 was contaminated with QPs3 or QPs2 and QPs3 had common epitopes. However, when the antibodies were treated with *E. coli* lysate before using them in Western blotting, the cross-reactivity of the antibodies with both *E. coli* lysate and QPs3 disappeared (Fig. 24), implying that the cross-reactivity mainly came from non-specific binding with *E. coli* cellular proteins, some of which might have the same epitope as QPs3. Therefore, preincubation of anti-QPs2 antibodies with *E. coli* lysate prior to use for screening a beef heart cDNA library was necessary.

Isolation of a cDNA Encoding QPs2 from Beef Heart cDNA Expression Library in λgt11

1.5×10^6 plaques of the beef heart cDNA expression library in λgt11 were screened with anti-QPs2 antibody. Four positive clones with the highest signal intensity were isolated and purified. The cDNA inserts were excised from recombinant phage DNA by digestion with EcoRI and subcloned into pGEM-3Zf(+) or amplified with PCR and subcloned into pCR II by using the TA cloning kit (Invitrogen). The approximate molecular sizes of the inserts were 0.9, 0.85, 0.65, and 0.45 kb.

This beef heart cDNA library had previously been screened for QPs2 cDNA clones with anti-QPs2 antibodies which were prepared by using QPs2 protein. Antigen had been obtained from SDS-PAGE system of Laemmli (11) in the presence of 7 M urea. Preincubation of antibody with *E. coli* lysate was not included during the screening.

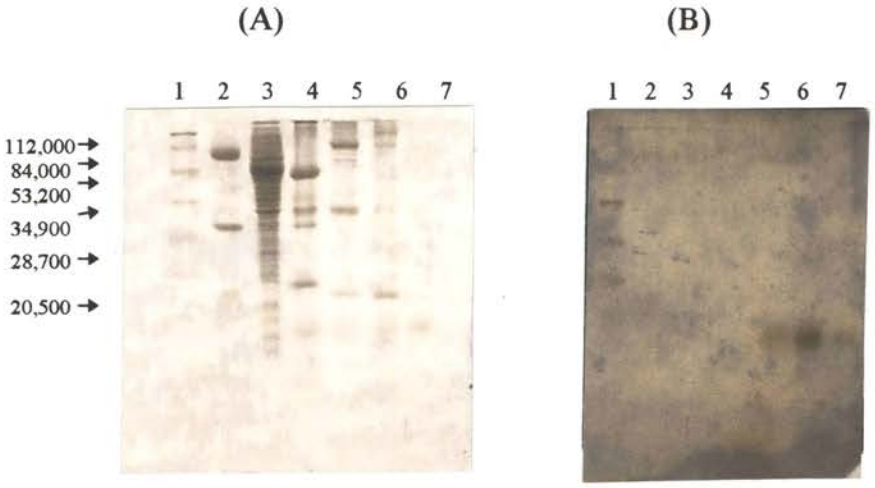


Figure 24. Western Blot Analysis of Antibodies Against QPs2. (a), SDS-PAGE of the presteined molecular weight standard (lane 1), *E.coli* SQR (lane 2), *E.coli* lysate (lane 3), ubiquinol-cytochrome c reductase (lane 4), succinate-Q reductase (lane 5), QPs eluted from hydrophobic column (lane 6), and the isolated QPs2 (lane 7). The prestained protein molecular weight standard contains phosphorylase *b* (112,000), bovine serum albumin (84,000), ovalbumin (53,200), carbonic anhydrase (34,900), soybean trypsin inhibitor (28,700), and lysozyme (20,500). SDS-PAGE was carried out according to the method of Schägger *et al.* containing 8 M urea in the separating gel (B), Western Blot of the proteins on gel (A).

One possible clone was isolated which had 1.1 kb of its insert size. For nucleotide sequencing of this clone, two EcoRI-HincII fragments were subcloned into pGEM-3Zf(+), and unidirectional deletions with Erase-a-Base System (Promega) (12) were generated.

Screening this beef heart cDNA library for QPs2 cDNA clones by a hybridization method had also been tried. Degenerate oligonucleotides were designed from the reported partial NH_2-terminal sequence (-SDSKAASL-) (14) or the partial sequence of a trypsin-digested peptide fragments (-AVAML-, -WTGER-) of QPs2. Unfortunately, no reasonable clone was obtained. Although several clones were found to have a part of the NH_2-terminal sequence, -S-D-S-, all of them resulted from false priming. Possible reasons for failure of obtaining a QPs2 cDNA by this hybridization method are: (a) the oligonucleotides have high degeneracy and are too short; (b) the partial peptide sequence of trypsin-digested fragments and the NH_2-terminal sequence are not of QPs2 peptides, but of contaminants. Isolated QPs2 is highly aggregated and may not be susceptible to protease digestion even after reductive carboxymethylation. The NH_2-terminal sequence for QPs2 reported by Cochran et al.(13), which is the same as that of QPs3, may be a result of contaminanation by QPs3.

The Nucleotide and Amino Acid Sequences Analysis of Obtained QPs2 cDNA clones

Figure 25-29 show the nucleotide and amino acid sequences of the five possible QPs cDNA clones obtained from the two rounds of immunological screening of the beef heart cDNA library in λgt11 with anti-QPs2 antibodies. The 0.9 kb insert has one possible ORF which codes 102 amino acids to give QPs2 molecular weight of 11,351 Da and a

```
    GAATTCGGGC GCGGGAGTAA ACAGCCCGAT CTGGGAAAGT GGAGCTGTGG CCACGTCCGG
    GTGCTGCAGG GCAGCGGCGG AGACGACGCA GGCCTATCCT CGGTGGCCGA C
112 ATG ACG GCC GCG GAT CAG AGC CCC CTG GCA CCG CTG TTG GAG ACT TTG GAA
1    M   T   A   A   D   Q   S   P   L   A   P   L   L   E   T   L   E
    GAC CCT TCC GCC TCC CTT GGA GAG CAG ACC GAC GCT TAC CTG ACC CTG ACC
18   D   P   S   A   S   L   G   E   Q   T   D   A   Y   L   T   L   T
    AGT CGT ATG ACT GGA GAA GAT GGA AAA GAA ATC ATT ACA GAA ATT GAG AAA
35   S   R   M   T   G   E   D   G   K   E   I   I   T   E   I   E   K
    AAA CTT CCT CAG CTA TTT AAA GTT TTA AAG ACT CAC ATT GCC AGT CAA AAC
52   K   L   P   Q   L   F   K   V   L   K   T   H   I   A   S   Q   N
    TCA GAC TCT AGA GGA CCC CGG GTA CCG AGC TCA GTA GTG CTG CTC TAC AGG
69   S   D   S   R   G   P   R   V   P   S   S   V   V   L   L   Y   R
    CCT TGG GGT TTT GCT TAT ATA ATC CCA AAA TTG CCT CTG GAT TAT CAG AGA
86   P   W   G   F   A   Y   I   I   P   K   L   P   L   D   Y   Q   R
    TAA
     *
    ATATTCAAGA ACTGCTTTCA AAACTGAATG ATATTGTTAA AAGTTCAGAT AAAAATGTAC
    GTACTAGGGC ACTTTGGGTG ATATCCAAGC AGACGTTTCC CACTGAAGTC GTTGGCAAAG
    TAGTTTCTAG TATCATTGAC TCACTAGAAA TAGTATTTAA TAGAGGAGAG

479 ATG CTT TCT GCT GTT GTG GAT TAT GAA GCA TTA AAT GTC ATT ATA AGA CTA
1    M   L   S   A   V   V   D   Y   E   A   L   N   V   I   I   R   L
    ATT GAA CAA GCC CCA GTT CAA ATG GGA GAA GAG TCA ATT AGG TGG GCA AAA
18   I   E   Q   A   P   V   Q   M   G   E   E   S   I   R   W   A   K
    TTG GTC ATA CCT TTA CTG GTT CAT TCA GCC CAA AAA GTA CAT TTG CGG GGA
35   L   V   I   P   L   L   V   H   S   A   Q   K   V   H   L   R   G
    GCC ACT GCT CTA GAG ATG GGA ATG CCT TTG TTG CTT CAG AAA CAG CAA GAA
52   A   T   A   L   E   M   G   M   P   L   L   L   Q   K   Q   Q   E
    ATA GCA TCT ATC ACA GAG CAG CTT ATG ACT ACC AAA TTA CTT TCA GAA CTC
69   I   A   S   I   T   E   Q   L   M   T   T   K   L   L   S   E   L
    CAG AAA CTG TTT ATG AGT AAA AAT GAG ACT TAT GTG TTA AAA TTG TGG CCT
86   Q   K   L   F   M   S   K   N   E   T   Y   V   L   K   L   W   P
    TTA TTT GTC AAA CTT CTC GGG AAG CCC CCG AAT TC
103  L   F   V   K   L   L   G   K
```

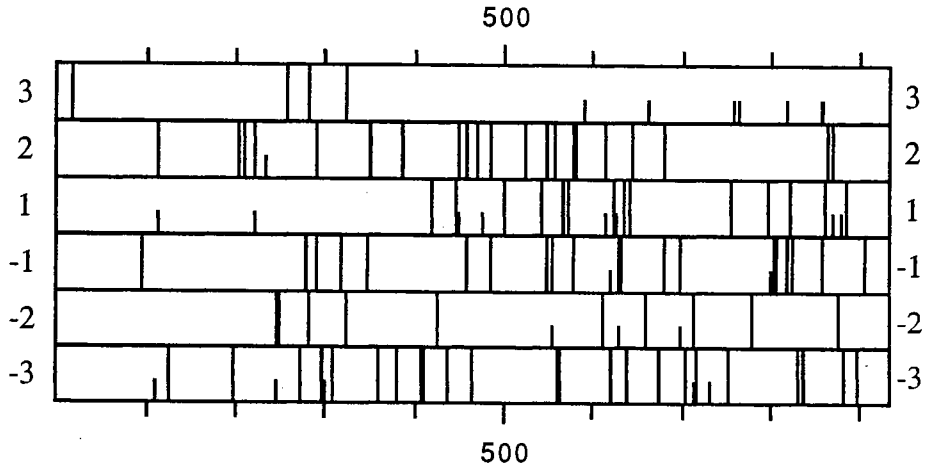


Figure 25. Nucleotide Sequence and ORF Map of a cDNA (0.9 kb insert) Encoding Bovine QPs2 and Deduced Amino Acid Sequence of Possible ORF.

```
1    GAATTCGGGC AGTTGTTCCC CCGGAGCTCT GGGAACATTG CCCTGCCTGA CAACATTCTT
61   GTCTGGGCAA GGGCAGTGTG ACCTTGAGTG GCACTTGCCA

101  ATG GGC CTC CTG CCT GGT GAC CAC TTA TGT CCC TTG CAT TTT TCT ATC TGT
1     M   G   L   L   P   G   D   H   L   C   P   L   H   F   S   I   C
152  GGT CAT GGC CCT GCC TCC TTG CGA AGT CTG GCT CTG TTG AGG ACC TGT CTG
18    G   H   G   P   A   S   L   R   S   L   A   L   L   R   T   C   L
203  GCA GAT GCC AGC GTC GGG GTC CAG GGC TCG TCT GGC CCT GGA GCA TCA TCA
35    A   D   A   S   V   G   V   Q   G   S   S   G   P   G   A   S   S
254  CAA GGT CTG GTG GGT ATG AAT ATG TCT GGT TCT CTG TCT ACC TGG AAG GAA
52    Q   G   L   V   G   M   N   M   S   G   S   L   S   T   W   K   E
305  GGA GGC AAC CAG GGG ACT GGT TCC CAG TCC TGG TTC CCA GCC TGC CCT GAG
69    G   G   N   Q   G   T   G   S   Q   S   W   F   P   A   C   P   E
356  GAT TCA GTC TCT TCT TGG GGT CAA GAA TGG ATT CCT GTG CAG TGA
86    D   S   V   S   S   W   G   Q   E   W   I   P   V   Q   *

401  ATCAGTCTTG GCTGTGACAG AGGAGGCCAG TCCTGATGAC CAAGATGAAT ATTCGCTGTG
461  TCTGGGCCAA GAAATTAGAA CCTTTAGGTG GGCAGGAACA GAAGGTCCCA CCGGCACACA
521  CAGTCCAGAG TCATGAAGAG AGTGAAGTGG AAAAGTTTCT GGGGGCTCAG CCTGGGGGGC
581  AGATGCCAAA GTCAAAAGCA GTCAAAAGTC AAAAATTATG ACTTCCGTGG TAGTCCAGTG
641  GTTAAGAATC TGCCTGCAGT GCCAGGGGAC ACGGGTTCCG ATTCCAGGTC CGGGAAGATC
701  CCACATGCTG CAGGGTGACT AAGCTCGGAC ACCACGACCG CTGGGCCCAC ACTGGAGAGC
761  CTGCAAGCTG TAACTGTTGA AGCCTGTCTG CCCACACACC ACAGTTAGAG AGTAGCCCCG
821  GCTCGCTGCA ACTGGAGAAA GCCTGTGAGC AGCAATGAAG ACCCAGCACA GCCAAAAAAA
881  TATTAAAAAA ATTTTAAGTT AAAAAAAAAC CCGAATTC
```

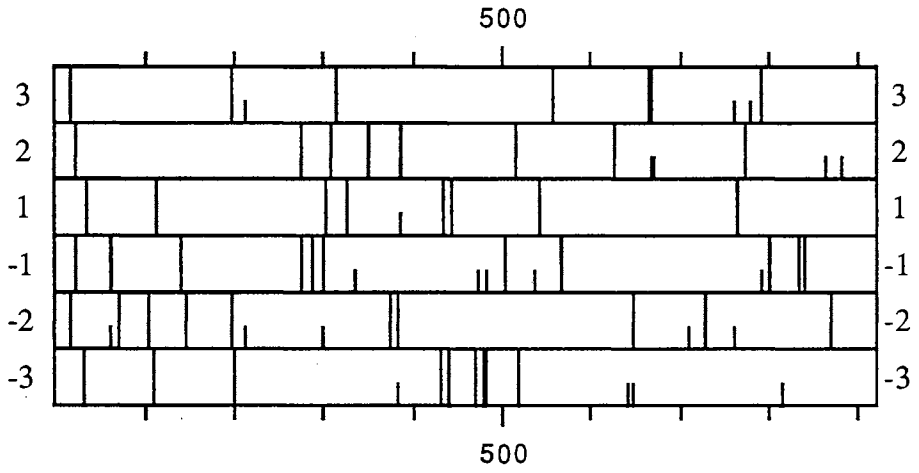


Figure 26. Nucleotide Sequence and ORF Map of a cDNA (0.85 kb insert) Encoding Bovine QPs2 and Deduced Amino Acid Sequence of Possible ORF.

```
1     GAATTCGGGG GGAAGCAAGG GGGAAGGACA GATACTGATT GGCTCAGCAA GTGCCTTATG
61    GGATCCATCA AACCATGCCC TAGAGGCTAG TTTTCTCTGT AGGTTACTTT TTTTTTCCTG
121   CCAACCAGAA TCTATGGAAG TCTAGGGACA GTTTTTACTT TACGCTGTAT ATTTTCTAGC
181   AGCTAGCATT ACAGTCTGGA GAAGGCAATG GCACCCCACT CCAGTACTCT TGCCTGGAAA
241   ATCCCATGGA TGGAGGAGCC TAGTAGGCTT CAGTCCATGG GGTCACTAAG AGTTGGACAC
301   GACTGAGCGA CTTCACTTTC ACTTTTGACT TTCATGCATT GGAGAAGGAA ATGGTAACTC
361   ACTCCAGTGT TCTTGCCTGG AGAATCCCAG GGATGGGGGA GCCTGGTGGG CTGCCGTCTA
421   TGGGGTCGCA CAGAGTCAGA CATGACTGAA GTGACTTAGC AGCAGTAGCA GCAGCATTAC
481   AGTCAGTGCA CAACCAATAT TAGCTAATTA GTAGCTAGTA CAAGGTTATG CCCATGCAAT
541   GCAAAAACTC TCTGCTTCAG TGGATGCTCT GTCTGCCTTC TTGGTTGTAC AGCATCCGAG
601   CCACATCTCA CATTTTCTCC AAGACCCCTT ATTTGAAAAC AAAATGTTCA GCTTTAAAAA
661   CTCTCTGCTT CAGTGGATGC TCTGTCTGCC TTCTTGGTTG TACAGCATCC GAGCCACATC
721   TCACATTTTC CCCGAATTC
```

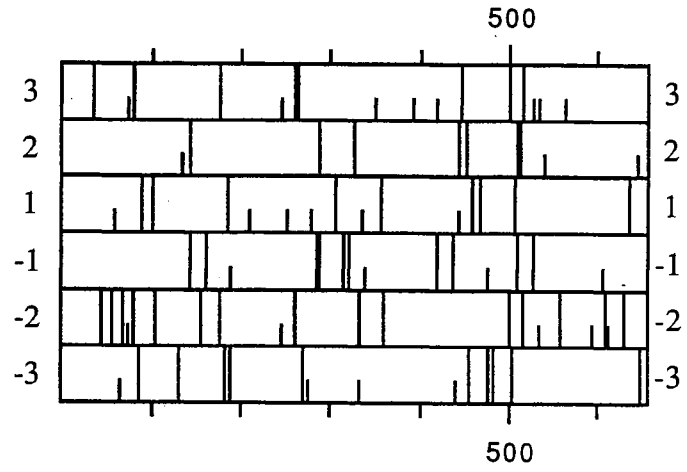


Figure 27. Nucleotide Sequence and ORF Map of a cDNA (0.65 kb insert) Encoding Bovine QPs2 and Deduced Amino Acid Sequence of Possible ORF.

```
1    GAATTCGGGC GCCAGCTGCA GCTGGGCGAG CGCTCCGTGC AGGAGGTGGC TGAGGTGGAA
61   GCTGTGCGGC GGGCCCTGCG GGGCTCGGGT GTCATTGCAG GCGTGTGGCT TGAGGAGGCG
121  AGGCAGAAAC TAAGCATTTA TGAGGCTCTG AAGAAAGAGT TGCTGCAGCC AGAGGCAGCT
181  GTCGCCCTGC TCGAGGCCCA GGCTGGCACC GGGCATGTCA TCGACCCTGC CACCAGCGCA
241  CGGGTGACTG TGGATGAGGC GGTGCGCGCC GGCCTGGTGG GCCCTGAGCT GCATGAGAAG
301  CTGCTATCGG CCGAGAAGGC TGTGACTGGC TACAAGGACC CCTACTCAGG GGAGAGCGTG
361  TCCCTGTTCC AGGCCCTGAA GAAGGGCCTC ATTCCCAGGG AGCAGGGCCT GCGTCTACTG
421  GACGCCCAGC TGTCCACGGG GGGCATCGTG GACCCCAGCA AGAGCCACCG TCTGCCCCTG
481  GATGTTGCCT GTGCCCGCGG CTACCTGGAT AAGGAAACCA TCACCGCCCT CACAGCGCCC
541  AGAGATGACG CCCCCGAATT C
```

```
1   RQLQLGERSV QEVAEVEAVR RALRGSGVIA GVWLEEARQK LSIYEALKKE
51  LLQPEAAVAL LEAQAGTGHV IDPATSARVT VDEAVRAGLV GPELHEKLLS
101 AEKAVTGYKD PYSGESVSLF QALKKGLIPR EQGLRLLDAQ LSTGGIVDPS
151 KSHRLPLDVA CARGYLDKET ITALTAPRDD A
```

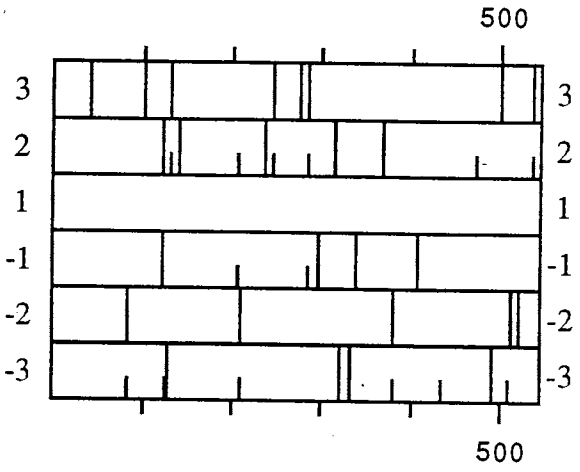


Figure 28. Nucleotide Sequence and ORF Map of a cDNA (0.45 kb insert) Encoding Bovine QPs2 and Deduced Amino Acid Sequence of Possible ORF.

```
1     GAATTCGGGG GGCTGTCCGC ATGAAGTTAA GTGCCGTTAA AGATGTTGTG CGTGGCAAAA
61    ATGTTGTCTT GGTGGATGAT TCAATTGTTC GTGGGACAAC CTCAATGTTT ATTGTACGGA
121   TGTTAAAAGA AGCCGGCGCC AAGTCAGTGC ATGTGCGTAT TGCCAGTCCA GTCTTTAAAT
181   TCCCATCATT TTATGGGATT GATATGCAAA CCACTGCTGA ATTAATGGGT GCTAATAATA
241   CGCTAGATGA GATGCGCGAC AAGATTGAGG CGGATTCGCT TGGTTTCTTG ACTGTCGATG
301   CGTTAGTCAA GGCGATTGAT TTACCCTATG ATGGTGAAGG CACAGGTCTG ACAACGGCGT
361   ACTTTGATGG TCACTATCCA AGTCCAATCT ATGATTATGA AGACAGTCTA GCTGACTTTG
421   TGGCAACGGG TGCGGTAGAC TTTACTGCTG AACCAACGAC CACCTATCAT CCCCGCCAAG
481   TCGCTTCGTT ACGCAACGAA GAAATCATGG CTGATGGGAC GATCCATTTA GATTCACAAA
541   CAATTCAGGG AGTAGAAGT

560   ATG ACC GAA CAA AAC GCT TAT CAG CAA GCC GGC GTC GAC ATT AAA GCC GGT
1      M   T   E   Q   N   A   Y   Q   Q   A   G   V   D   I   K   A   G
611   GAA GAA GCC GTC GAA TTG ATG AAA GAT GCG GTC GCT GAT ACG TAT AAT GCA
18     E   E   A   V   E   L   M   K   D   A   V   A   D   T   Y   N   A
662   CAA GTT TTG GAT GGC TTA GGC GGC TTT GGG GCA GCT TTT GCT TTA GGT AAT
35     Q   V   L   D   G   L   G   G   F   G   A   A   F   A   L   G   N
713   CAG TAC CAA AAC CCT GTC TTG GTA TCG GGT GCT GAT GGT GTC GGG ACA AAG
52     Q   Y   Q   N   P   V   L   V   S   G   A   D   G   V   G   T   K
764   TTG TTA CTA GCG ATT GCG GCT GAT AAA CAC GAT ACG ATT GGA CAA CAT CTT
69     L   L   L   A   I   A   A   D   K   H   D   T   I   G   Q   H   L
815   GTG GCG ATG GTG GCT AAC GAT ATT TTG GCA CAA GGG CCA AGC CGG CAT TTA
86     V   A   M   V   A   N   D   I   L   A   Q   G   P   S   R   H   L
866   TGT TGG ATT ATC TTG CCG TGG ATA AGA TGC GAC CAG CAG TCG TGG CAG AAA
103    C   W   I   I   L   P   W   I   R   C   D   Q   Q   S   W   Q   K
917   TTG TCA CGG GTG TGG CCA AGG CTG CCA AAG CTT CAG GGA TGG CGT TAA
120    L   S   R   V   W   P   R   L   P   K   L   Q   G   W   R   *

965   TTGGTGGTGA AAGTGCAGAA TTACCAGGTC TTTATGCACC TAAGCACTAT GATTTGGCGG
1025  CATTTGCTGT TGGTGTGGCT GAGCAAGACC AACTCTTAAA TCCTAAAAAC GTGGCGGTTG
1085  GCGATGTTTT GATTGGTTTG CCATCTTCTG GAATTC
```

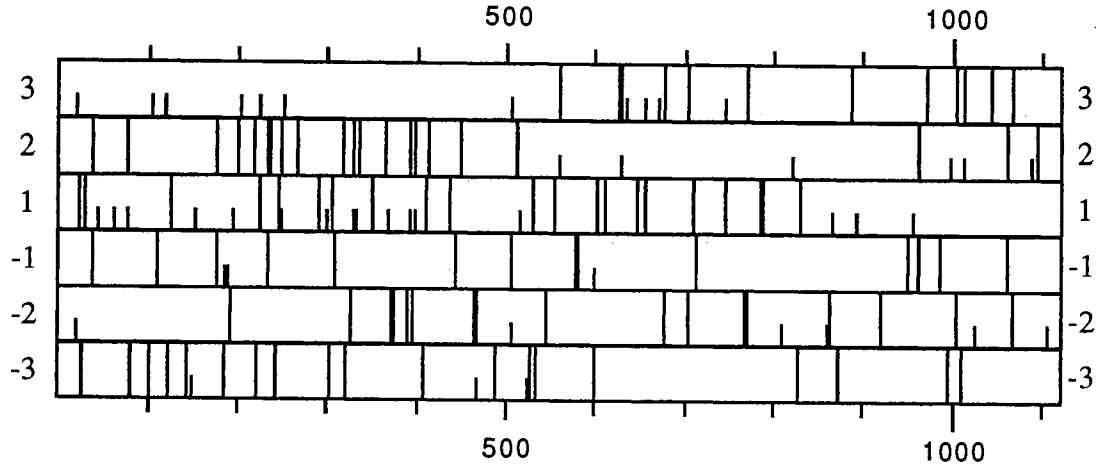


Figure 29. Nucleotide Sequence and ORF Map of a cDNA (1.1 kb insert) Encoding Bovine QPs2 and Deduced Amino Acid Sequence of Possible ORF.

partial ORF which codes 110 amino acids. The 0.85 kb insert shows one reasonable ORF which codes 99 amino acids to give QPs2 molecular weight of 10,120 Da. The 0.65 kb insert does not give any reasonable ORF, but the possibility of partial clone can not be excluded. The 0.45 kb insert, also, generates one full reading frame without any start or stop codon. The 1.1 kb insert shows a reasonable ORF which codes 134 amino acids to give QPs2 molecular weight of 14,320 Da. The possibility that a presequence exists may reduce the size of the true mature protein.

QPs2 is a highly hydrophobic protein and believed to have transmembrane helix(ces) in its structure. Possibility for transmembrane helix formation and hydrophobicity of the protein could be partly examined by hydropathy plot of its amino acid sequence. The hydropathy plots of the five possible ORF are shown in Figure 30. The first ORF of 0.9kb insert yields a relatively hydrophilic protein with a small hydrophobic portion at COOH-terminus and the partial ORF of 0.9kb insert gives QPs2 two possible transmembrane helices. The ORF of 0.85kb insert allows QPs2 to have one hydrophobic part at the NH_2-terminus and the 0.45kb insert produces a large protein without any possible transmembrane helix. The ORF of 1.1kb insert yields four QPs2 hydrophobic portions with possible helices structure.

However, the amino acid sequence of the ORF in 0.85 kb insert was found to have weak similarity to fibrinogen, 0.45 kb insert 60% identity to rat Plectin, and 1.1 kb insert 71% identity to chicken 5-aminoimidazole ribotide synthase according to BLASTp searches. Therefore, the possibility of these three polypeptides to represent QPs2 may be ruled out.

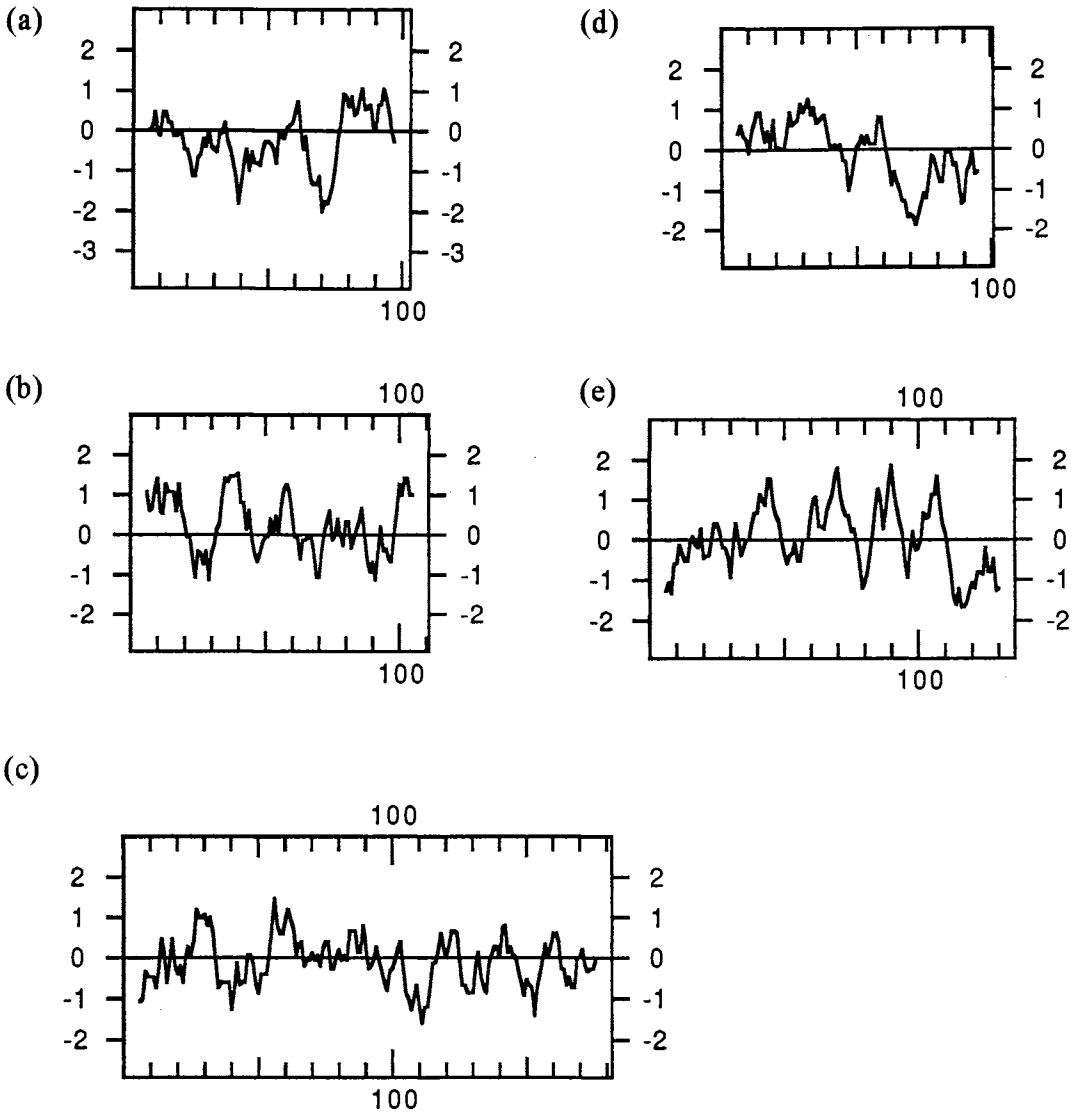


Figure 30. Hydropathy plots of Five Possible QPs2 Sequences: (a), the first ORF of 0.9kb insert; (b), the partial ORF of 0.9kb insert; (c), the full region of 0.45kb insert; (d), the ORF of 0.85kb insert; and (e) the ORF of 1.1kb insert.

Confirmation of QPs2 cDNA Clone

Although we have all five possible cDNA inserts for QPs2 sequenced, at present , no conclusion can be made because confirmation of QPs2 cDNA clone met with experimental difficulty. Several confirmation methods have been tried, but failed. These are: determination of the partial NH_2-terminal sequence of QPs2 either by microsequencing of the protein blotted on a PVDF membrane or by regular NH_2-terminal sequencing. Since SQR was used as a starting material for isolation of QPs2, a blue-native gel electrophoretic step (14) was introduced to further purify the SQR. The blue gel containing SQR was sliced and subjected to high resolution SDS-PAGE. Pure QPs2 blotted on a PVDF membrane or eluted from gel slices of SDS-PAGE of SQR was used for NH_2-terminal amino acid sequence analysis.

All the NH_2-terminal amino acid sequence obtained showed the similar results with two heterogeneous sequences (Fig. 31). SDS-PAGE of SQR, sometimes, showed two bands with similar sizes on the QPs2 position. These two bands reacted with anti-QPs2 with equal intensity. The observation of heterogeneous NH_2-terminal amino acid sequences on QPs2 preparation and of two bands on SDS-PAGE can be explained as follows: (a) QPs2 preparation has the other impurity with similar size and amount; (b) these two NH_2-terminal amino acid sequences obtained may originate from two other contaminants and the NH_2-terminus of QPs2 is modified post-translationally; or (c) some of QPs2 has been degraded during purification. It should be noted that the partial NH_2-terminal sequences observed in QPs2 preparation do not match with any of the NH_2-

(A) QPs2 isolated from PVDF membrane of SQR obtained form calcium phosphate-cellulose column chromatography

primary:	Ser	Pro	Ser	Lys	Glu	Ala	?	Leu
secondary:	Ile	Gly	Leu	Thr				

(B) QPs2 isolated from PVDF membrane of SQR obtained by blue native SDS-PAGE after calcium phosphate-cellulose column chromatography

primary:	Ser	Pro	Ser	?	Ala
secondary:	Ile	Gly	Leu		

(C) QPs2 isolated from the gel slices of SDS-PAGE of SQR obtained by blue native SDS-PAGE after calcium phosphate-cellulose column chromatography

primary:	Gly	His	Ser	His	Pro
secondary:	Ile	Pro	Leu	Lys	Ala
				Ala	His

Figure 31. The Partial NH_2-Amino Acid Sequence Analysis of QPs2.

terminal amino acid sequences of QCR subunits, indicating that SQR preparation is not contaminated with QCR.

A great effort has also been directed to confirm the isolated cDNA clones by internal peptide sequence of QPs2. Pure QPs2 obtained by the described method is highly aggregated and resistant to protease digestion. We have tried to subject the protein to reductive carboxymethylation before being digested by trypsin or chymotrypsin. When the digested, soluble fraction of the sample was applied onto Synchropak RP-8 column (0.46 x 25 cm) using a gradient formed from 0.1 % trifluoroacetic acid and 90 % acetonitrile containing 0.1 % trifluoroacetic acid with a flow rate of 1 ml/min, several hydrophilic peptide peaks were revealed. However, their peptide sequences do not match with any of the obtained QPs2 sequences deduced from nucleotide sequencings. It is highly possible that these peptides are contaminants and the QPs2 protein was totally unsusceptible to trypsin or chymotrypsin digestion.

References

1. Jenner, E. L. (1973) U. S. Patent 3737516.
2. Sambrook, J., Fritsh, E. F., and Maniatis, Y. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
3. Harmon, H. J., and Crane, F. L. (1976) *Biochim. Biophys. Acta*, **440**, 45-58.
4. Yu, C. A., and Yu, L. (1980) *Biochemistry*, **19**, 5715-5720.
5. Yu, L, and Yu, C. A. (1982) *J. Biol. Chem.*, **257**, 2016-2021.

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

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

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

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

10. Sanger, F., Nicklen, S., and Coulson, A. R. (1979) *Proc. Natl. Acad. Sci. USA*, **74**, 5463-5476.

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

12. Generation of Unidirectional Deletions with the Erase-a-Base System, (1991) Promega Protocols and Applications Guide, Promega Corporation.

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

14. Schägger, H., and von Jagow, G. (1991) *Anal. Biochem.*, **199**, 223-231.

SUMMARY

The amino acid sequence of QPs1 was revised by addding 8 amino acid residues, LGTTAKEE-, to the NH_2-terminus of reported sequence. This revision was made through the success in obtaining the partial NH_2-terminal amino acid sequence of QPs1. The use of pre-electrophoresed SDS-PAGE gel and inclusion of 0.1 mM sodium thioglycolate in the running buffer prevented blockage of the NH_2-terminus during electrophoresis, thus make sequencing possible. The molecular weight of QPs1 is 15,149.

The cDNA encoding QPs1 is 1003 bp long with a partial open reading frame of 492 bp which encoded a 140-amino acid mature protein and a 24-amino acid presequence. The presequence of QPs1 has typical mitochondrial signal peptide sequence. It has a preponderance of basic (Arg and Lys) and hydroxylated (Ser and Thr) residues, and the lack of acidic residues (Glu and Asp) to pass the mitochondrial membrane posttranslationally. QPs1 is more likely to be processed by a two-step cleavage showing the similar consensus sequence (RAHLS) without arginine on -2 upstream position.

The identification of QPs1 as the Q-binding protein in SQR is achieved by photoaffinity labeling technique using azido-Q derivatives. When succinate-free SQR was incubated with 3-azido-2-methyl-5-methoxy-6-(3,7-dimethyl[^{3}H]-octyl)-1,4-benzoquinone ([^{3}H]azido-Q) in the dark for 20 min at 0 °C and then subjected to illumination with a long

wavelength UV light for 7 min at 0°C, 35% of SQR activity was inactivated and radioactivity was found predominantly in QPs1. Since the extent of SQR inactivation upon illumination correlated with the amount of radioactivity labeling on QPs1, the participation of QPs1 in Q-binding of SQR is obvious.

The Q-binding domain in QPs1 is identified by peptide sequencing of a tryptic [^{3}H]azido-Q peptide. The [^{3}H]azido-Q peptide was obtained by HPLC separation of the tryptic digested, carboxymethylated, [^{3}H]azido-Q labeled QPs1. The [^{3}H]azido-Q labeled QPs1 was obtained from [^{3}H]azido-Q labeled SQR by preparative SDS-PAGE followed by electrophoretic elution of QPs1 protein from gel slice. The partial NH_2-terminal sequence of tryptic [^{3}H]azido-Q peptide are GLTISQL-, which corresponds to residues 113-119 of QPs1 sequence. The Q-binding domain is most likely located in the sequence connecting transmembrane helices 2 and 3.

QPs1 has been successfully expressed in *E. coli* DH5α cells as a GST fusion protein using a constructed QPs1 expression vector, pGEX/QPs1. Production of recombinant GST-QPs1 fusion protein in *E. coli* DH5 α cells harboring pGEX/QPs1 plasmid is temperature, induction time, and growth media dependent. Maximum production of GST-QPs1 fusion protein, which accounts for 10.2 % of total cellular protein, was obtained when cells were grown for 3 hrs at 27 °C in enriched medium containing 440 mM sorbitol and 2.5 mM betaine after addition of IPTG. About 40 % of the total recombinant GST-QPs1 fusion protein produced are in the soluble form; the rest are in inclusion body precicipates. The GST-QPs1 fusion protein in inclusion body precipiate can be solubilized by 8 M urea treatment followed by dialysis. The recombinant

soluble GST-QPs1 fusion either produced in cell-free extract or solubilized from inclusion body precipitates can be purified to homogeneity with a apparent molecular weight of 41 kDa by a glutathione agarose gel column chromatography.

Although QPs1 has been believed to be cytochrome b_{560} and the heme is thought to be ligated to the protein moiety by two conserved histidines located on the surface of the membrane, the recombinant GST-QPs1 fusion protein contains little cytochrome b_{560} heme moiety. Cytochrome b_{560} could be reconstituted by addition of hemin chloride in DMSO to isolated soluble recombinant GST-QPs1 fusion protein. Maximum incorporation of heme to the isolated GST-QPs1 fusion was less than 0.5 mole of heme per mole protein which corresponded to the purity of the isolated protein. The cytochrome b_{560} heme in recombinant heme-reconstituted GST-QPs1 was reactive to carbon monoxide.

The heme-reconstituted recombinant QPs1 was obtained from reconstituted recombinant GST-QPs1 by thrombin treatment at room temperature for 1hr in the presence of 0.01% n-dodecylmaltoside followed by removing GST and uncleaved GST-QPs1 fusion protein from the sample by Superose-12 (Pharmacia) gel filtration. Purified recombinant heme-containing QPs1 is highly aggregated with an apparent molecular weight of over one million in 50 mM Tris-Cl, pH 8.0, containing 150 mM sodium chloride and 0.01% dodecylmaltoside. This result indicates that the two histidines involved in ligation of heme exists in QPs1.

Possible cDNA clones encoding QPs2 have been obtained by immunologically screening of the beef heart cDNA library in λgt11 with antibodies against QPs2.

Antibodies against QPs2 were raised in rabbits and purified. Purified antibodies against QPs2 have high titer as detemined by ELISA and are immunologically specific to mitochondrial QPs2 as determined by Western Blot. Five clones with highest signal intensity were isolated, purified, and sequenced. However, confirmation of cDNA clone for QPs2 has met with difficulty due to the inability to obtain the NH_2-terminal partial amino acid sequence or internal peptide sequences of QPs2.

VITA

Gyesoon Yoon Lee

Candidate for Degree of

Doctor of Philosophy

Thesis: QUINONE-BINDING PROTEINS IN BOVINE HEART MITOCHONDRIAL SUCCINATE-UBIQUINONE REDUCTASE

Major Field: Biochemistry and Molecular Biology

Biographical:

Personal Data: Born in Seoul, Korea, On January, 1962, the daughter of Young Yoon and Jung-Whan Kim.

Education: Graduated from Han-Sung Women's High School, Seoul, Korea in Febrary 1980; received Bachelor of Pharmacy degree from Kyung-Hee University, Seoul, Korea in Febrary 1984; received Master of Pharmacy degree from Kyung-Hee University, Seoul, Korea in Febrary 1986. Completed the requirements for the requirements for the Doctor of Philosophy degree with a major in Biochemistry and Molecular Biology at Oklahoma State University in May 1996.

Abstracts Presented at Meetings: Cloning and Sequencing of a cDNA Encoding QPs2 of Bovine Heart Mitochondrial Succinate-Ubiquinone Reductase. (1994) *Biophysical Journal* **66**, A364; Ubiquinone Binding Domain in Cytochrome b_{560} (QPs1) of Bovine Heart Mitochondrial Succinate-Ubiquinone Reductase. (1995) *Biophysical Journal* **68**, A319. and (1994) *37th Annual West Central States Bioch. & Mole. Biol. Conference*. P-19.

Publications: Identification of the Ubiquinone-binding Domain in QPs1 of Succinate-Ubiquinone Reductase. (1995) *J. Biol. Chem.* **270**, 6193-6198.

Professional Memberships: Korean Pharmacist Association; American Association For the Advancement of Science; Korean-American Scientist and Engineers in America.