PROPERTIES OF THE UBISEMIQUINONE RADICAL IN ISOLATED UBIQUINOL-CYTOCHROME *c* REDUCTASE FROM *RHODOPSEUDOMONAS SPHAEROIDES*, R-26

By

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NOMENCLATURE

aa	Antimycin A
ATP	Adenosine 5'-triphosphate
°C	degree centigrade
DMSO	dimethylsulfoxide
EDTA	ethylenediaminetetraacetic acid
E_h	apparent midpoint potential
$E_{M,7}$	midpoint potential at pH 7
EPR	electron paramagnetic resonance
g	force of gravity
G	gauss
GHz	gigahertz
Gpp	gauss, peak-to-peak
gm	gram
ISP	reiske type iron-sulfur protein
°K	Kelvin
K_s	stability constant of semiquinone radical
KHz	kilohertz
µg	microgram
µl	microliter
µM	micromolar
µW	microwatt
mM	millimolar
ml	milliliter

mW	milliwatt
M	molar
Mr	relative molecular weight
MOPS	(3-[N-Morpholino]propanesulfonic acid)
nm	nanometer
nM	nanomolar
NADH	reduced nicotinamide adenine dinucleotide
$P^{1/2}$	power at 1/2 (Amplitude/(Power)$^{1/2}$)
pH	-log [H^+]
PQ	plastoquinone
psi	pounds per square inch
Q	ubiquinone
QH^-	deprotonated quinol
QH_2	ubiquinol (reduced ubiquinone)
$Q^{\cdot-}$	ubisemiquinone radical anion
QCR	Ubiquinol-cytochrome *c* reductase
Qps	quinone binding protein in the succinate-Q reductase region
$Q_oC_{10}Br(H_2)$	reduced 6-(10'-Bromodecyl)-2,3-dimethoxy-5-methyl-1,4 benzoquinone
Rs	Rhodobacter sphaeroides
Rs.*b*-c_1	Ubiquinol-cytochrome *c* reductase from *Rhodobacter sphaeroides*
TTFA	Thenoyltrifluoroacetone

CHAPTER I

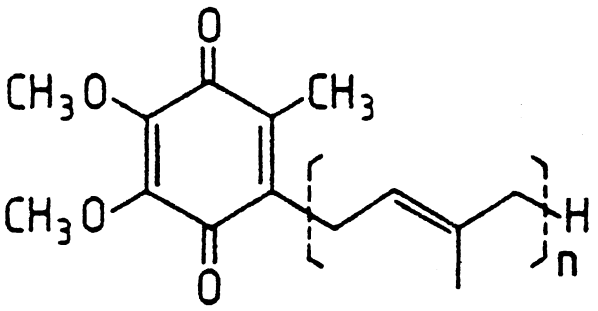
INTRODUCTION

Recent research of the protonmotive respiratory, photosynthetic, electron transport chain systems of mitochondria, photosynthetic bacteria and chloroplasts has provided a wealth of information and a better understanding of the molecular mechanisms of these systems which transfer electrons and protons in the process producing a driving force for the synthesis of ATP by ATP synthetase. In these systems ubiquinone (Q) or plastoquinone (PQ), is an integral component. Q acts as an electron and proton acceptor and donor in these systems. The transfer of hydrogen occurs heterolytically by the successive transfer of protons and electrons. The transfer of hydrogen requires that the Q and QH_2 molecules be bound to specific enzyme domains, not only do the Q and QH_2 need to be bound but also the reactive intermediates, semiquinone radical anion ($Q^{\cdot-}$) and the deprotonated quinol (QH^-).[1] The necessity of this containment is to maintain an environment with respect to E_m and pK_a to which a favorable condition exists for the rapid transfer of electrons and protons.

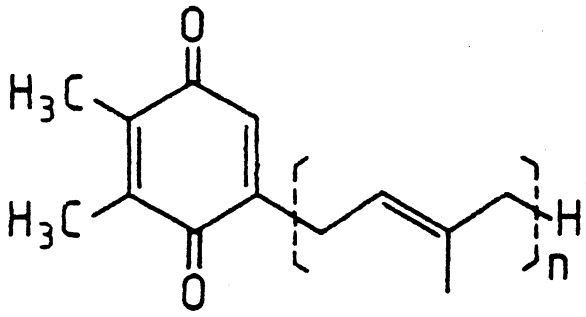
Ubiquinone or Coenzyme Q was first discovered in the late 1950's by two different groups, namely Green *et al.* at the University of Wisconsin and Morton *et al.* at the University of Liverpool.[2, 3] The structure of ubiquinone was determined by Folkers and associates at Merck Laboratories.[4] Figure 1 shows the structure of ubiquinone and plastoquinone.

The name ubiquinone (Q) was chosen to describe the apparent ubiquitous distribution of this chemical compound throughout the realm of biological systems. By 1959 the list of biological species containing Q or analogues of Q encompassed animal

Figure 1. Structure of Ubiquinone and Plastoquinone.



ubiquinone (Q)



plastoquinone (PQ)

n= 9 , 10

tissues, insects, higher plants, algae and microorganisms, although the presence of Q is widespread in nature, it is not in fact ubiquitous. The amount of Q can be correlated to the aerobic respiratory capacity and therefore species which have little or none show an absence of Q. But a high level of vitamin K[5] is found in species which do not contain coenzyme Q.

In mitochondria the electron transport system consists of four protein complexes, namely NADH-Q reductase (NQR), succinate-Q reductase (SQR), ubiquinol-cytochrome *c* reductase (QCR) and cytochrome *c* oxidase, complex I, II, III and IV, respectively. Ubiquinone is involved in the electron transfer process in the complexes I, II and III. All of the oxidative phosphorylation system of eukaryotes is found in the mitochondrial inner membrane. The inner membrane is approximately 70% protein and 30% lipid.

Complex I catalyzes the oxidation of substrate NADH and reduces Q to QH_2. This reaction is coupled to a vectorial proton translocation across the membrane.[6, 7] The prosthetic groups of complex I are flavin mononucleotide and an iron-sulfur protein (ISP). The enzyme complex is inhibited by rotenone, piericidin A, barbiturates, demerol and mercurials. All of these inhibitors appear to act upon the same site to prevent the reduction of Q and the oxidation of the ISP.[8]

Complex II catalyzes the oxidation of succinate to fumarate and the reduction of Q to QH_2. This enzyme complex is the only enzyme in the Krebs cycle which is bound to the inner mitochondrial membrane. The prosthetic groups contained in complex II are a covalently bound flavin adenine dinucleotide, three iron sulfur centers and a cytochrome *b* protein which is closely associated with the Q-binding protein, (QPs).[9-11]

Complex III catalyzes the oxidation of QH_2 to Q and the reduction of cytochrome *c* and is coupled to the transmembraneous proton translocation. The prosthetic groups of complex III are two cytochrome *b*, one cytochrome c_1, and an ISP. Complex III is discussed in more detail in subsequent chapters.

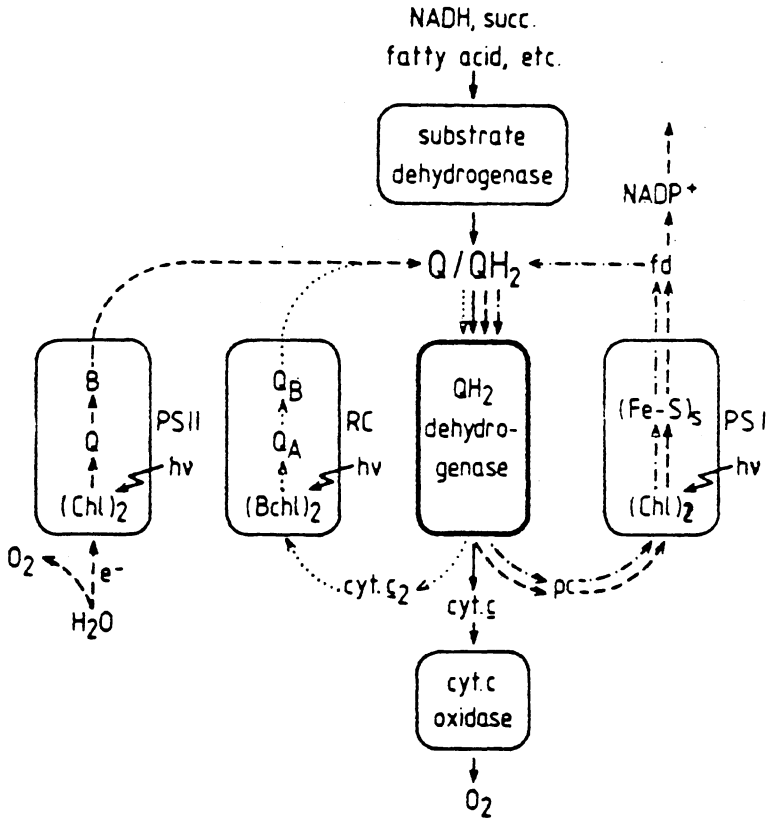
The amount of ubiquinone present in the complexes I, II, III is reported to be 4.2-4.5, 0, 1-2, nanomoles/mg protein, respectively.[9]

Photosynthetic systems also contain Q or PQ. The Q analogue plastoquinone is found in higher plants, algae and the cyanobacteria,[12, 13] mainly in the thylakoid membrane, but also a small amount is found in the chloroplast envelope.[14] Plastoquinone has several functions in the non-cyclic electron transfer in the thylakoid membrane, 1) connects photosystem II to photosystem I as a pool function designated Q pool, 2) Q_A acts as a quencher of fluorescence, 3) Q_B is a secondary acceptor of photosystem II, 4) PQH_2 is oxidized by b_6-f complex, 5) proton translocator, 6) electron transfer out of stacked grana.[15]

In photosynthetic bacteria Q is found in the cytoplasmic membrane. There are three species of Q which are found in the cyclic electron transfer scheme. The first, Q_A, is found to be tightly bound in the reaction center of this photosystem, Q_B is also found in the reaction center but is bound at least five times less tightly.[16] The role of Q_A and Q_B is to act as electron traps from the excited photoactive bacterochlorophyll dimer present in the complex, thus preventing a fast charge recombination competing with the secondary electron transfer reactors. Another Q is found in the QCR region of the photosynthetic bacteria. The QCR complex has the same prosthetic groups and functions the same as in the mitochondrial system. The pathways of electrons in the different species containing ubiquinone, or plastoquinone, are shown in Figure 2.

The isolated QCR complex of the photosynthetic bacteria *Rhodospeudomonas sphaeroides* R-26, (recently changed nomenclature designates this species as *Rhodobacter sphaeroides* R-26), was studied to determine if the physical properties of Q in the resolved electron transfer system were comparable to the physical properties of Q in the native organism. Specifically the reduction-oxidation behavior was studied with the combined techniques of electron paramagnetic resonance and reduction-oxidation potentiometry. The results of these studies are reported herein.

Figure 2. Electron Flow Schemes. A generalized schematic shows the electron flow in the mitochondria respiratory chain, cyclic electron flow in purple photo synthetic bacteria, and the cyclic and non-cyclic electron flow in chloroplast.



——→ electron flow in the mitochondrial respiratory chain
·····→ cyclic electron flow in purple photosynthetic bacteria
- - - → non-cyclic electron flow in chloroplasts
-·-·→ cyclic electron flow in chloroplasts

CHAPTER II

LITERATURE REVIEW

Ubiquinol-cytochrome *c* Reductase

The enzyme complex ubiquinol-cytochrome *c* reductase, as stated above, catalyzes the oxidation of QH_2 and the reduction of cytochrome *c*. In the native membrane QCR also pumps two protons across the mitochondrial inner membrane for every one electron which is transferred through the enzyme. As can be seen from Table I, QCR has been isolated from a large variety of organisms, all contain the same essential prosthetic groups namely, two cytochrome *b* type proteins, one cytochrome *c* type protein and an ISP.[8] Among the biological species from which QCR enzymes have been isolated are beef,[17-19] rat,[20] pigeon,[21] yeast,[22, 23] *Rhodobacter sphaeroides*,[24-26] plants,[27] Anabaena variabilis,[28] and Neurospora.[29]

The electron flow through the cytochrome b-c_1 complex can not be explained by a simple linear path. The most widely accepted electron transfer mechanism is known as the Q-cycle which was originally proposed by Peter Mitchell.[1] The key feature of this mechanism is a branched pathway which was earlier proposed by Wikstrom and Berden to explain a phenomenon called oxidant-induced reduction of the cytochrome *b*.[30] The principle feature here is that one electron is passed from QH_2 to a *b* type cytochrome and the other electron is passed to the *c* type cytochrome. The difference between the Wikstrom-Berden model and the Q-cycle is that in the Q-cycle the electron transfer from QH_2 to cytochrome *b* is returned to a Q molecule instead of being passed on through the electron transport chain. This makes it possible for only one of the electrons to pass to a more positive potential via the ISP, while two protons are pumped outside of the inner

TABLE I

VARIOUS SOURCES AND PREPARATIONS OF PURIFIED MITOCHONDRIAL COMPLEX III AND ANALOGOUS COMPLEXES FROM PHOTOSYNTHETIC MEMBRANES (FROM REFERENCE 77)

Source	Preparative procedure	Content of redox subunits (μ moles/g protein)			SDS-PAGE bands (kDa)
		c_1 type cyt.	b type cyt.	$(FeS)_2$	
MITOCHONDRIA:					
Bovine heart	Bile salt–salt fractionation	4.1	8.5	–	–
"	"	3.4±0.2	6.8±0.4	3.1±0.2	–
"	"	3.0–3.5	5.0–6.5	–	47.5, 45.5, 29.5, 27.8, 24.8, 13.9, 10.7, 4.8–9.0
"	"	"	"	"	50, 46, 37, 31, 27, 16, 13, 11, 7–8, 2–5
"	"	4.1	6.5	3.0	53, 50, 37, 30, 28, 17, 15
"	Alkali treatment, bile salt-ammonium acetate	5.7–6.0	10–10.5	3.3–3.6	9 bands
"	Triton X-100, hydroxyapatite chromatography	4.0	8.0	3.5–4.0	49, 47, 30, 25, 12, 11, 6
"	Triton X-100, chromatography with cyt c - linked Sepharose	3.3	6.5	partly removed	50, 48, 40, 30, 25, 13.5, 12.5, 12.5
"	Complex co-crystallized with cytochrome c	4.0	7.0	–	–
Pigeon breast muscle	Triton X-100, deoxycholate-salt fractionation	2.6–3.1	4.9–5.4	2.5–2.9	–
Rat liver	Triton X-100, hydroxyapatite chromatography (antimycin stabilized).	3.9	8.2	0.9	50, 46, 33, 25, 12.5, 10, 5.6
"	As above (stabilized by dithionite + dithiothreitol)	4.4	6.5	–	50.7, 46.2, 33.5, 24.5, 12, 10.2, 5.7
Yeast (*Saccharomyces cerevisiae*)	Bile salt–salt fractionation (antimycin stabilized)	3.0	6.0	Present	49, 40, 32, 17, 14, 11
"	Bile salt–salt fractionation (stabilized by dithioerythritol)	(heme Fe = 8.0)		Nonheme Fe = 15	43, 40, 32, 24, 22, 20, 18
"	Bile salt–salt fractionation (stabilized with phenylmethyl sulphonyl fluoride (PMSF)	4.6	9.3	4.6	– 47.5, 42.7, 32.7, 22.6, 17.6, 14.5, 11.5, 7.9, 5.8
"	Above procedure	4.24	7.03	–	49, 50; 39, 41; 30, 29.5, 29, 30; 24.5, 24.25; 17.5, 17.7; 15.5, 15.5
Neurospora crassa	Triton X-100, affinity chromatography on immobilized cytochrome c	3.5	7.5	Removed	50, 45, 31, 27, 14, 10, 8
"	Modification of above procedure	3.5	7.0	Present	52, 45, 31, 30, 25, 14, 11,
PHOTOSYNTHETIC APPARATUS:					
Rhodopseudomonas sphaeroides chromatophores (ubiquinol-cyt c_2 reductase)	Ammonium sulphate fractionation from combined octylglucoside, Triton X-100, cholate solutions	5.0	10.0	Present	40, 34, 25 (major bands) < 10 (2 minor bands)
"	Ammonium sulphate fractionation of solution in Triton X-100 and cholate	8.3	8.3	Nonheme Fe = 15	48, 30, 24, 12
Spinach chloroplasts (plastoquinol-plastocyanin reductase)	Cholate-ammonium sulphate fractionation; density gradient separation in Triton X-100	Cyt f 7.25	Cyt b_6 13.1	8.3	34, 33, 23.5, 20, 17.5
"	Modification of above	–	–	–	34, 33, 23, 20, 17
Anabaena variabilis (thylakoid membranes)	Modification of above	Cyt f 4.5	Cyt b_6 9.0		38 or 31, 22.5, (2 bands), 16.5, < 10 (2 bands)

membrane. The result is a net positive charge of one, i.e., $H^+/e^- = 2$, whereas in a simple redox reaction the ratio would be one.

The protonmotive Q cycle model specifies that first an electron from QH_2 is passed through the ISP to the cytochrome *c*, resulting in a Q^- intermediate and the second electron is passed to cytochrome b_{566}. This leaves the Q molecule in an oxidized state with two protons being pumped out of the mitochondria. The second electron is then transferred to a cytochrome b_{562} and then is transferred to an oxidized Q producing a ubisemiquinone radical $Q_i^{\cdot-}$ which can react with two more protons and another $Q^{\cdot-}$ to produce a Q and QH_2 molecule. Figure 3 shows the schematic for the Q-cycle and the *b*-cycle.

To understand the mechanism of the QCR enzyme complex it is essential to study the basic redox components of the electron transfer system.

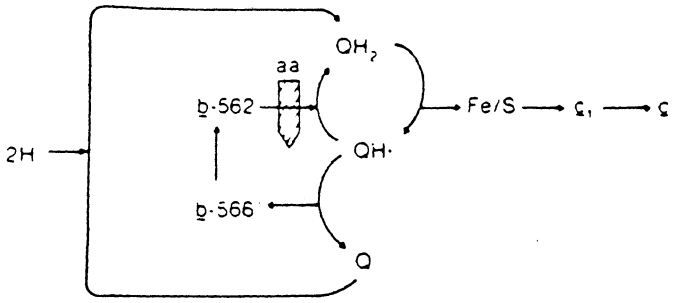
The iron-sulfur protein (ISP) is a protein which contains an iron-sulfur cluster of the 2Fe-2S type. The molecular weight of this polypeptide is approximately 25 kD.[31] The $E_{M,7}$ of the ISP is the most positive in the cytochrome b-c_1 complex at +280mV, with a one electron transfer.[32] Since the potential of this prosthetic group is the most positive this is logically the first electron acceptor from the reduced Q. According to the Q cycle this results in the translocation of the two protons to the cytoplasmic side of the membrane, resulting in a ubisemiquinone anion $Q^{\cdot-}$ which is a powerful reductant, (E_{M7} for $Q^{\cdot-}/Q$ ~ -240 mV), for reducing the lower potential cytochrome *b*.

Cytochrome c_1 is the direct recipient of the electron donating ISP. Cytochrome c_1 is a two polypeptide protein with molecular weights of 28 kD and 9 kD.[34, 35] The prosthetic heme group is covalently bound to the larger subunit and has an $E_{M,7}$ of +225 - +245 mV.[36-38] The midpoint potential of cytochrome c_1 is much lower than that of the ISP and almost the same as that of the electron acceptor cytochrome *c*.

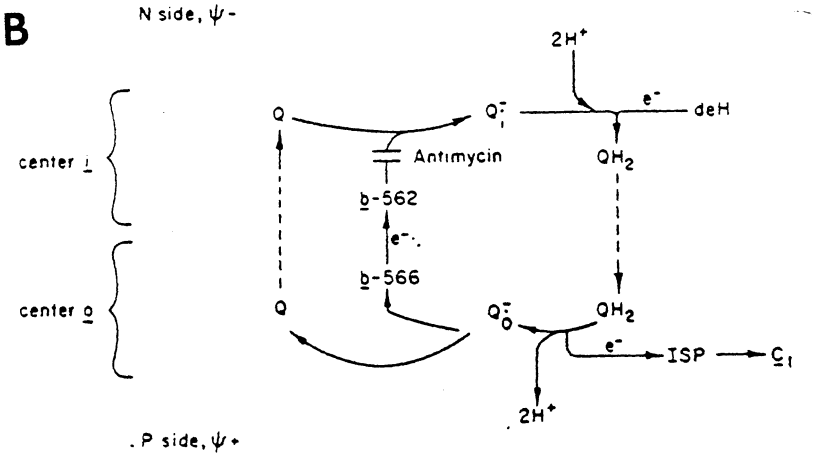
The other branch of the electron transfer pathway consists of the *b* type cytochromes. For simplicity only two distinct species shall be discussed here although there are several reports of variations to this. In general two *b* hemes are considered to be

Figure 3. Q-Cycle and Cytochrome *b* Cycles. A) shows the cyt. *b* cycle proposed by Wikstrom and Kreb. B) shows a generalized Q cycle proposed by Mitchell.

A


2H
QH2
aa
b-562
Fe/S
c1
c
QH·
b-566
Q

B


N side, ψ-
2H+
e-
deH
Q
Q·i
Antimycin
QH2
center i
b-562
e-
b-566
center o
Q
Q·o
QH2
e-
ISP
c1
2H+
P side, ψ+

associated with a single polypeptide of Mr ~ 43 kD although it is possible that two apoproteins containing one heme each exists. The midpoint potential for these two heme groups has been reported as +30 mV and -30 mV.[39]

Ubiquinone binding proteins were first postulated by Mitchell[1] to stabilize the semiquinone radical. Evidence of ubiquinone binding proteins (Qp) in the cytochrome b–c_1 complex was first described by Yu and Yu.[40] The use of a radiolabelled azido-Q derivative to label a specific site(s) indicated that a protein with a Mr of about 17 Kd and another protein with a Mr of about 37 kD are responsible for Q binding. The 37 Kd protein has been identified as that of cytochrome b.[41]

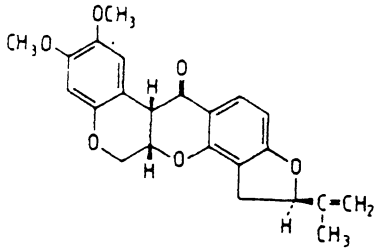
The inhibitors of the cytochrome b-c_1 complex act upon different locations of the complex. Antimycin A[42] is a potent inhibitor which blocks electron flow from cytochrome b_{562} to ubiquinone, thus preventing any semiquinone formation. UHDBT acts upon the ISP and inhibits the reduction of cytochrome c_1.[43] Myxothiazol prevents the reduction of the ISP and therefore acts directly upon the ISP.[44] In Figure 4 we see a list of the more common inhibitors of the electron transport system.

Ubisemiquinone Radical

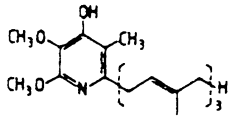
The discovery of a biological free radical in mitochondria was made by Sands and Beinert[45] in 1960 with the use of EPR spectroscopy. But the notion of a ubisemiquinone in the transfer of electrons was not suggested until 1967 by Raikhman and Blumenfeld.[46] Through the use of inhibitors they were able to localize the site of formation to the succinate and/or NADH oxidase region of the respiratory chain. Upon depletion of ubiquinone and the reincorporation they were able to deduce that some of the semiquinone signal was due to ubisemiquinone and that the flavin also contributed to the radical signal. The quantity of ubisemiquinone was also found to be only 2% of the total ubiquinone in the mitochondria. The detection of ubisemiquinone in submitochondrial particles, by EPR later confirmed their findings.[47]

Figure 4. Inhibitors of the Electron Transport System.

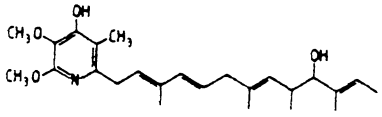
rotenone



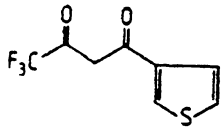
ubicidin-3



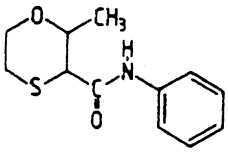
piericidin A



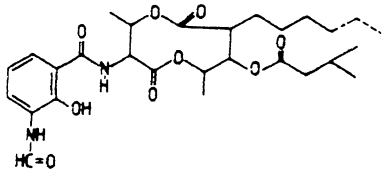
TTFA



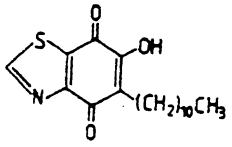
carboxin



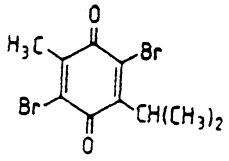
antimycin



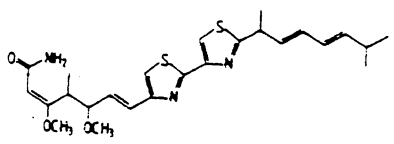
UHDBT



DBMIB



myxothiazol



A specific role for the ubisemiquinone radical in the cytochrome *b*-c_1 complex was first introduced by Wikstrom and Berden[30] in 1972 as previously discussed, and the idea of a protein stabilized ubisemiquinone was postulated by Mitchell.[1]

The mechanism by which ubiquinone undergoes a redox reaction is of central importance in the understanding of the QCR complex as a whole. There are many theories as to the exact pathway of electrons in QCR but an essential feature in all these schemes is the formation of a ubisemiquinone anion. A representation of the reaction at physiological pH may be shown as follows: $QH_2 \underset{H^+}{\rightarrow} QH^- \underset{e^-}{\rightarrow} QH^\bullet \underset{H^+}{\rightarrow} Q^{\dot{-}} \underset{e^-}{\rightarrow} Q.$

This consists of two one-electron transfers and is considered as a reversible reaction. The necessity of a stabilized ubisemiquinone was first suggested by Mitchell,[1] to coincide with the evidence he used in formulation of the Q cycle. The two redox couples E_1 and E_2 correspond to the ubisemiquinone couples ($Q/QH^\bullet$) and ($QH^\bullet/QH_2$). The stability constant is defined as $K_s = \left[\frac{[QH^\bullet]^2}{[Q]\,[QH_2]}\right]$.

There are four ubisemiquinone radical species in the respiratory electron transfer chain namely Q_N, Q_S, $Q_{c,i}$, and $Q_{c,o}$, these subscripts correspond to the different segments of the respiratory chain, NADH-Q reductase, succinate-Q reductase and QH_2-cytochrome *c* reductase respectively.

Q_N is the least understood of the four ubisemiquinones. The Q_N was detected by King *et al.* and found to be insensitive to antimycin and TTFA,[48] which is indicitive of not being associated with the other Q^- species. The $E_{M,\,8.0}$ has been reported to be about −110mV,[49] which is the lowest of the four species of $Q^{\dot{-}}$.

$Q_s^{\dot{-}}$ accepts electrons from succinate dehydrogenase, and is stabilized by a protein designated Qps. The Qps protein isolation has been reported in several papers.[50-52] The Qps can be reconstituted with isolated succinate dehydrogenase to regain succinate-Q reductase activity. In addition to the Q_S species, there appears to be a $Q^{\dot{-}} - Q^{\dot{-}}$ pair interaction which has been observed and extensively studied, (see ref. 49), which arises

from the $Q^{\cdot}_S$ pool. The Q_S and $Q^{\cdot} - Q^{\cdot}$ are both affected by the presence of TTFA, an inhibitor of the succinate-Q reductase enzyme, and both also have an $E_{M,7}$ of about 110 mV. According to the work done by Salerno[53], *et al.* using oriented multilayers of membranes from submitochondrial particles, the $Q^{\cdot}$ pair span the membrane with the quinone rings perpendicular to the membrane plane and with the oxygen - oxygen direction probably also perpendicular to the membrane plane.

Two different semiquinones have been found in the cytochrome *b*-c_1 region, namely, $Q_{c,i}$ and $Q_{c,o}$. The subscript (c) refers to the semiquinone radicals formed in the cytochrome *b*-c_1 region. The subscripts (i) and (o) refer to the location of the semiquinone in the Q cycle scheme as seen in Fig. 3. $Q_{c,o}$ is the most recent addition to the ubisemiquinone group. Considered to be the least stable, it is detected in the cytochrome *b*-c_1 complex under conditions required for the oxidant-induced reduction of cytochrome *b*, specifically by addition of antimycin, TTFA and NADH to a submitochondrial particle suspension. The signal observed by EPR is insensitive to antimycin and TTFA but is abolished by the addition of BAL, a compound which destroys the iron-sulfur moiety.[54] The Q_o is formed after the reduction of the ISP and is believed to be the reductant for cytochrome *b*. As previously mentioned this $Q^{\cdot}$ is considered to be the least stable thermodynamically, the amount which can be generated has been estimated at 0.02 mole per mole of cytochrome c_1, at a pH 7.0, which is even lower than would be expected for unbound ubisemiquinone.

$Q_{c,i}$ is the antimycin-sensitive ubisemiquinone radical found in the cytochrome *b*-c_1 complex. It is also the ubisemiquinone of interest in this thesis. $Q_{c,i}$, which will be labeled Q_c herein, is the predominant ubisemiquinone in the cytochrome *b*-c_1 complex. The function of this species is to act as an oxidant to the reduced cytochrome *b*. In the Q cycle we can see that the Q at center i is reduced completely to QH_2, via the semiquinone, by cytochrome *b* and/or a dehydrogenase. This leads to two situations which must be considered as the mechanism for Q reduction, the first being as described above with one

electron donated from the cytochrome b_{562} and the second electron from a dehydrogenase. The second situation is when there is an absence of donation of both electrons from the dehydrogenase or the cytochrome *b*, in this case it is possible for the donor to pass two electrons and the process completed by dismutation of the ubisemiquinone. The first situation is the most probable in the native respiratory system when considering the Q cycle, indeed the succinate dehydrogenase redox center S-3 is found in close proximity to cytochrome *b*.[56]

The second case is probably what is seen to happen in the case where there is an isolated cytochrome *b*-c_1 complex, which does not contain any succinate dehydrogenase.

Several inhibitors appear to bind at the site of the Q_c formation, but the most well known and studied is antimycin. The signal in EPR which arises from $Q_c^{\cdot}$-formation is abolished by the inhibitor.[57] Antimycin has also been reported to bind to the cytochrome b_{562}.[58] Another aspect of the effect that antimycin has on the cytochrome *b*-c_1 complex is that it causes a conformational change in the complex.[59] This conformational change cannot be excluded as possible evidence that the destabilization of the Q_c may be due to this structural difference and not by binding of the inhibitor in the exact location of the Q molecule.

The redox behavior of Q_c in submitochondrial particles,[55, 60] succinate-cytochrome *c* reductase,[57] mitochondrial QCR,[52] and chromatophores from *Rhodobacter sphaeroides* GA,[61] has been reported. In mitochondrial systems the $E_{M,7}$ of the Q/QH_2 couple is between 80-110 mV which is dependent on the preparation. The value of $E_{M,7}$ for the chromotophores was about +150 mV.

EPR of the QCR Complex

The earliest studies of the mitochondrial respiratory chain with the electron paramagnetic spectroscopy technique were done in 1960,[45] fifteen years after the development of the EPR instrument.[62] The use of EPR to study the QCR complex has

proven to be a powerful technique. The benefit of EPR in the study of QCR is that all of the functionally active prosthetic groups are detectable under certain redox states and observation temperatures. Numerous studies have been done on the QCR system using EPR and indeed has become somewhat of a routine if not a necessary part of a thorough study of the complex. Figure 5 shows the spectra of the cytochromes and the ISP.

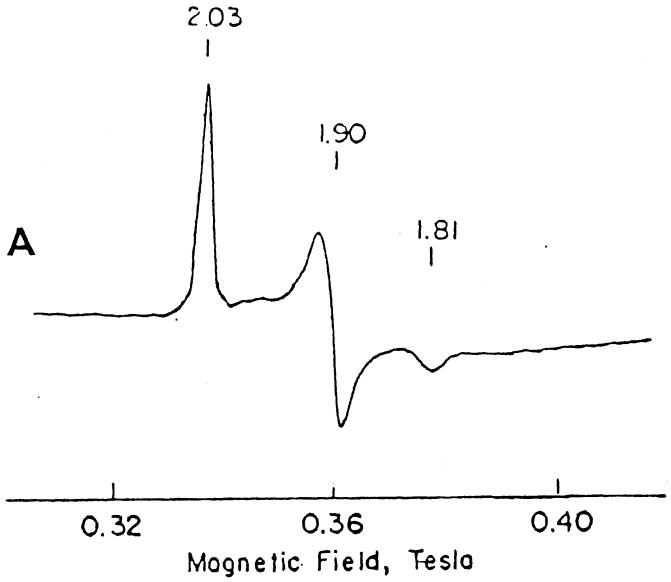
Cytochromes. The EPR spectra of heme proteins is usually observed at temperatures in the range of 5°-15°K. This requirement of temperature is due to the rapid relaxation characteristics of the iron center. The identification of the cytochrome components in mitochondria were first reported in 1974 by Orme-Johnson, *et al.*[63] They reported the existance of two *b* type and one *c* type of cytochrome with g-values of 3.7, 3.44, and 3.28 respectively. The two cytochrome *b* peaks were later assigned to the spectrophotometrically distinguishable b_{562} and b_{566}. In *Rhodobacter sphaeroides* R-26 the cytochrome *b* peaks were found to be at g=3.75 and 3.40 and the cytochrome c_1 was at g=3.40.[67]

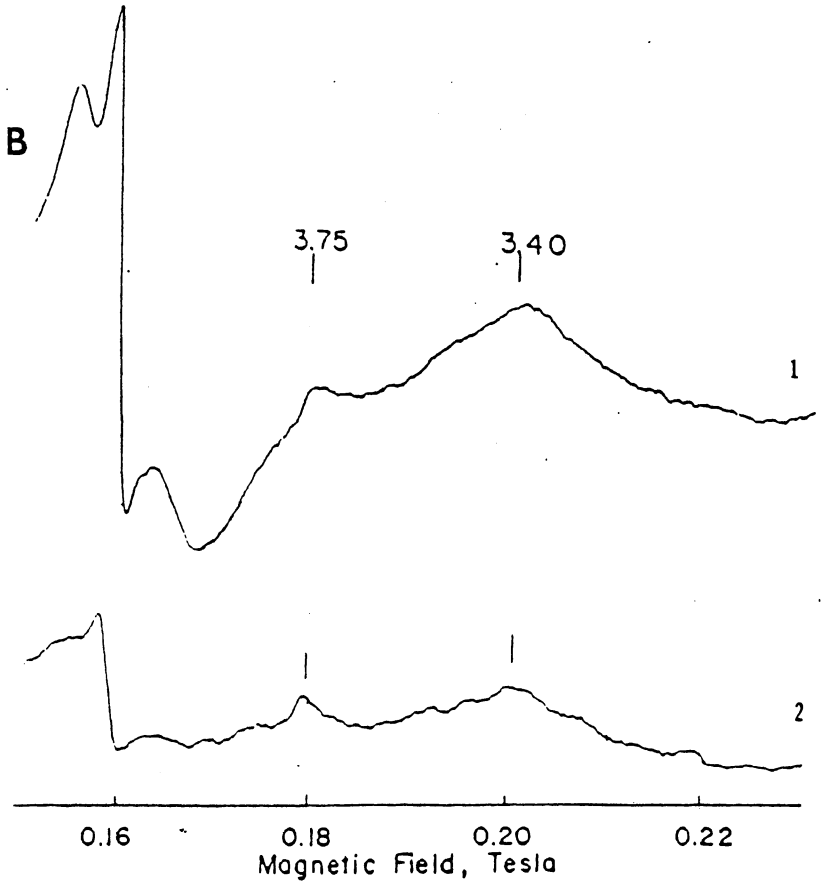
Several reviews and articles[64-66] have been published on heme proteins to which the reader is referred for further discussion.

Iron-sulfur Protein. The iron-sulfur protein of QCR displays a three line spectra when observed by EPR at temperatures below 100°K. The reduced ISP is paramagnetic while the oxidized ISP is EPR silent. The central g-value of the ISP is at approximately 1.90 with $g_x = 1.81$ and $g_z = 2.03$ in the *Rhodobacter sphaeroides*,[67] which is typical of g values found in all cytochrome b-c_1 complexes. The spectra is affected by oxidation-reduction state of ubiquinol[68] and quinone type inhibitors.[69]

Ubisemiquinone Radicals. The use of EPR to study ubisemiquinone radicals has allowed the differentiation of these radicals based on g values, spectral line shape, linewidth and power saturation characteristics. The two $Q^{\cdot-}$ species in QCR, as previously described above, are Q_i and Q_o. The prominent Q_c species is $Q^{\cdot-}_i$ which has a g-value of

Figure 5. EPR Spectra of the Heme Proteins and Iron-Sulfur Protein of QCR. A) The ascorbate reduced spectra of the iron-sulfur protein of Rs.*b*-c_1. B) The oxidized cytochrome spectra is shown in 1) and the ascorbate reduced spectra in 2).


2.03
1.90
1.81
A
0.32
0.36
0.40
Magnetic Field, Tesla


B
3.75
3.40
1
2
0.16
0.18
0.20
0.22
Magnetic Field, Tesla

about 2.005 and a linewidth of 8-10G. The species shows a $P^{1/2}$ of around 0.12-0.28 mW at 60°K in mitochondrial cytochrome b-c_1 complex which is close to that of the ubisemiquinone in liposomes at alkaline pH.[70] This slow relaxation behavior indicates that the Q_i is not located extremely close to a transition metal redox center. The line shape of the Q_i is gaussian, suggesting that it is in an aprotic environment. In the photobacterium *Rhodobacter sphaeroides* GA, Q_i has been reported to have a g value of 2.005, with a linewidth of 8G[61]

Q_o has a reported g-value of 2.006 with a linewidth of 8.3G.[54] The power saturation characteristics of Q_o were reported to be 0.11 mW, this would indicate that it also is in an "isolated" location. This result seems to contradict the evidence of other researchers[68, 69] that contend that QH_2 is the direct reductant of the ISP as noted by the effect on the ISP by Q. If Q_o is located close to the ISP it would be expected to have a faster relaxation rate. Further research on this anamolous behavior may provide a better understanding of the mechanism of Q in this region. Table 2 summarizes the properties of the semiquinone radicals.

TABLE II

EPR AND THERMODYNAMIC PROPERTIES OF UBISEMIQUINONES IN BOVINE MITOCHONDRIA

Radical	*g* Factor	$\Delta H_{pp}(10^{-4}T)$	$P_{1/2}$(mW)	*K* (pH 7)	E_m for QH_2/Q at pH 7 (mV)
Q_c	2.005	9.5–10	0.12–0.28 (50 K)	5×10^{-2}	80 (200 K)
Q_s	2.005	12	Not saturated at 50 K	10	134 (12 K)
Q_{out}	2.006	8.3	0.11 (50 K)	Too low to measure	Not determined
Q_N	2.004	6.8			

K = stability constant.
E_m= midpoint potential versus the standard hydrogen electrode.
ΔH_{pp} = width from maximal amplitude in spectrum to minimum.
Data for Q_c are from refs. 54, 55, 57, 60; data for Q_s are from refs. 57, 60; data for Q_{out} are from ref. 54.

CHAPTER III

EXPERIMENTAL MATERIALS AND METHODS

Isolation of Rs.b-c_1

Materials

The cell culture of *Rhodobacter sphaeroides* R-26 was a gift from Drs. Okamura and Feher, Department of Physics, University of California at San Diego. The Q analogues used in the assays were synthesized in this laboratory by the reported methods.[71] Calcium phosphate was prepared according to method of Jenner.[72] Calcium phosphate was mixed 1:1 (w/w) with cellulose powder obtained from Whatman. Triton X-100 was obtained from Rohn and Haas Co. Horse heart cytochrome c, Type III, was obtained from Sigma. Cholic acid was purchased from the Aldrich Chemical Co.

Method

The cytochrome b-c_1 complex of *Rhodobacter sphaeroides* R-26 (Rs.b-c_1) was prepared according to the previously reported methods[26] with some minor modifications. Figure 6 summarizes the isolation procedure. All operations were performed at 0°C unless otherwise noted. Seventy grams of cell paste was suspended in 200 ml of 20 mM tris-succinate buffer, pH 8.0. The suspension was then centrifuged at 3000 x g to remove any particulate matter. The precipitate was resuspended leaving the light colored, hard precipitate behind. The suspension was passed through a french pressure cell operated at a cell pressure of 18,000 psi. After passing through the french pressure cell the solution was received in a beaker in an ice/salt bath. Immediately added to the solution was a 7.7

ml solution of ethanol containing 0.180 gr of phenylmethylsulfonyl fluoride. The solution was then brought to 0.15M KCl and stirred for thirty minutes, after which the solution was centrifuged at 130,000 x g for one hour. The supernatent is then discarded and the precipitate was suspended in approximately 200 ml and stirred overnight in 20 mM Tris-succinate, pH 8.0 containing 1 mM EDTA. The suspension was then brought to a final volume of 380 ml and Triton X-100 was added at a concentration of 60 ml/liter, the solution was stirred for 30 min and then centrifuged at 130,000 xg for one hour. The supernatent was collected and diluted with 0.5 volumes of 20 mM Tris-succinate, pH 7.4 containing 1 mM EDTA. The solution was then subjected to ammonium sulfate fractionation. The first fraction was made at 22% ammonium sulfate. The solution was centrifuged at 40,000 x g for 20 minutes and the precipitate is discarded. The supernatant was then brought to 36% ammonium sulfate saturation and centrifuged as before. The precipitate was then dissolved in 200 ml of Tris-succinate, pH 7.4 containing 1 mM EDTA, and to this was added 400 ml of 50 mM K/K phosphate buffer pH 7.4 containing 20% glycerol and 1 mM EDTA. This solution was then centrifuged at 40,000 x g, for 20 minutes to remove any precipitate and the supernatant is collected. The solution was then applied to a calcium phosphate column which had been equilibrated with the phosphate buffer described above. After the sample was applied the column was washed with 2 bed volumes of the equilibrating buffer containing 0.2% sodium cholate. The crude $RSbc_1$ was eluted from the calcium phosphate column with 0.3 M K/K phosphate, pH 8.0 containing 20% glycerol, 0.3% sodium cholate and 1 mM EDTA. The eluate was then subjected to an ammonium sulfate fractionation using a cold, neutralized, saturated solution of ammonium sulfate. The first precipitate is formed at 30% ammonium sulfate saturation, and centrifuged at 40,000 x g for 15 minutes. The precipitate was discarded and the supernatent is brought to 40% ammonium sulfate saturation and centrifuged as before. Once again the precipitate was discarded and the supernatant brought to 58% ammonium sulfate saturation. The purified Rs.b-c_1 is collected as the precipitate and

dissolved in 50 mM K/K phosphate, pH 8.0 containing 20% glycerol and 1 mM EDTA, and stored at -80°C until used.

The enzymatic activity assay of the ubiquinol-cytochrome *c* reductase were done at 23°C in a Cary 219 spectrophotometer. The assay solution contained, 50 mM Na/K phosphate, pH 7.0, 0.5 mM EDTA and 100 μM cytochrome *c*. To one ml of this solution 5 μl of $Q_oC_{10}Br(H_2)$ in 95% ethanol was added, the autooxidation of the $Q_oC_{10}Br(H_2)$ was then monitored at 550 nm for 1 minute. After this background was measured, a 5 μl aliquot of enzyme solution containing between 5-20 μg of protein was added, the absorbance change was observed for 1 to 2 minutes. The specific activity was then calculated using a millimolar extinction coefficient for cytochrome *c* of 18. The concentration of cytochromes *b* and *c*, were determined spectrophotometrically using dithionite and ascorbate reduced samples, respectively. The millimolar extinction coefficients for cytochrome *b* at 560 nm and c_1 at 550 nm were 28.5 and 17.5 for the reduced minus oxidized sample. Protein concentration was determined by spectrophotometric means using a factor of 0.3 times the absorbance at 278 nm, while the enzyme was in an oxidized state.

Redox Titrations

Materials

Rs.*b*-c_1 was prepared as described above. The redox mediators used consisted of 1,4 benzoquinone, 1,4 napthoquinone, duroquinone, indigo carmine, indigo tetrasulfonic acid, phenosafranin and safranin O, all were obtained from commerical sources and at the highest grade of purity available. The oxygen scavenger was obtained from Supelco, Inc. Co. The saturated calomel electrode Model 4112 was obtained from Radiometer, Denmark. Antimycin A was obtained from United States Biochemical Corp. Sodium dithionite, potassium ferricyanide, dimethyl sulfoxide, Tricine, glycyl-glycine, glycine, Trizma and MOPS were obtained from various sources at reagent grade purity or as

Figure 6. Flow Sheet for the Isolation of QCR from *Rhodopseuddomonas sphaeroides* R-26.

Preparation of Cytochrome *b*-c_1 Complex from *Rhodospeudomonas sphaeroides*

Frozen cell paste, 72 g
↓ 216 ml of 20 mM TrisSucc, pH 8.0
French Press, 1000 psi, flow rate 20 ml/min.
DNase 1.44 mg, PMSF 180 mg in 7.2 ml ethanol

Centrifuge, 5K, 5 min.
↓
Sup, + 14.4 ml 3N KCl, Stirring for 30 min.
↓
Centrifuge, 45K, 1 hr (Ti-45)
↓
Ppt, homogenizes with 192 ml of 20 mM TrisSucc, pH 8.0.
↓
Chromatophores
↓ Triton-X-100, 15% 60 µl/ml, Stirring for 30 min at 0°C

Centrifuge, 45K, 1 hr
↓
Sup + 1/2 vol of 20 mM TrisSucc pH 7.4 (S1)
↓ + 0.25 vol (SI) sat'd ammonium sulfate solution

Centrifuge, 18K, 20 min.
↓
Sup + 0.35 vol(S1) sat'd ammonium sulfate solution
↓
Centrifuge, 18K, 20 min.
↓
Ppt, dissolve in 96 ml TrisSucc buffer dilute with 192 ml 50 ml K/KPi buffer, pH 7.4
↓ containing 20% glycerol
Centrifuge, 18K, 20 min.
↓
Sup
↓
2 CaPO4 columns (3.2 x 6)
↓ Wash with 2 column vol 50 mM K/KPi, pH 7.4 containing 20% glycerol & 0.2% SC. Elute with 0.3M K/KPi pH 7.4 containing 20% glycerol and 0.3% SC
Eluate ....ml.

$Amso_4$ fractionation -- 0.43 vol; 0.17 vol; 0.15 vol.

Ppt dissolve in 50 mM K/Kpi pH 8.0 containing 20% glycerol & freeze in -80°C
↓
Rs *b*-c_1

available. The potential measurements were taken with the use of a Beckman Altex Model 71 pH meter. The reaction flask used was fabricated in the OSU glass shop by Mr. Tom Denton. Temperature control was obtained through the use of a Haake Model A81 Temperature Control Unit.

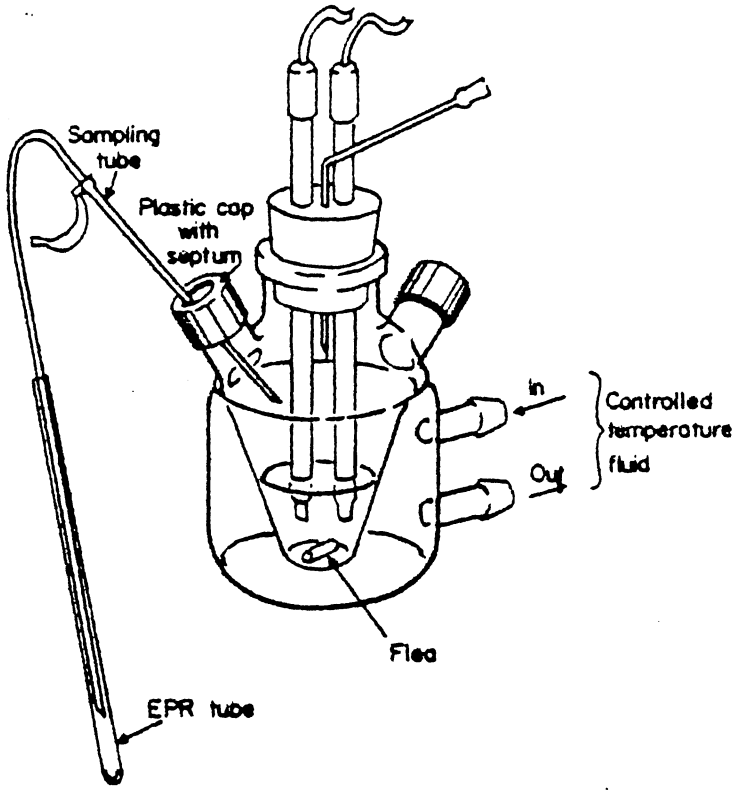
Apparatus

The apparatus used was essentially the same as that described by Dutton.[73] As seen in Figure 7 the flask is fitted with a saturated calomel electrode and a platinum electrode. A small stirring bar is placed in the bottom of the flask to facilitate the mixing after additions of reductant and oxidant. The gas train consisted of an argon cylinder fitted with a regulator and oxygen scavenger. After the oxygen scavenger the gas was bubbled through a flask which contained distilled water at 0°C, which "saturates" the gas at this temperature so that the sample solution at 0°C is not evaporated or diluted over the course of the titration. The gas then flows through the reaction flask and out via a stainless steel syringe tip fitted with tygon tubing which is bubbled into a container of water to create a back pressure. The temperature is controlled by placing the reaction flask in a water bath which is cooled by the Haake temperature control unit. This arrangement allows the observation of the contents of the reaction flask as opposed to using an ice-water bath which would obscure the view.

Method

Redox titrations were performed essentially as described by Dutton.[73] The separate Rs.*b*-c_1 preparations were combined to create one large uniform batch of the enzyme. The pooled solution was then divided into 1.5 ml fractions and frozen at -80°C until ready to use. At the time of titration a sample was removed and thawed, the sample was then measured for concentration of cytochromes, protein and assayed for specific activity. This was done for the first titration and the remaining fractions were considered to have the

Figure 7. Apparatus for Redox Titration.


Sampling tube
Plastic cap with septum
In
Out
Controlled temperature fluid
Flea
EPR tube

same activity and concentration. The pH of the enzyme solution was then adjusted with 2M Tris-base to the pH of the titration. After adjusting the pH, a concentrated solution of the buffer to be used was added along with cold water to bring the final concentration of buffer to 100 mM. The buffers used were Tricine pH=7.6, 8.2 and 8.4, glycyl-glycine pH=9.0 and 8.5, K/K phosphate pH=7.1, 7.6 and 8.9, glycine pH 9=9.5, MOPS pH=7.8. The sample was then placed in the reaction flask and stirred under an argon atmosphere at 0°C for 45 minutes. After the incubation period either 6 µl of DMSO or 6 µl of 25 mM antimycin in DMSO was added and the solution was allowed to incubate for 5 minutes. The amount of antimycin was in excess of the cytochrome c_1 content. The redox mediators were then added to the reaction flask to make a final concentration = 30 µM of mediators. This was allowed to equilibrate for 10 minutes before the addition of any oxidant or reductant. The oxidant, potassium ferricyanide, and the reductant, sodium dithionite, were prepared in a buffered solution to a concentration of 10 and 100 mM each. The solution buffer was the same as that used in the titration and was kept anaerobic and at 0°C. The additions of oxidant and reductant were usually in microliter quantities through a teflon septum with a Hamilton syringe with the overall volume added usually less than 25 µl for a titration. After an addition of either oxidant or reductant the solution was allowed to equilibrate to a point where the potential was changing by 2-3 mV per minute or less. At that time a syringe needle fitted with teflon tubing was inserted into the solution and 0.3 ml of the solution was forced by the argon pressure into the EPR sample tube and quickly frozen in an isopentane/liquid nitrogen bath. The EPR tube was then stored in liquid nitrogen. In a titration, ten or more samples were taken in the mV range of interest.

The saturated calomel platinum electrode was by using a series of 1 mM solutions of potassium ferricyanide and following the absorbance at 420 nm.

A background titration was performed to check for any interfering EPR signals. The control contained 100 mM Tricine, pH 8.4, 30 µM mediator dyes, 50 µM antimycin and 25 mg of bovine serum albumin.

Correlation Between $Q^{\cdot}$ and Enzyme Activity

Method

Six portions containing 0.15 ml of Rs.b-c_1 were diluted up to 0.3 ml with a buffer containing 300 mM fumarate, pH 7.0, 4 mM succinate, pH 7.0, and 200 mM MOPS, pH 7.8. The samples were then placed in a 30°C water bath. At a certain time 20 µl was removed and 5 µl of succinate dehydrogenase 20 mg/ml was added to the major portion. This was allowed to equilibrate for 5 minutes while the aliquot removed was checked for activity. The sample was then frozen in an EPR tube in liquid nitrogen. This process was repeated for times of 0, 30, 60, 90, 180 and 300 minutes. The EPR signal was then checked for intensity. After this the samples were thawed, antimycin added (excess to cytochrome c_1) and the EPR signal was checked again for background contributions to the spectra from the succinate dehydrogenase.

EPR Measurements of $Q^{\cdot}$

Materials

The EPR instrument used was a Bruker ER-200D equipped with an IBM Aspect 2000 computer system. The temperature was controlled with a Bruker ER 4111 VT flowing nitrogen system above 100°K, or a finger dewar for liquid nitrogen temperature measurements. Calibration of the magnetic field was done using a weak pitch standard, in combination with a Varian NMR gaussmeter. The specific microwave frequency was obtained by the use of a Hewlett-Packard Model 5340A frequency counter. The spin quantitation standard used was the spin label 3-Maleimidomethyl-1-proxyl obtained from Aldrich.

Method

Redox Titrations. The samples from the redox titrations were observed at 77°K using a finger dewar. The instrument settings were as follows: specific microwave frequency = 9.21GHz, modulation Amplitude = 10 Gpp, Modulation frequency = 100 KHz, Time constant = 0.2 seconds, Scan Width = 50 G, Scan time = 100 seconds and microwave power = 10 μW. Each sample was recorded and the intensity was measured as the height from peak to trough of the signal.

Linewidth and g-Value. The linewidth and g-value were calculated by using a standard of weak pitch (g=2.0028) and a gaussmeter to calibrate the magnetic field. The instrument settings used were the same as above except the modulation amplitude was 5 Gpp and microwave power was 0.063 mW.

Power Saturation. The relaxation behavior of the radical species was determined at 77°K by varying the microwave power from 10 μW to 200 mW and measuring the signal intensity. The other instrument settings were the same as in the redox titrations.

Temperature Dependence. The signal intensity as a function of temperature was determined by using a flowing nitrogen variable temperature control unit. After every temperature change the sample was allowed to equilibrate for five minutes before a measurement was made. EPR settings were the same as those for the redox titration except for the microwave power which was 0.063 mW.

Activity Dependence of $Q^{\cdot}$. The signal intensity as a function of the enzyme activity was determined with the same EPR settings as the redox titration except the modulation amplitude was 6.3 Gpp.

$Q^{\cdot-}$ Quantitation. The concentration of the $Q^{\cdot-}$ was estimated by double integration of the maximum signal intensity at pH 8.4 and comparing this to that of a 10 μM spin label standard 3-(Maleimidomethyl)-Proxyl.

CHAPTER IV

RESULTS AND DISCUSSION

Properties of Isolated Rs.b-c_1

The preparation of Rs.b-c_1 consisted principally of these main steps; rupture of the bacteria cell wall, KCL wash, triton-x-100 solubilization, ammonium sulfate fractionation calcium-phosphate column chromatography and an ammonium sulfate fractionation. The isolated enzyme typically contained 8 nanomoles of cytochrome b and 8 nanomoles of cytochrome c_1 per mg of protein. The activity of the enzyme was determined by the amount of horse heart cytochrome c that was reduced by the enzyme in the presence of an excess amount of reduced $Q_0C_{10}Br(H)_2$. The activity of the enzyme used in the experiments ranged varied from 4.0-9.0 μmole cytochrome c reduced/minute/mg of Rs.b–c_1 at 23°C. As stated previously in the Materials and Methods section, for a series of experiments the Rs.b-c_1 concentration and activity were consistent. The maximum activity reported for this enzyme[26] is 12.6 μmole cytochrome c reduced/minute/nanomole cytochrome c_1. This is approximately 10% of the activity of QCR from beef heart mitochondria.

The absorption spectra of the Rs.b-c_1 showed an α-band cytochrome b peak at 560 nm when the enzyme was reduced with dithionite, and an α-band cytochrome c_1 peak at 552 nm when reduced by ascorbate. The soret region absorption shows a peak at 413 nm in the fully oxidized state; upon reduction with ascorbate an absorption peak appears at 418 nm, when the enzyme is fully reduced with dithionite an absorption peak is seen at 430 nm.

The purified Rs.*b*-c_1 contains four major polypeptides as seen by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the molecular weight of these polypeptides were estimated to be 48, 30, 24 and 12 Kd.[26] The 48 Kd protein was found to correspond to cytochrome *b*, the 30 Kd protein to cytochrome c_1 and the 24 Kd protein to the Reiske type iron-sulfur protein. The protein subunit composition of Rs.*b*-c_1 is less complicated than the cytochrome *b*-c_1 complex isolated from beef heart mitochondria. The Rs.*b*-c_1 lacks two higher molecular weight "core proteins" found in the beef heart enzyme complex.

The pH stability of the Rs.*b*-c_1 has previously been reported.[26] Over the range of pH 6-9 the enzyme is about equally stable, with a 20-25% loss of activity after 19 hours at 0°C. This stability is an important consideration in the redox titration experiments, since the titrations were performed in this range of pH values.

Spectral Characteristics of Q_c

The EPR spectra of Q_c is characteristic of that reported for this radical species.[54, 55, 57, 60] The lineshape observed is gaussian due to a g-value anisotropy. This anisotropy is due to the crossover between the three principle g values, which are so close that they cannot be resolved at the frequency at which a conventional EPR experiment is done, (X-band), although these values have been resolved using a higher frequency.[74] The linewidth at 77°K was found to be 8.0G. The g-value was centered at 2.0046. Figure 8 shows the spectra of Q_c in the presence and absence of antimycin.

Temperature Dependence of the EPR Signal of Q_c

The Q_c signal measured at various temperatures was found to be linear when plotted as signal intensity versus - log (1/T) as seen in Figure 9. This may not seem surprising since the Boltzmann energy distribution equation $\frac{Ni}{N} = e^{-(\Delta E/RT)}$ predicts such a behavior.

Figure 8. EPR Spectra of the Semiquinone Radical. A) shows the spectra of the antimycin-sensitive semiquinol. B) is the spectra after addition of antimycin. C) is a computer simulated spectra for a Gaussian shaped curve. EPR instrument settings are detailed in the text.

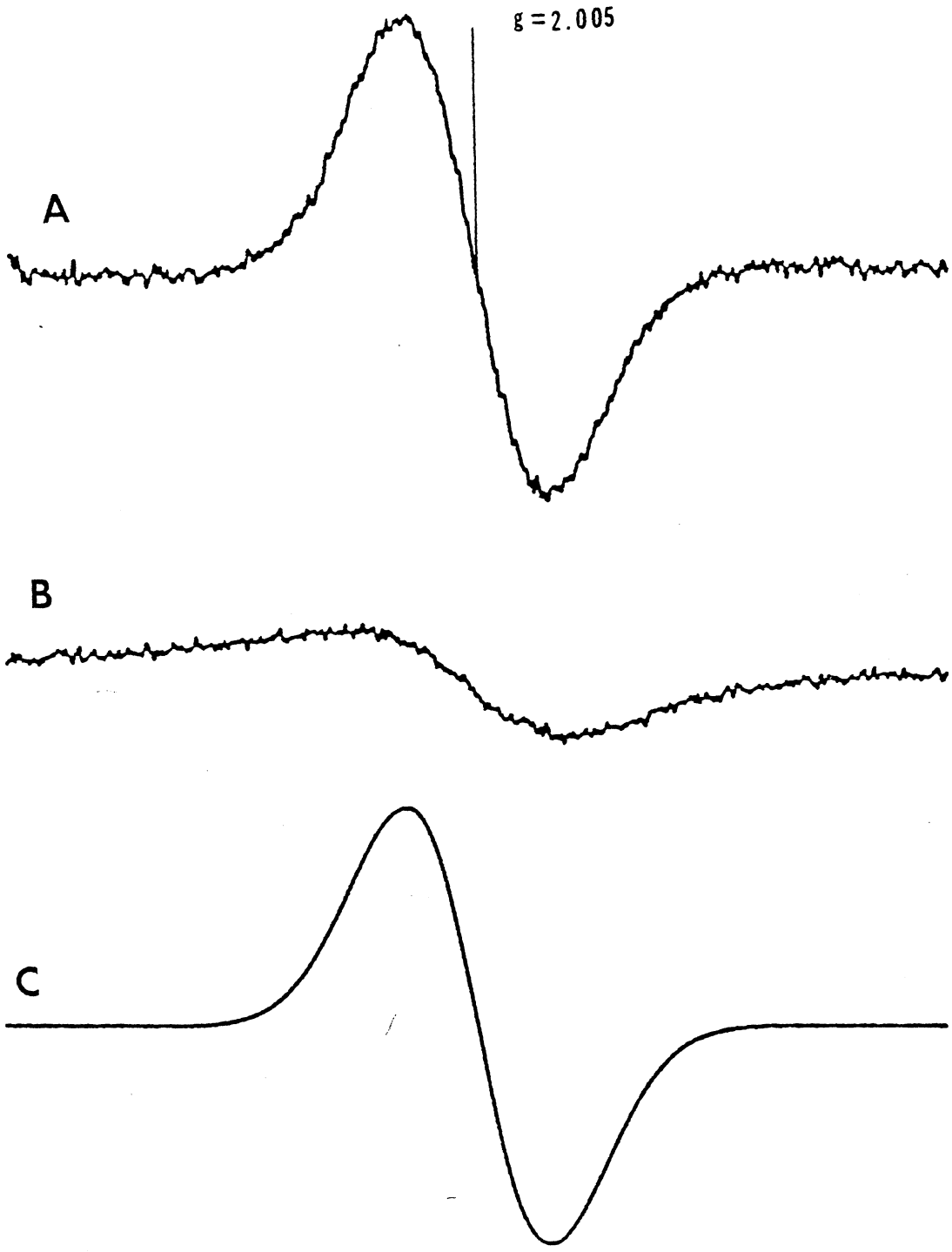
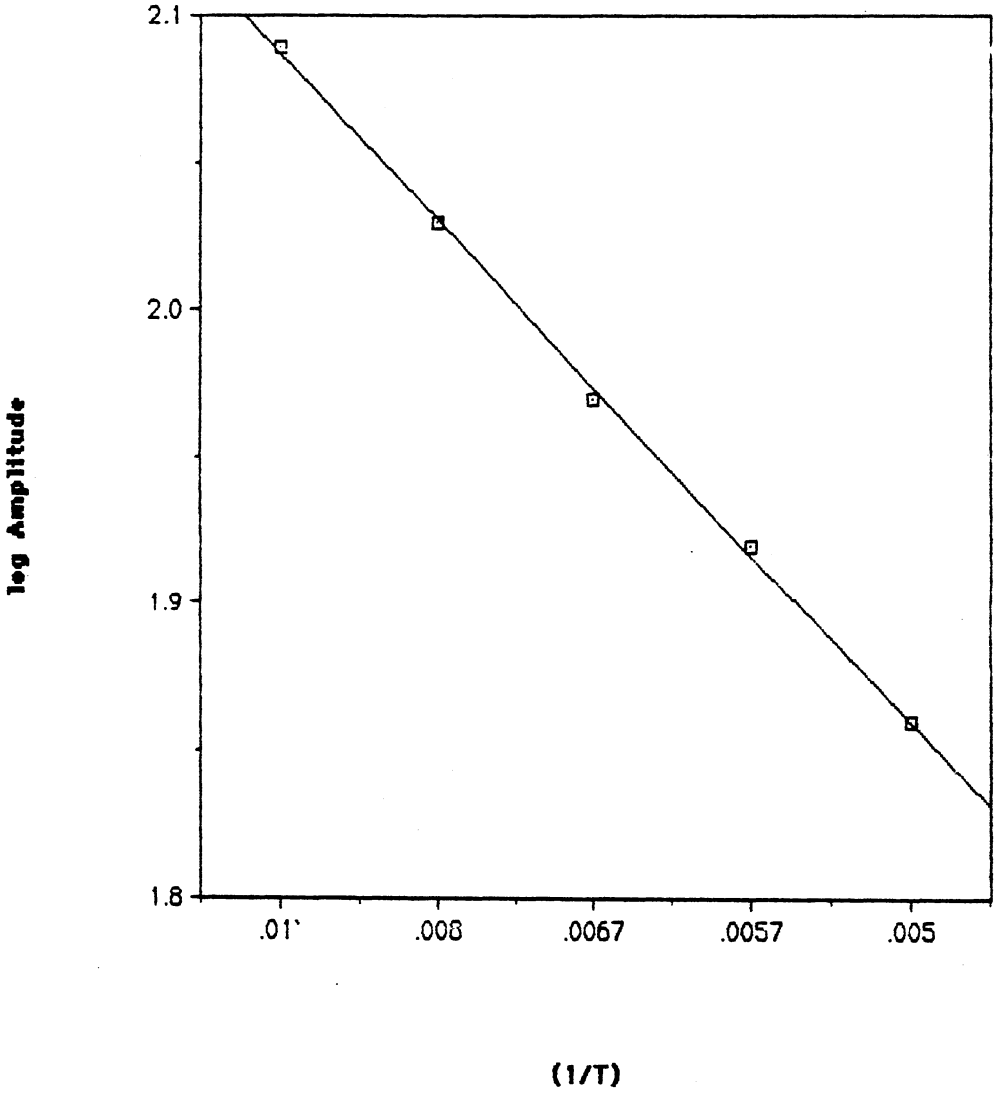

g = 2.005
A
B
C

Figure 9. Temperature Dependence of Radical Signal.

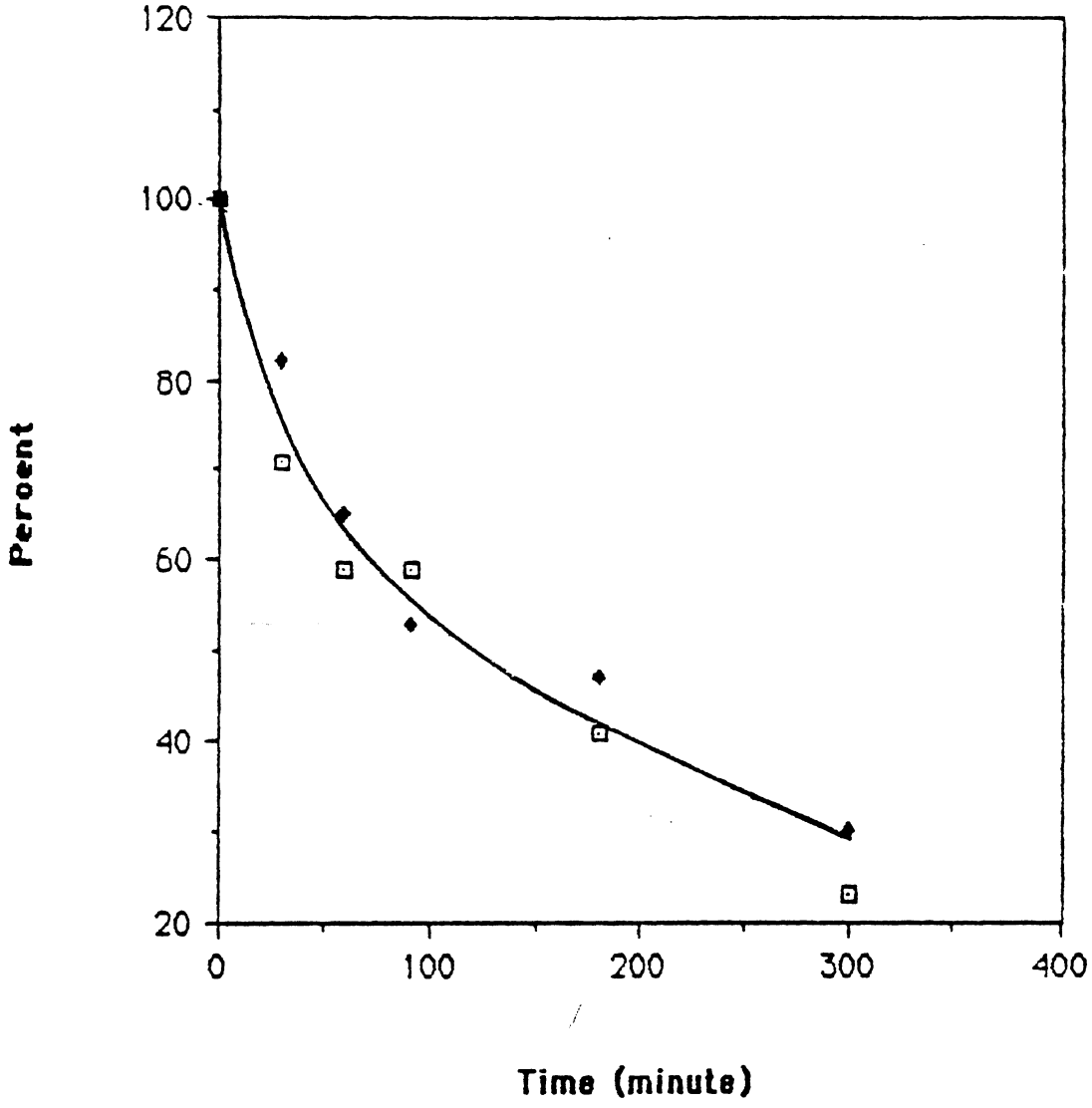

2.1
2.0
1.9
1.8
log Amplitude
.01
.008
.0067
.0057
.005
(1/T)

Yet the possibility of interference from the iron-sulfur protein at 77°K needed to be investigated. The concentration at which the enzyme sample was studied was not sufficient to display any ISP contribution. This lack of interference allowed the use of liquid nitrogen temperature (77°K) which provides a better signal to noise ratio than at 200°K, which is the temperature used in more complex systems such as submitochondrial particles and chromatophores.

The Relationship Between Enzymatic Activity and Q_c Signal

The EPR signal of Q_c was investigated as a function of the natural thermal denaturation process of the enzyme. To provide a consistent set of redox potentials from one sample to the next, in a short period of time, the use of succinate dehydrogenase in a succinate/fumarate solution provided a redox potential of around 90 mV. The stability of Rs.*b*-c_1 at 0°C has already been reported.[26] From the reported data, the enzyme complex showed no loss of activity over the course of three hours. The typical time required for a redox titration was 1.5-2 hours. However at 30°C the activity remaining was only 25% of the original activity after 3 hrs. The EPR signal intensity was taken as the untreated sample intensity minus the intensity of the antimycin-treated sample intensity. The results of this study shows clearly that the signal intensity decreases proportionally to the enzyme activity as seen in Figure 10. The observation that the enzymatic activity is proportional to the EPR signal intensity is of interest in two aspects, the first being that, of the studies which have previously[54, 55, 57, 60] been done on Q_c only one[57] reports the specific activity of the enzyme complex. This is disturbing as such, due to calculations done which involve quantitation of $Q_c^{\cdot-}$. There is an underlying assumption that all these preparations are at maximal activity and thereby maximal $Q_c^{\cdot-}$ formation. The second item that is noted by the enzymatic dependence of the $Q_c^{\cdot-}$ signal is that, albeit indirectly, there is a good possibility that antimycin acts as an antagonist of the structural integrity of the enzyme,[59] as discussed in the literature review, rather than a direct block of electron flow.

Figure 10. Correlation Between Enzymatic Activity and EPR Signal Intensity. The percent of activity remaining is shown as (•) and the EPR signal at that time as (o).


120
100
80
60
40
20
0
100
200
300
400
Percent
Time (minute)

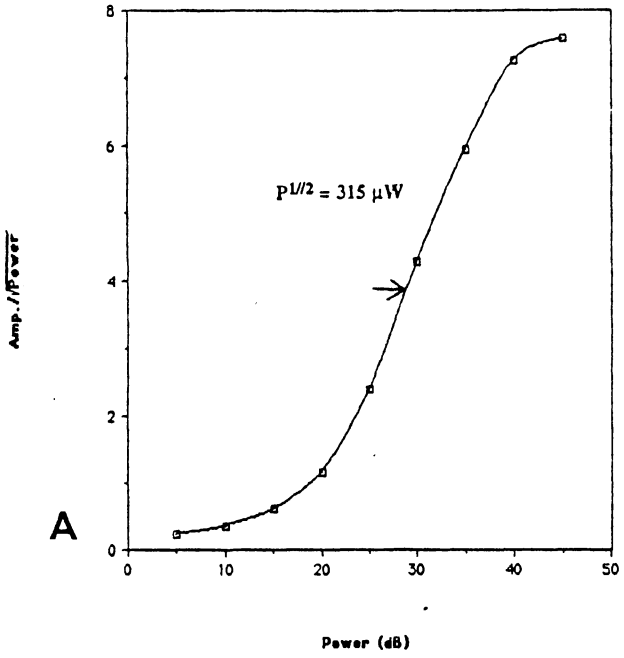
Relaxation Characteristic of Q_c

The power saturation curve is made by plotting the amplitude divided by the square root of the power versus the log power i.e. dB, where the maxiumum power is 200 mW at OdB, as seen in Figure 11. The value most commonly reported is $P^{1/2}$, this is the point where the amplitude factor is 1/2 the maximum. In the $Rsbc_1$ samples there appears to be two different radical species which are distinguishable by power saturation characteristics. The Q_c signal which is monitored at relatively low power, 10 μW, shows saturation around 630 μW while an unknown signal which is insensitive to antimycin is not saturated even at 200 mW. This indicates that the Q_c is in an isolated location since it cannot cross-relax with another redox center. The unknown signal may be a contaminant or possibly a mediator dye, but in background control titrations the signal seen is only half of that seen when antimycin treated Rs.*b*-c_1 was titrated.

Redox Behavior of Q_c

From the redox potential and EPR signal height measurements the Q/QH_2 couple midpoint can be determined. By plotting the redox potential (mV) versus the EPR height of the Q_c signal the maximum signal height corresponds to the midpoint of the redox couple as seen for pH 8.4 in Figure 12. From the data, at various pH values the midpoint potential is seen to vary by approximately -60 mV per pH unit as seen in Figure 13. This is typical of an ubisemiquinone radical. It has been estimated [75] that the Q/QH_2 couple in the Q pool of *Rhodobacter sphaeroides* is 90 mV at pH 7. The $E_{M,7}$ in the isolated Rs.*b*-c_1 was found to be 140 mV. This value is very close to that reported for Q_c in the chromatophore preparation,[61] $E_{M,7}$ = 150 mV. This increase in the $E_{M,7}$ over that of the Q pool is indicative of the fact that Q_c is not in equilibrium with a Q pool and that the binding site preferentially binds the quinol form rather than the quinone.[61] The width of the titration curve at half the height is approximately 125 mV, this data when compared to the values calculated in reference 61 indicate that the fractional amount of $Q_c^{\cdot}/c_1$ is 0.6 and .

Figure 11. Power Saturation Curve. A) The signal intensity/$\sqrt{}$ power is plotted versus dB where OdB = 200 μW. B) Amplitude is plotted versus dB.


A
$P^{1/2}$ = 315 μW
Amp./√Power
Power (dB)
0
2
4
6
8
0
10
20
30
40
50

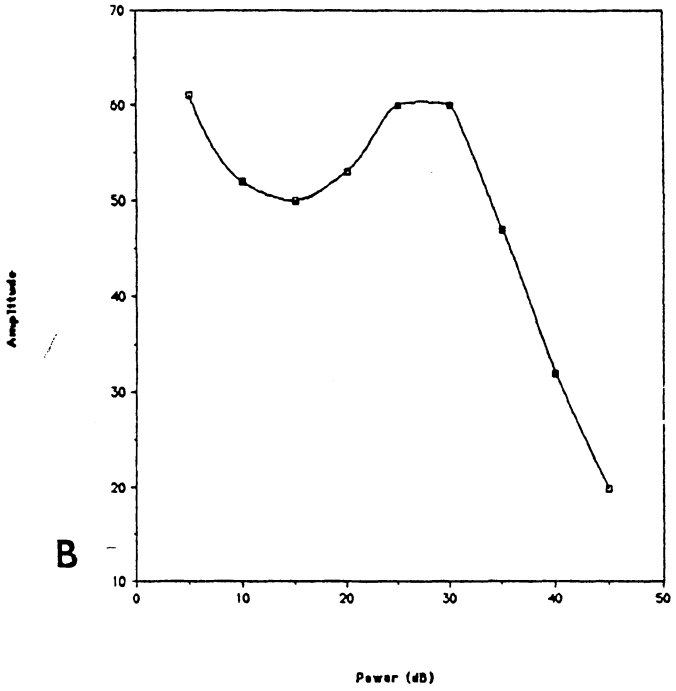

B
Amplitude
Power (dB)
10
20
30
40
50
60
70
0
10
20
30
40
50

Figure 12. E_h vs. Signal Intensity at pH 8.4.

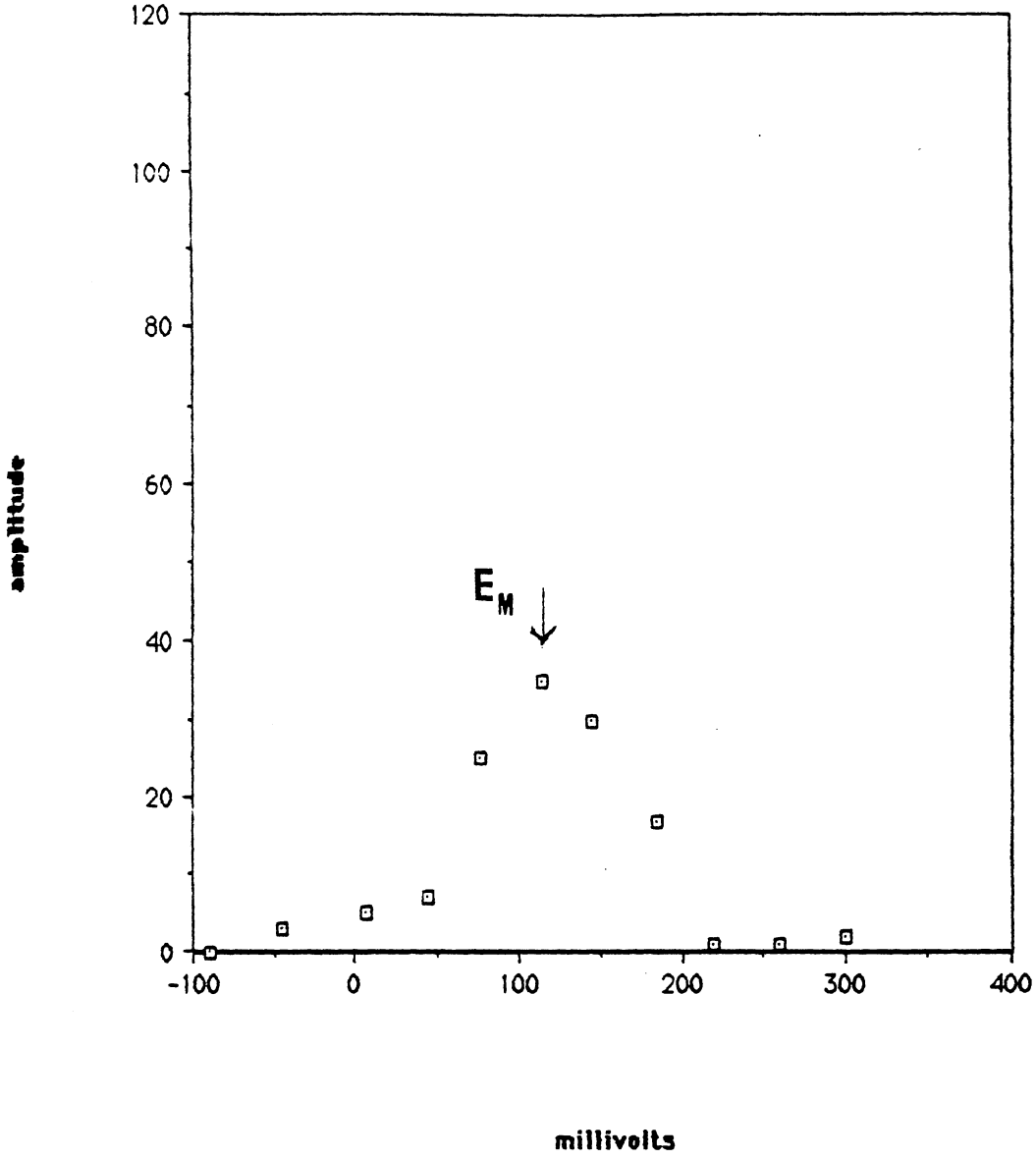
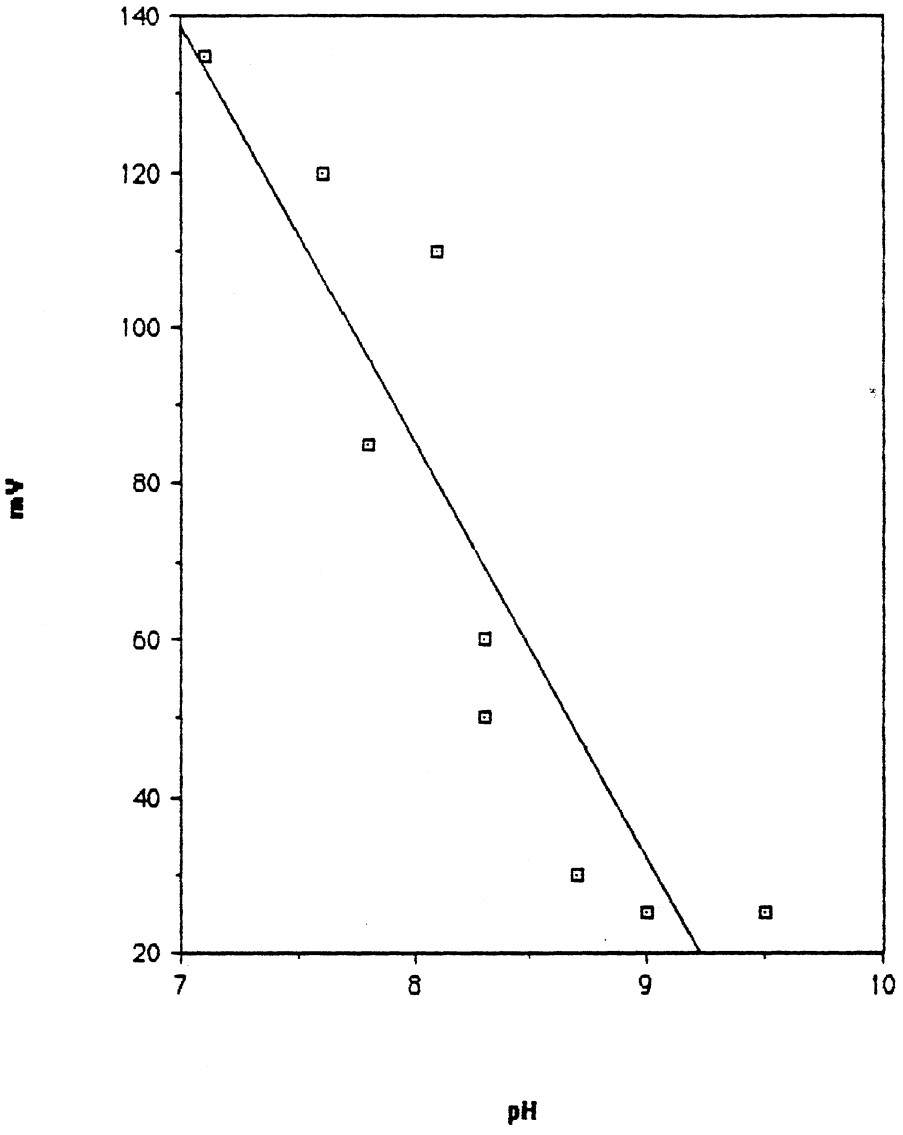

120
100
80
60
40
20
0
amplitude
E_M
-100
0
100
200
300
400
millivolts

Figure 13. E_m vs. pH.


140
120
100
80
60
40
20
mV
7
8
9
10
pH

E_1-E_2 is about 55 mV at pH 8.4, where $Q_c^{\cdot-}$ is maximal. The stability constant Ks was estimated to be 2.5 from the equation E_1-$E_2 \equiv 60$ ln Ks at pH 8.4. This value is comparable to that obtained in the *Rhodobacter sphaeroides* chromatophore.[61]

Quantitation of the Q_c

The double integration of the Q_c radical signal yielded a value of 4μm for the $Q_c^{\cdot-}$ when compared to a 10 μM sample of the spin label 3-(Maleimidomethyl)-Proxyl. This gave a ratio of 0.2 for $Q^{\cdot-}/c_1$, but the activity of the enzyme was about one-third of the most active preparations. When the effect of denaturation is taken into consideration, the value approaches 0.6 $Q_i^{\cdot-}/c_1$. This is in agreement with the calculation obtained from the redox titration data.

CHAPTER V

SUMMARY

The ubisemiquinone radical anion, Q_c, was studied utilizing electron paramagnetic resonance spectroscopy in combination with redox potentiometry. The main objective of this study was to determine the redox behavior of the $Q_c^{\cdot}$ in the isolated *b*-c_1 complex and compare this to previously reported studies. The data obtained allows the qualitative assumption that the isolated *b*-c_1 complex is very similar thermodynamically to the *b*-c_1 complex in the native system.

Specifically, the experiments showed that the Q_c radical spectra is gaussian in shape, has a linewidth of 8G, a g-value of 2.0046 and saturates at approximately 630 μW. From this information it was concluded that indeed this was a $Q^{\cdot}$ radical and that it was isolated from any redox centers. It was also determined that the signal intensity of the radical was proportional to the QCR enzyme activity.

The redox behavior of the $Q_c^{\cdot}$ was found to be typical of reported ubisemiquinone radical anion behavior. The $E_{M,7}$ was extrapolated to be about +140 mV and changed by –60 mV/pH. From the width at half the maximum signal intensity of the titration curve it was determined that E_1-E_2 = 55 mV and that the ratio of $Q_c^{\cdot}/c_1$ was about 0.6 at pH 8.4. This value obtained was also substantiated by spin quantitation using a spin label as a standard.

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