SUPEROXIDE PRODUCTION IN RESPIRATORY ELECTRON TRANSPORT MINIMIZATION AND UTILIZATION

By

ISAAC PAUL FORQUER

A dissertation submitted in partial fulfillment of
the requirements for the degree of

DOCTOR OF PHILOSOPHY

WASHINGTON STATE UNIVERSITY
School of Molecular Biosciences

December 2008

To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of ISAAC PAUL FORQUER find it satisfactory and recommend that it be accepted.

_____
Chair

_____

_____

_____

ACKNOWLEDGMENT

I would like to acknowledge first and foremost my mentor and advisor Dr. David M. Kramer, who has shown patience and compassion with me over the years of our working relationship. In addition, he had the courage to be tough and demanding when I needed it the most. Most of all, Dave has taught me science, not in the way of the trivial matters of day to day working (though he did do that too), but in the way that science is not a job or school subject, but rather an approach to life in general. At some point the details of this document will be outdated at best, but the means by which the ideas presented herein were generated and tested are the true achievement that I will boast. I have no one more than Dave to thank for the intellectual tools learned during this journey.

It is a funny thing in the Kramer Lab, even amongst other graduate students enrolled in the SMB, our lab contained some unusual characters that sort of defines the culture of our workplace. To my very good and life long friends, Tom Avenson, Jeff Cruz, Atsuko Kanazawa, Kenji Takizawa, Kristy Podelnyk and Aaron Livingston, I thank you for your dedication and support over the years. To Jonathan Cape, to whom I am truly in debt, for being an inspirational and motivating person to work with, in addition to being a first class friend, I thank you.

The work done on *Plasmodium* in this dissertation would not have been possible without the extraordinary efforts of the workers in Dr. Michael Riscoe's Lab. To Dr. Riscoe, Marty Smilkstein, Jane Kelly and Rolf Winters I thank you for the support you provided me.

My daughter Anna Forquer provided me with a new form of motivation and pride that actually helped me to see the importance of doing my work. Her joy and passion for life that I see as a reflection of myself has been a true gift in life.

Last, Jenn Cotter saved me from the pits of despair in my darkest days. She not only picked me up, but propelled me to see me for who I really am and what I could be. She believed in me when I did not believe in myself and has shown me a form of beauty I had not yet experienced.

SUPEROXIDE PRODUCTION IN RESPIRATORY ELECTRON TRANSPORT

MINIMIZATION AND UTILIZATION

Abstract

by ISAAC PAUL FORQUER
Washington State University
December 2008

Chair: David M. Kramer

The oxidation of ubiquinol by the cytochrome bc_1 complex is usually a tightly controlled process, however there are instances when the reaction is compromised by mutation or other external factors such as inhibitors that leads to superoxide production. The first part of this dissertation compares the electron transfer reactions between when the enzyme is performing efficiently and when superoxide production is taking place. The results of this section suggest that the same rate limiting step governs the two reactions, which allows for the elimination of several models explaining these critical reactions.

The second part of this thesis deals with the electron transport chain of mitochondria in *Plasmodium* spp., the causative protozoan of malaria. First, it was hypothesized that a series of modified acridones were inhibitors of the cytochrome bc_1 complex in *Plasmodium* spp. To validate the target of these compounds, they were tested first to see if in fact they inhibited the enzyme in mitochondrial extracts of *Plasmodium yoelli.* Then to conduct more detailed studies, the compounds were tested against the cytochrome bc_1 complex from rat heart tissue. The results showed that acridones with an alkyl tail in the 3-position of the main ring were 1. potent inhibitors of the enzyme and 2. inhibited the site of ubiquinol oxidation.

The last part of this dissertation explores a controversy in the field of malaria research. An interesting observation was made by some groups that *Plasmodium* spp. contained an 'alterative oxidase', leading to cyanide resistant respiratory oxygen consumption. However, no such gene was found in any of the several species of *Plasmodium* spp. for which the genome has been sequenced. It was hypothesized that dihydroorotate dehydrogenase, a critical enzyme in terms of pyrimidine biosynthesis in *Plasmodium* spp., was the source of the apparent cyanide sensitive oxygen consumption, in the form of superoxide production. The data presented suggests this is in fact the case, and that the very activity leading to this phenomenon may explain a major group of drug resistant parasites found in nature.

TABLE OF CONTENTS

TABLE OF FIGURES

Chapter 1:

Introduction

Isaac Forquer

Since the advent of oxygenic photosynthesis, oxygen has been the major source of potential energy to drive aerobic metabolism. The increase in driving force that using oxygen as a terminal electron acceptor affords organisms greatly expanded genetic arenas for biota to evolve in (1). Almost all major clades of Eukaryotic life on earth utilize oxygen as the terminal electron acceptor in the mitochondrial electron transport chain.

Living in environments where such a high energy electron acceptor such as oxygen does not come without risks. Recent advances in our understanding of how deleterious side reactions involving reactive enzyme intermediates in oxygen redox chemistry have highlighted our understanding of aging and other mitopathies that are lethal or significantly decrease the quality of life for humans (2-7). Enzymes that utilize high-energy intermediates capable of reducing oxygen are therefore under selection to minimize the interaction of these intermediates with oxygen.

Perhaps it follows then that some systems have evolved mechanisms of coping with, and perhaps utilizing small amounts of reactive oxygen species. There are salient examples of organisms using reactive oxygen species as a signaling molecule to up-regulate (8), among other things, detoxifying mechanisms for reactive oxygen (9).

The cytochrome bc1 complex (cyt bc1 complex) in mitochondria and some bacteria is the third major respiratory enzyme on the electron transport chain (10-12). It acts as a quinol oxidase, which involves two-electron transfers from the quinol to generate quinone. In doing so,

there is the risk of stably generating the one-electron semiquinone that is relatively reactive and capable of reducing oxygen (13). Despite this possibility, most investigators observed that the semiquinone was either not present or present in sufficiently small steady state concentrations so as to not present any real threat in terms of superoxide production, in addition to the fact that no one had observed the semiquinone spectroscopically.

Several controversial models attempted to explain the observed efficiency of quinol oxidation. They all operated within the general confines of the Q-cycle, first proposed by Mitchell (14). In the Q-cycle, quinol (depending on the organism, different species of quinol are used, the most frequently encountered is ubiquinol) is bound at the quinol oxidase (Q_o) site and oxidized by the Rieske [2Fe2S] cluster. The electron continues down the high potential chain where it is ultimately transferred to the docked water-soluble cyt c. Cyt c then undocks and proceeds to cyt c oxidase to provide reducing equivalents for oxygen reduction. The second electron from quinol at the Q_o site is transferred to the low potential chain, consisting of two hemes, cyt b_L and b_H, with b_L being the "low-potential" and b_H the relatively "high-potential" heme. Electrons are transferred from cyt b_L to cyt b_H, and then to a bound quinone at the quinone reductase (Q_i) site, forming a stable semiquinone. Once another quinol is processed at the Q_o site, a second electron transfer from cyt b_H to the semiquinone generates a quinol.

One major division in the models to account for the highly efficient bifurcated electron transfer event at the Q_o site was found in the timing of the reaction. One model, the double concerted electron transfer (DCET) model, proposed in (15), implicated simultaneous electron transfers from the quinol to the high and low potential chains. The DCET model could easily account for the for the lack of an observed semiquinone because.

Within the sequential models, numerous strategies were presented to account for the observed activity of the enzyme (12). All of them were tethered to the idea that quinol was first oxidized by [2Fe2S], and then somehow the semiquinone was safely oxidized by the low-potential chain, and not by oxygen or any other exogenous electron acceptors.

All of the models considered here were nicely described (12), and are briefly summarized below. In kinetic steering models such as presented by (16), the semiquinone is kinetically trapped into reaction coordinates that make only the desirable reaction probable compared to other reactions. Gated models (17,18) provide a mechanical barrier to the first quinol oxidation until the enzyme is prepared to efficiently catalyze the bifurcated electron transfer. In this way the first electron transfer won't take place until the low potential chain is capable accepting the electron, thereby avoiding the generation of substantial amounts of semiquinone. In semiquinone stabilization models (19,20), the typical view of enzyme catalysis is employed and the activation energy of quinol oxidation is lowered, leaving a relatively stable semiquinone, that would leave the semiquinone refractory to oxygen but in steady state concentrations that can support the Q-cycle. On the other hand, semiquinone destabilization models (21) make the unusual prediction that the activation energy for the first quinol reaction is purposefully high, thus generating a very low concentration of quite reactive semiquinone. The advantage here is that the semiquinone is never in high enough concentrations to significantly interact with oxygen to support superoxide production.

Related to the details of quinol oxidation was the open question concerning the identity of the oxygen reductant when the cyt bc1 complex is partially inhibited by both Q_i and Q_o site inhibitors leading to superoxide production. Clearly, in the DCET model, if a semiquinone acted as the reductant, then a dramatically different reaction mechanism of quinol oxidation would

have to take place during normal Q-cycle reactions and superoxide production. However in sequential electron transfer models, the semiquinone generated by the initial electron transfer to the high-potential chain could be the reductant for oxygen.

Because of the central role that the cyt bc_1 complex plays in organisms employing aerobic metabolism (i.e. oxidative metabolism), it is not surprising that if an opportunity to generate an inhibitor that could shut down the enzyme in an infectious organism without affecting the mammalian enzyme would present itself, the infectious disease community would embrace it as a valuable drug. In fact this is exactly the case of drug known as Atovaquone.

Malaria is probably responsible for more premature human deaths than any other natural phenomenon in the history of humankind. Over 1 million people will die from malaria this year, out of about 500-700 million people who will be afflicted with the disease (22). Today, malaria persists in regions of the world where habitat is plentiful for the mosquito vector, *Anopheles* spp., and where there are geographic and especially economic barriers to treatment (23).

One of the biggest challenges the scientific and medical communities face in combating the malaria epidemic is the ever-increasing populations of drug-resistant strains of *Plasmodium* spp., the infectious protozoan of malaria. Numerous reports from around the world have verified that many of the traditionally effective drugs have elicited resistant strains of the parasite, causing shifts in drug distribution strategies (24-29). The most widely adopted strategy is to always treat individuals with at least two independently acting drugs to lessen the possibility of a resistance response by the infecting population of parasites.

Bearing the current situation in mind, it is obvious that new generations of anti-malarial drugs are needed in this fight. It is also imperative that we expand our list of potential targets to combat resistance.

The mitochondria of *Plasmodium* spp. are distinct from well-known mammalian and other eukaryotic mitochondria. These differences appear to be manifested at several levels, including biochemical organization, where a functional TCA cycle has not been observed (30-32). Perhaps most importantly, at least some of the major respiratory complexes on the mitochondrial electron transport chain show subtle structural modifications (with respect to the mammalian system) that have allowed the use of one drug, Atovaquone, to be used to selectively inhibit respiration in *Plasmodium falciparum* (33-35).

Atovaquone (a quinone analog) is a competitive inhibitor of the quinol oxidase site Q_o site of the cytochrome bc_1 complex (E.C. 1.10.2.2) (cyt bc_1 complex). Subtle differences in the Q_o site primary structure results in order of magnitude sized differences in K_D for atovaquone between the *Plasmodium* spp. and mammalian systems (35).

Other mitochondrial targets have been exploited in *Plasmodium* spp. Dihydroorotate dehydrogenase (E.C. 1.3.3.1) has been targeted in the treatment of malaria (30) as the enzyme is vital to the survival of *Plasmodium* spp., which, unlike mammals, has no known pyrimidine salvage mechanism.

Plasmodium spp. was also thought to have a cyanide insensitive alternative quinol oxidase (AO) (E.C. 1.10.X.X) , resulting in a branched (and at least somewhat redundant) electron transport chain (30). The evidence that AO exists was is based on the observation that cyanide does not fully (only 75%) inhibit oxygen reduction. The subject has become controversial with the publication of several species and sub-species of *Plasmodium* spp. that do not contain any transcript of an alternative oxidase (36). The value of such a target is obvious, with no such enzyme in humans, it would be a sure-fire drug target for malaria treatment.

1. Koch, L. G., and Britton, S. L. (2008) *J Physiol* **586**, 83-95
2. Genova, M. L., Pich, M. M., Biondi, A., Bernacchia, A., Falasca, A., Bovina, C., Formiggini, G., Castelli, G. P., and Lenaz, G. (2003) *Exp Biol Med (Maywood)* **228**, 506-513
3. Kristal, B. S., Jackson, C. T., Chung, H. Y., Matsuda, M., Nguyen, H. D., and Yu, B. P. (1997) *Free Radic Biol Med* **22**, 823-833
4. Lebovitz, R. M., Zhang, H., Vogel, H., Cartwright, J., Jr., Dionne, L., Lu, N., Huang, S., and Matzuk, M. M. (1996) *Proc Natl Acad Sci U S A* **93**, 9782-9787
5. Lin, Y., Berg, A. H., Iyengar, P., Lam, T. K. T., Giacca, A., Combs, T. P., Rajala, M. W., Du, X., Rollman, B., Li, W., Hawkins, M., Barzilai, N., Rhodes, C. J., Fantus, I. G., Brownlee, M., and Scherer, P. E. (2005) *J. Biol. Chem.* **280**, 4617-4626
6. Schmiedel, J., Jackson, S., Schafer, J., and Reichmann, H. (2003) *J Neurol* **250**, 267-277
7. Young, T. A., Cunningham, C. C., and Bailey, S. M. (2002) *Arch Biochem Biophys* **405**, 65-72
8. Carmen Wolf, F. H., Harald Kusch, Dirk Albrecht, Michael Hecker, Susanne Engelmann,. (2008) *PROTEOMICS* **8**, 3139-3153
9. Brune, B., and Zhou, J. (2007) *Cardiovasc Res* **75**, 275-282
10. Crofts, A. R. (2004) *Annu Rev Physiol* **66**, 689-733
11. Trumpower, B. L., and Gennis, R. B. (1994) *Annu Rev Biochem* **63**, 675-716
12. Cape, J. L., Bowman, M. K., and Kramer, D. M. (2006) *Trends Plant Sci* **11**, 46-55
13. Kramer, D. M., Roberts, A. G., Muller, F., Cape, J., and Bowman, M. K. (2004) *Methods Enzymol* **382**, 21-45
14. Mitchell, P. (1975) *FEBS Lett* **56**, 1-6
15. Osyczka, A., Moser, C. C., Daldal, F., and Dutton, P. L. (2004) *Nature* **427**, 607-612
16. Snyder, C. H., Gutierrez-Cirlos, E. B., and Trumpower, B. L. (2000) *J Biol Chem* **275**, 13535-13541
17. Darrouzet, E., Moser, C. C., Dutton, P. L., and Daldal, F. (2001) *Trends Biochem Sci* **26**, 445-451
18. Zhang, Z., Huang, L., Shulmeister, V. M., Chi, Y. I., Kim, K. K., Hung, L. W., Crofts, A. R., Berry, E. A., and Kim, S. H. (1998) *Nature* **392**, 677-684
19. Link, T. A. (1997) *FEBS Lett* **412**, 257-264
20. Berry, E. A., and Huang, L. S. (2003) *FEBS Lett* **555**, 13-20
21. Crofts, A. R. (2004) *Biochim Biophys Acta* **1655**, 77-92
22. Korenromp, E., Miller, J., Nahlen, B., Wardlaw, T., Young, M. (2005), World Health Organization
23. WHO. (2005), World Health Organization
24. Mi-Ichi, F., Takeo, S., Takashima, E., Kobayashi, T., Kim, H., Wataya, Y., Matsuda, A., Torii, M,. Tsuboi, T. and Kita, K. (2003) in *Tropical Diseases From Molecule to Bedside* (Marzui, V. a. S., ed) Vol. 531, pp. 117-133, Kluwer Academic/ Plenum Publishers, New York
25. Syafruddin, D., Asih, P.B.S., Siregar, J. E. and Tjitra, E. (2003) in *Tropical Diseases From Molecule to Bedside* (Marzuki, S., Verhoef and Snippe, ed) Vol. 531, pp. 103-115, Kluwer Academic/ Plenum Publishers, New York
26. Syafruddin, D., Siregar, J. E., and Marzuki, S. (1999) *Mol Biochem Parasitol* **104**, 185-194

27. Korsinczky, M., Chen, N., Kotecka, B., Saul, A., Rieckmann, K., and Cheng, Q. (2000) *Antimicrob Agents Chemother* **44**, 2100-2108
28. Peters, J. M., Chen, N., Gatton, M., Korsinczky, M., Fowler, E. V., Manzetti, S., Saul, A., and Cheng, Q. (2002) *Antimicrob. Agents Chemother.* **46**, 2435-2441
29. Gil, J. P., Nogueira, F., Stromberg-Norklit, J., Lindberg, J., Carrolo, M., Casimiro, C., Lopes, D., Arez, A. P., Cravo, P. V., and Rosario, V. E. (2003) *Molecular and Cellular Probes* **17**, 85-89
30. Krungkrai, J. (2004) *Parasitology* **129**, 511-524
31. Fry, M., and Beesley, J. E. (1991) *Parasitology* **102 Pt 1**, 17-26
32. Vaidya, A. B., and Mather, M. W. (2005) *Curr Top Microbiol Immunol* **295**, 233-250
33. Kessl, J. J., Ha, K. H., Merritt, A. K., Lange, B. B., Hill, P., Meunier, B., Meshnick, S. R., and Trumpower, B. L. (2005) *J. Biol. Chem.* **280**, 17142-17148
34. Kessl, J. J., Lange, B. B., Merbitz-Zahradnik, T., Zwicker, K., Hill, P., Meunier, B., Palsdottir, H., Hunte, C., Meshnick, S., and Trumpower, B. L. (2003) *J. Biol. Chem.* **278**, 31312-31318
35. Mather, M. W., Darrouzet, E., Valkova-Valchanova, M., Cooley, J. W., McIntosh, M. T., Daldal, F., and Vaidya, A. B. (2005) *J. Biol. Chem.* **280**, 27458-27465
36. Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W., Carlton, J. M., Pain, A., Nelson, K. E., Bowman, S., Paulsen, I. T., James, K., Eisen, J. A., Rutherford, K., Salzberg, S. L., Craig, A., Kyes, S., Chan, M.-S., Nene, V., Shallom, S. J., Suh, B., Peterson, J., Angiuoli, S., Pertea, M., Allen, J., Selengut, J., Haft, D., Mather, M. W., Vaidya, A. B., Martin, D. M. A., Fairlamb, A. H., Fraunholz, M. J., Roos, D. S., Ralph, S. A., McFadden, G. I., Cummings, L. M., Subramanian, G. M., Mungall, C., Venter, J. C., Carucci, D. J., Hoffman, S. L., Newbold, C., Davis, R. W., Fraser, C. M., and Barrell, B. (2002) **419**, 498-511

Chapter 2:

Similar Transition States Mediate the Q-cycle and Superoxide Production by the Cytochrome bc_1 Complex*

Isaac Forquer[1], Raul Covian[2], Michael K. Bowman[3], Bernard L. Trumpower[2] and David M. Kramer[1]

[1]Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340
[2]Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755
[3]Structural Biology and Microimaging, Battelle Northwest Laboratory, PO Box 999 K8-98, Richland, Washington 99352-0999

Abstract

The cytochrome *bc* complexes found in mitochondria, chloroplasts and many bacteria play critical roles in their respective electron transport chains. The quinol oxidase (Q_o) site in this complex oxidizes a hydroquinone (quinol), reducing two one-electron carriers, a low-potential cytochrome *b* heme and the 'Rieske' iron-sulfur cluster. The overall electron transfer reactions are coupled to transmembrane translocation of protons via a 'Q-cycle' mechanism, which generates proton motive force for ATP synthesis. Since semiquinone intermediates of quinol oxidation are generally highly reactive, one of the key questions in this field is: how does the Q_o site oxidize quinol without the production of deleterious side reactions including superoxide production? We attempt to test three possible general models to account for this behavior: 1) The Q_o site semiquinone (or quinol:imidazolate complex) is unstable and thus occurs at a very low steady-state concentration, limiting O_2 reduction; 2) the Q_o site semiquinone is highly stabilized making it unreactive towards oxygen; and 3) the Q_o site catalyzes a quantum mechanically-coupled two-electron/two proton transfer without a semiquinone intermediate. Enthalpies of activation were found to be almost identical between the uninhibited Q-cycle and

superoxide production in the presence of Antimycin A in wild type. This behavior was also preserved in a series of mutants with altered driving forces for quinol oxidation. Overall, the data supports models where the rate-limiting step for both Q-cycle and superoxide production is essentially identical, consistent with model 1 but requiring modifications to models 2 and 3.

Introduction

The cytochrome (cyt) bc_1 complex (E.C. 1.10.2.2) (cyt bc_1 complex) is found on the inner-membrane of mitochondria and energy transducing membranes of many bacteria (1-3). It is structurally and functionally homologous to a taxonomically wide spread group, collectively referred to as "*bc* complexes", additionally consisting of the cyt b_6f complex and the menaquinol oxidizing complexes in many bacteria (4-6). In all well studied cases, cyt *bc* complexes couple the oxidation of a substrate quinol (QH_2) to the formation of a proton motive force (*pmf*) across the energy transducing membrane in which the given complex resides, the energy of which is ultimately stored as ATP .

Cytochrome bc_1 complexes contain four redox active metal centers, arranged in two separate chains (2,5,7-9). The "high-potential chain" consists of the Rieske iron-sulfur [2Fe2S] cluster (in the "Rieske", or "Iron-Sulfur subunit" – hereafter "the [2Fe2S] cluster"), and a *c*-type cyt, known in mitochondria and gram negative bacteria as cyt c_1. The "low-potential chain", which is liganded to the cyt *b* subunit of ubiquinol oxidizing complexes, consists of two *b*-type hemes, cyt b_L and b_H labeled for their relatively lower and higher electrochemical potentials. Crystal structures from several taxonomic sources (2,8,10,11) suggest a well conserved placement of the cofactors throughout the various cyt *bc* complexes.

Three enzymatic binding sites participate in catalysis on the cyt bc_1 complex, the quinol oxidase (Q_o) site, quinol reductase (Q_i) site and a docking site for soluble cyt *c* on cyt c_1. The Q_o

site is located toward the positively-charged side of the membrane, where protons are released during turnover of the enzyme. The Q_i site is located toward the negatively-charged surface of the membrane, where protons are taken up during catalysis. The water-soluble cyt *c* docking site is located on the *c*-type cyt representing the terminal electron carrier within the cyt bc_1 complex on the positive side of the membrane.

Cyt bc_1 complex catalysis is thought to occur by a "Q-cycle" mechanism (1,12-14). Perhaps the key reaction of the Q-cycle is the 'bifurcated' electron transfer at the Q_o site, the mechanism of which is still controversial (1,4,7,15-17). In the bifurcated reaction, QH_2 is oxidized at the Q_o site, with one electron being transferred through the high-potential chain to reduce cyt *c*, and the other electron is transferred through the low potential chain, to reduce a quinoid species (Q or SQ, depending on the state of the two-electron gate) at the Q_i site. Two turnovers of the Q_o site are required to reduce a Q_i site Q to a QH_2.

Under some conditions, the Q-cycle can be short circuited by various "bypass" reactions, some of which yield the physiologically deleterious superoxide (18-22). The bypass reactions are typically observed *in vitro* under 'partially inhibited' conditions, e.g. in the presence of Antimycin A (AA) or under high proton motive force, where it is thought that the [2Fe2S] cluster can oxidize QH_2 to a semiquinone (SQ), but processing of electrons by the low-potential chain is hindered, resulting in the accumulation of a SQ intermediate which in turn can reduce O_2 to superoxide (18,20,21). Under such conditions, superoxide production can occur at 2-10% of the uninhibited rates. Superoxide production rates approaching 100% of normal turnover were observed when the natural substrate, ubiquinol, was replaced with rhodoquinol (23).

We consider here the three classes of models, illustrated in Scheme I, proposed to account for the high efficiency of the bifurcated reaction under uninhibited conditions. The

models differ in the nature of the reactive intermediates of quinol oxidation (Reviewed in (24)). In the first class (Scheme I, panel A), SQ is destabilized so that its concentration is kept very low, preventing rapid rates of O_2 reduction (25). In the second category (Scheme I, panel B), the SQ intermediate is stabilized by the Q_o site, making it less reactive to O_2 (26,27). In the third category (Scheme I, panel C), the SQ is avoided completely by forcing both quinol electrons to be transferred simultaneously (28,29). In addition to the above models, a number of 'gating' mechanisms have been proposed where the Q_o site is modified during key stages of catalysis, preventing the escape of reactive intermediates (30-32).

In this work, we attempt to discriminate among these models by comparing the activation energetics for the uninhibited Q-cycle and superoxide production in the presence of AA in *Saccharomyces cerivisae* complexes with a range of [2Fe2S] cluster redox potentials (33,34).

Materials and Methods

Yeast Strains- Yeast strains with site-directed mutations in the Rieske subunit of the cyt bc_1 complex (22,33,34), were grown in a synthetic mixture of amino acids excluding tryptophan (Sigma-Aldrich Chemical Co., St. Louis) and with dextrose (Fisher) as the carbon source. Ammonium sulfate (Fisher) and Yeast Nitrogen Base (Sigma-Aldrich Chemical Co., St. Louis) without amino acids were added as a source of nitrogen.

Sub-mitochondrial Particles Preparation- Sub-mitochondrial particles (SMP's) were prepared as described Muller et al. (20) and used as prepared, or after dispersal in dodecyl maltoside (5 mg/mg protein/dodecyl maltoside) (Anatrace) on ice for 1 hour, followed by centrifugation for 5 min at 16,000 X g to remove insoluble material. The concentration of cyt bc_1 complex was estimated by measuring the sodium dithionite/ascorbate absorbance difference spectrum of the cyt *b* hemes at 562-578 nm using an extinction coefficient of 25.6 $mM \cdot cm^{-1}$ (35).

Enzyme Assays- All assays were carried out in buffer containing 50 mM MOPS [3-(*N*-morpholino)-propanesulfonic acid], 100 mM KCl, 20 mM NaN_3, 50 μM horse heart cyt c^{+3} (Fisher), 50 μM decylubiquinol at pH = 8.0. Sample temperature was set using a flowing temperature controller and water jacketed cell holder, and measured *in situ* using a thermocouple thermometer. Rates are reported as mol cyt c^{+3} reduced·s^{-1}·mol^{-1} cyt bc_1 complex. In all experiments, the kinetic traces were initiated before the addition of enzyme (0.5-20 nM cyt *b*) to allow for the subtraction of background cyt *c* reduction (or background superoxide production) by free decylubiquinol. Decylubiquinol was freshly generated as needed using a modification of the technique reported in Kramer et al. (36) by reducing decylubiquinone (Sigma) dissolved in 1:1 ethanol:ethylene glycol with ~2mg of sodium borohydride, followed by the addition of 0.1 N HCl to quench unreacted sodium borohydride. For the superoxide production experiments, the cyt bc_1 complex was treated with a saturating (20 μM) concentration of AA (Sigma).

For measuring superoxide production in the presence of 20 μM AA, we used the Amplex Red assay (Molecular Probes, Eugene, OR, product no 12212) (This assay was found to be much more sensitive than the cyt *c* reduction experiments described in (18,20). Resorufin fluorescence was measured using an in-house constructed fluorimeter. Fluorescence was excited at 525 nm using 2.3 KHz modulated green light emitting diodes and detected at 90^o from the direction of excitation via a photodiode (Hamamatsu 1223) covered by a 590 ± 30 nm band pass filter. Signals were amplified using a laboratory built amplifier and processed with a Thor Labs Inc. Model LIA 100 lock-in amplifier. The assay was calibrated by titrating known amounts of hydrogen peroxide into the assay buffer with the full complement of enzymes and buffers. The calibration parameters were independent of temperature within the experimental range. All superoxide production rates are quoted as mol superoxide generated·s^{-1}·mol^{-1} cyt bc_1 complex.

This system yielded results that allowed us to detect as little as 100 pM superoxide produced·s^{-1} in our 500 μL volume. The wild-type SMP's were tested using the cyt *c* reduction method (18,20) and found to yield rates within 5% of that determined by the Amplex Red system.

Pre-steady state kinetics- Pre-steady state reduction of the cyt bc_1 complex was followed by stopped flow rapid scanning spectroscopy using an OLIS Rapid Scanning Monochromator. The temperature of the mixing chamber was varied from 10-35 ^{o}C with a thermostatically controlled Julabo F12 circulating water bath, and the temperature of the enzyme sample was equilibrated to that of the mixing chamber before each reaction. Reactions were started by mixing 3 μM cyt bc_1 complex in assay buffer containing 50 mM potassium phosphate, pH 7.0 plus 1 mM sodium azide, 1 mM EDTA and 0.05% Tween-20 against an equal volume of the same buffer containing decylubiquinol. A fresh solution of decylubiquinol substrate was prepared before each experiment, and the concentration was varied from 20-180 μM as indicated in the legend to Fig. 2. Two equivalents of AA per cyt bc_1 were mixed with the enzyme prior to the reaction to block any reduction of cyt *b* through the Q_i site. A spectrum of oxidized bc_1 complex was obtained by mixing the oxidized bc_1 complex against assay buffer and averaging the data sets to a single scan. For each experiment, seven or eight data sets were averaged, and the oxidized spectrum was subtracted from each scan. From the three-dimensional data set comprised of wavelength, absorbance and time, the time course and amplitude change for cyt *b* reduction at 563 nm minus 578 nm or cyt c_1 reduction at 553 nm minus 539 nm was extracted using the OLIS software.

Results

General Enzymatic Data- The apparent Michaelis constant (K_m) for decylubiquinol at the cyt bc_1 complex Q_o site in detergent solubilized SMP's was 4-12 μM for WT and each of the mutants (see Table 1), consistent with previous determinations (19,23,37-41). In the presence of

saturating concentrations of myxothiazol, we found a raised K_m of approximately 40 μM decylubiquinol, consistent with previous observations (19), implying that the measured K_m values reflect saturation of the Q_o site with substrate (and not other processes such as micelle breaking). We concluded that 50 μM decylubiquinol essentially saturated the Q_o site under steady-state uninhibited conditions.

Table 1 shows the uninhibited V_{MAX} (V_{QC}) and turnover number for superoxide production (V_{SOP}) measured for wild type and three mutant complexes with altered [2Fe2S] redox potentials. Our results on uninhibited rates were comparable with those made previously made by Denke et al. (33). With saturating concentrations of AA, superoxide production was measured at about 1.4-2.7% the respective uninhibited rate of cyt *c* reduction, with essentially the same dependence on 2Fe2S redox potential as previously reported (33,42).

Activation Energy Measurements- The Arrhenius activation energy (E_a) for the uninhibited turnover of the WT yeast enzyme was found to be 57.0 ± 3.0 kJ·mol^{-1} at pH 8.0 (See Fig. 1), consistent with previous reports from the cyt bc_1 complex in *Rhodobacter*, *Bos* and *Saccharomyces* (17,25,43,44).

We also measured the rates of pre-steady state reduction of cyt *b* and cyt c_1 at a range of temperatures and decylubiquinol concentrations in cyt bc_1 complex isolated from wild-type yeast as shown in Fig. 2. These measurements have the advantage that they are not complicated by considerations of ionic strength or detergent concentration (which did alter the apparent K_m for ubiquinol in the Q_o site) as in the catalytic assay. As shown in Fig. 3, when these measurements were used to calculate activation energies, they gave values of 53 kJ mol^{-1} for cyt *b* reduction and 50 kJ mol^{-1} for c_1 reduction. When corrected for pH, these values are in good agreement with the activation energy for the catalytic turnover of the enzyme. The fact that cyt *b* and cyt c_1

reduction have the same activation energy is noteworthy since these rates likely reflect the first and second oxidation steps of the ubiquinol at the Q_o site. This has implications as regards the rate-limiting step in ubiquinol oxidation as discussed below.

The E_a increased linearly with decreasing driving force for QH_2 oxidation, i.e. with decreasing E_m for the [2Fe2S] cluster (Fig. 4). The slope of E_a vs. E_m was similar for the uninhibited Q-cycle and superoxide production in the presence of AA (see Fig. 4), and, importantly, the value of this slope was near one. Over the entire range of complexes, the E_a measured for the AA bypass reaction appeared to be about 3.5 $kJ \cdot mol^{-1}$ higher than that for the uninhibited Q-cycle. This difference was significant to the 95% confidence limit.

When experiments were repeated in absence of added detergents, Arrhenius plots deviated from linearity at higher temperatures (data not shown), likely reflecting a shift in rate-limiting step from QH_2 oxidation at the Q_o site at low temperature to other processes, as suggested for similar trends seen in chromatophores from *Rhodobacter sphaeroides* (17). However, the E_a values at lower temperatures were similar to those shown in Fig. 1, suggesting QH_2 oxidation was not strongly modified by addition of detergent.

Discussion

We found that the activation energies for the uninhibited Q-cycle and superoxide production in the presence of AA were identical within the noise (Fig. 4). Changing the driving force for QH_2 oxidation by changing the redox properties of the [2Fe2S] cluster resulted in linear changes in E_a for both the Q-cycle and superoxide production, with a slope near unity (Fig. 4).

The similarities in E_a, and its dependence on the E_m of the [2Fe2S] cluster indicates that both the normal Q-cycle and superoxide production are rate-limited by a process which involves electron transfer from QH_2 to the [2Fe2S] cluster, most likely involving very similar chemistry

(See Fig. 5). The fact that the activation energies for reduction of cyt *b* and cyt c_1 under pre-steady state conditions are identical allows us to exclude the possibility that movement of the Rieske protein prerequisite to reduction of cyt c_1 is the rate-limiting step at the Q_o site, and it is consistent with our conclusion that electron transfer to the 2Fe2S cluster is rate-limiting. In these experiments, conducted at pH 7.0, oxidation of the first QH_2 at the Q_o site reduces cyt *b* and the [2Fe2S] cluster, with only partial cyt c_1 reduction, owing to its relatively low midpoint potential in comparison to that of the [2Fe2S] cluster. During the second QH_2 oxidation, cyt c_1 becomes reduced. The observed rate of cyt c_1 reduction is slower than cyt *b* reduction due to the fast equilibration with the [2Fe2S] cluster, which favors the electron residing in the [2Fe2S] cluster. The fact that cyt *b* and cyt c_1 reduction have the same activation energy, reflecting sequential turnovers of the Q_o site, indicates that they share the same rate-limiting step, QH_2 oxidation, as previously suggested by (17) probing flash-induced kinetics in the *Rhodobacter sphaeroides* chromatophore system.

Besides the hypothetical Q_o site SQ, the most reducing species is cyt b_L^{+2} with an E_m between -30 and -50 mV, probably insufficiently reducing to support rapid rates of O_2 reduction (20,21), as also suggested by slow rates of cyt b oxidation in the presence of O_2 (45). Furthermore, superoxide production is readily observed in the presence of Q_o site 'proximal niche' inhibitors (e.g. myxothiazol, MOA-stilbene, mucidin) that completely inhibit cyt *b* reduction (19,20,46,47). These inhibitors probably allow QH_2 to bind to the 'distal' niche of the Q_o pocket, allowing electron transfer to the [2Fe2S] cluster, forming SQ which in turn reduces O_2 (18-20,24). Taken together, these data indicate that a Q_o site intermediate, likely a SQ, is the reductant for superoxide production.

Our data thus imply that production of the low potential reductant that reduces cyt b_L in the Q-cycle is also involved in superoxide production and is formed via the same (or very similar) routes (i.e. these two processes probably share the same reactive intermediates – see Scheme I, panel A). The low potential reductant may be SQ, as discussed above. An alternative suggested by Trumpower (48) is that the cyt b_L reductant is a quinol:imidazolate complex formed when an electron from the quinol oxygen is delocalized into the orbitals of the imidazole ring and [2Fe2S] cluster, though the energetics for delocalization would still follow the relative redox potential of the [2Fe2S] cluster, and thus such a species would be energetically indistinguishable from a discrete SQ. In either case, we conclude that the formation of the low potential reductant occurs during both the normal Q-cycle and during superoxide production and that the reductant is formed through the same transition barrier (see Scheme I, panel A).

This conclusion is in contradiction with Q_o site models involving truly simultaneous electron transfer from QH_2 to directly form Q, because they require radically different Q_o site chemistries for Q-cycle superoxide production (18,20,24,28). Theoretical treatments by Zusman (49) predict distinct transition barriers and reorganization energies for sequential vs. concerted (DCET) electron transfers reactions, which should be reflected in different E_a values and reorganization energies, i.e. their dependencies on driving force (compare the solid lines, representing the DCET reaction with the dashed lines representing the sequential reaction in Scheme I, panel C). In contrast, our data shows that normal Q-cycle turnover and superoxide production have very similar activation barriers and their response to the redox properties of the [2Fe2S] cluster, which clearly alters the driving force for the reactions. Overall, the simplest interpretation of our data is that DCET does not operate within the Q_o site. Alternatively, one could argue that AA-inhibited enzyme acts in a completely different way, with fortuitously

similar energetics, but this seems very improbable. We also cannot at present rule out another possibility that the reductant for superoxide production occurs after the Q_o site activated intermediate, e.g. a special form of cyt b_L with a very low redox potential able to reduce O_2, thus giving similar activation behavior for both processes. In this case, though, we would have to invoke a separate mechanism for superoxide production in the presence of myxothiazol or other Q_o site proximal niche inhibitors (20,24,28), making this option more complex.

Class 2 models invoke high stabilization of the Q_o site SQ, preventing it from being an effective O_2 reductant (Scheme I, panel B). We argue that our data places certain limits on the stability of the Q_o site SQ. If, for instance, the Q_o site SQ is highly stabilized its formation would be energetically favored ($\Delta G < 0$), while its oxidation would require the input of energy (26). In this case, we would expect the rate-limiting and energy-requiring step to be shifted from QH_2 oxidation by the [2Fe2S] cluster to SQ oxidation by cyt b_L, and consequently little dependence on the redox properties of the [2Fe2S] cluster. Instead, we observed linear dependence of E_a on changing the E_m of the [2Fe2S] cluster, consistent with previous work (33,34,50) indicating that the rate-limiting step for the Q_o site reaction involves electron transfer to the [2Fe2S] cluster. This conclusion is consistent with work by Crofts and coworkers (1) suggesting that the fractional occupancy of the intermediate states of Q_o site catalyzed QH_2 oxidation could not exceed 0.1. We add, though, that this conclusion does not exclude stabilization of a SQ species after it is formed as long as the processes involved are significantly more rapid that the initial formation of SQ from QH_2.

We next consider a simple 'unstable' SQ model (Scheme I, panel A), where QH_2 bound at the Q_o site comes into equilibrium with a Q_o site SQ. If the SQ is highly unstable, its formation at the Q_o site would be energetically unfavorable ($\Delta G > 0$) and its concentration

should be roughly proportional to the logarithm of driving force for the one-electron transfer to the 2Fe2S cluster, as indeed we observe (Fig. 4). Further, if superoxide production at the Q_o site is pseudo-first order (with respect to [SQ]), then the rate of superoxide production also should depend linearly on the logarithm of changes in driving force for SQ formation. Again, as shown in Fig. 5, this is precisely what we have observed, leading us to suggest that an unstable Q_o site SQ comes into equilibrium with the ground state of the enzyme and that this SQ can be oxidized either by cyt *b*, resulting in normal Q-cycle activity, or by other oxidants including O_2, resulting in superoxide production or other bypass reactions. In principle, the SQ could appear either near the transition state for QH_2 oxidation, as suggested by Hong and coworkers (17), or as a species which has a concentration that is tightly coupled to the concentration of the transition state.

Because the E_a values for superoxide production and Q-cycle turnover are quite similar, the reactive intermediate of the Q-cycle could reduce either cyt *b* or O_2 without a large difference in enthalpic terms. This likely places the midpoint potential of the O_2/cyt b_L reductant similar to or more negetic than that of the $O_2/O_2^{\cdot -}$ couple. If the solution potential for this couple operates in our system, this would imply a potential of around -150 mV or lower for the cyt b_L (or O_2) reductant, again suggesting a very unstable intermediate.

Although this simple model accounts for the data in this paper, an important caveat needs to be considered. It has been pointed out by several authors (20,24,28) that superoxide production by the cyt bc_1 complex occurs at measurable rates only when the complex is partially inhibited, e.g. by AA or high proton motive force. (It should be noted, however, there are reports of significant superoxide production by uninhibited cyt bc_1 complexes (22,51)). Our own experiments on the *Saccharomyces* cyt bc_1 complex did not show rates of superoxide production above the background (20) and there appears to be general consensus that superoxide production

is slow in the uninhibited complex. In conflict with these observations, the simple, unstable SQ model described above predicts similar transition states under conditions favoring uninhibited Q-cycle and SO production.

In one possible model to reconcile these observations, the Q_o site could act primarily as a sealed 'reaction chamber' (24). Under uninhibited conditions, where the lifetime of the SQ intermediate is short, the chamber can effectively shield the SQ from oxygen. However, when the Q-cycle is blocked, SQ can escape from the reaction chamber or oxygen can diffuse into the chamber, perhaps following slow (on the time scale of the overall reaction), large-scale conformational fluctuations of the complex.

Several groups have suggested reasonable models that entail conformational or electrostatic gating of the Q_o site reactions(52-57). In these models, the activity of the Q_o site is gated by the properties of other components of the complex. For example, the Q_o site might not be able to bind substrate or becomes redox-inactive when cyt b_L is reduced (28), the Q_i site is altered (54), substrate occupancy in one half of the operational dimer (58) may inhibit activity in the other half of the dimer (53), Under normal conditions, such gating could effectively prevent side reactions, but might be susceptible to slippage over large time scales, leading to the observed differences in SO production rates in the uninhibited and inhibited cases.

In both the 'leaky reaction chamber' and the 'leaky gating' models, superoxide production is at least partly controlled by conformational changes.

References

1. Crofts, A. R. (2004) *Annu Rev Physiol* **66**, 689-733
2. Lange C., H. C. (2002) *Proc Natl Acad Sci U S A* **99**, 2800-2805.
3. Zhang, Z., Berry, E. A., Huang, L. S., and Kim, S. H. (2000) *Subcell Biochem* **35**, 541-580
4. Darrouzet, E., Cooley, J. W., and Daldal, F. (2004) *Photosynthesis Research* **79**, 25-44

5. Berry, E. A., Guergova-Kuras, M., Huang, L. S., and Crofts, A. R. (2000) *Annu Rev Biochem* **69**, 1005-1075
6. Schütz, M., Brugna, M., Lebrun, E., Baymann, F., Huber, R., Stetter, K., Hauska, G., Toci, R., Lemesle-Meunier, D., Tron1, P., Schmidt, C., Nitschke, W. (2000) *Journal of Molecular Biology* **300**, 663-675
7. Crofts, A. R., and Berry, E. A. (1998) *Curr Opin Struct Biol* **8**, 501-509
8. Iwata, S., Lee, J. W., Okada, K., Lee, J. K., Iwata, M., Rasmussen, B., Link, T. A., Ramaswamy, S., and Jap, B. K. (1998) *Science* **281**, 64-71
9. Zhang, H., and Cramer, W. A. (2004) *Methods Mol Biol* **274**, 67-78
10. Berry, E. A., Huang, L. S., Zhang, Z., and Kim, S. H. (1999) *J Bioenerg Biomembr* **31**, 177-190
11. Berry, E. A., Huang, L.-S., Saechao, L. K., Pon, N. G., Valkova-Valchanova, M., and Daldal, F. (2004) *Photosynthesis Research* **81**, 251-275
12. Mitchell, P. (1975) *FEBS Lett* **56**, 1-6
13. Trumpower, B. L. (1990) *J Biol Chem* **265**, 11409-11412
14. Mitchell, P. (1976) *J Theor Biol* **62**, 327-367
15. Rich, P. R. (1984) *Biochim Biophys Acta* **768**, 53-79
16. Crofts, A. R., Barquera, B., Gennis, R. B., Kuras, R., Guergova-Kuras, M., and Berry, E. A. (1999) *Biochemistry* **38**, 15807-15826
17. Hong, S., Ugulava, N., Guergova-Kuras, M., and Crofts, A. R. (1999) *J Biol Chem* **274**, 33931-33944
18. Kramer, D. M., Roberts, A. G., Muller, F., Cape, J., and Bowman, M. K. (2004) *Methods Enzymol* **382**, 21-45
19. Muller, F., Roberts AG, Bowman, MK and Kramer, DM. (2003) *Biochemistry* **42**, 6493 - 6499
20. Muller, F. L., Crofts A.R., Kramer,D. M. (2002) *Biochemistry* **41**, 7866 -7874
21. Turrens, J. F., Alexandre, A., and Lehninger, A. L. (1985) *Arch Biochem Biophys* **237**, 408-414
22. Sun, J., and Trumpower, B. L. (2003) *Arch Biochem Biophys* **419**, 198-206
23. Cape, J. L., Strahan, J. R., Lenaeus, M. J., Yuknis, B. A., Le, T. T., Shepherd, J. N., Bowman, M. K., and Kramer, D. M. (2005) *J Biol Chem* **280**, 34654-34660
24. Cape, J. L., Bowman, M. K., and Kramer, D. M. (2006) *Trends Plant Sci* **11**, 46-55
25. Crofts, A. R. (2004) *Biochim Biophys Acta* **1655**, 77-92
26. Berry, E. A., and Huang, L. S. (2003) *FEBS Lett* **555**, 13-20
27. Link, T. A. (1997) *FEBS Lett* **412**, 257-264
28. Osyczka, A., Moser, C. C., Daldal, F., and Dutton, P. L. (2004) *Nature* **427**, 607-612
29. Snyder, C. H., Gutierrez-Cirlos, E. B., and Trumpower, B. L. (2000) *J Biol Chem* **275**, 13535-13541
30. Darrouzet, E., Moser, C. C., Dutton, P. L., and Daldal, F. (2001) *Trends Biochem Sci* **26**, 445-451
31. Zhang, Z., Huang, L., Shulmeister, V. M., Chi, Y. I., Kim, K. K., Hung, L. W., Crofts, A. R., Berry, E. A., and Kim, S. H. (1998) *Nature* **392**, 677-684
32. Osyczka, A., Moser, C. C., and Dutton, P. L. (2005) *Trends in Biochemical Sciences* **30**, 176-182
33. Denke, E., Merbitz-Zahradnik, T., Hatzfeld, O.M., Snyder, C.H., Link, T.A., and Trumpower, B.L. (1998) *J Biol Chem* **273**, 9085-9093

34. Snyder, C. H., Merbitz-Zahradnik, T., Link, T. A., and Trumpower, B. L. (1999) *J Bioenerg Biomembr* **31**, 235-242
35. Ljungdahl, P. O., Pennoyer, J. D., Robertson, D. E., and Trumpower, B. L. (1987) *Biochim Biophys Acta* **891**, 227-241
36. Kramer, D. M., Joliot, A., Joliot, P., and Crofts, A. R. (1994) *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1184**, 251-262
37. Nett, J. H., Hunte, C., and Trumpower, B. L. (2000) *Eur J Biochem* **267**, 5777-5782
38. Brasseur, G., Di Rago, J.-P., Slonimski, P. P., and Lemesle-Meunier, D. (2001) *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1506**, 89-102
39. Palsdottir, H., Lojero, C. G., Trumpower, B. L., and Hunte, C. (2003) *J Biol Chem* **278**, 31303-31311
40. Brasseur, G., Lemesle-Meunier, D., Reinaud, F., and Meunier, B. (2004) *J Biol Chem* **279**, 24203-24211
41. Kessl, J. J., Ha, K. H., Merritt, A. K., Lange, B. B., Hill, P., Meunier, B., Meshnick, S. R., and Trumpower, B. L. (2005) *J. Biol. Chem.* **280**, 17142-17148
42. Guergova-Kuras, M., Kuras, R., Ugulava, N., Hadad, I., and Crofts, A.R. (2000) *Biochemistry* **39**, 7436 -7444
43. Crofts, A. R., Guergova-Kuras, M., Kuras, R., Ugulava, N., Li, J., and Hong, S. (2000) *Biochim Biophys Acta* **1459**, 456-466
44. Brandt, U., and Okun, J. G. (1997) *Biochemistry* **36**, 11234-11240
45. Forquer, I., Cape, J., Bowman, M.K. and Kramer, D.M. (2004) in *Photosynthesis: Fundamental Aspects to Global Perspectives*, ACG Publishing, Montreal
46. Starkov, A. A., and Fiskum, G. (2001) *Biochem Biophys Res Commun* **281**, 645-650
47. Crofts, A. R., Shinkarev, V. P., Dikanov, S. A., Samoilova, R. I., and Kolling, D. (2002) *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1555**, 48-53
48. Trumpower, B. L. (2002) *Biochim Biophys Acta* **1555**, 166-173
49. Zusman, L. D., Beratan, D.N. (1996) *Journal of Chemical Physics* **105**, 165-176
50. Guergova-Kuras, M., Kuras, R., Ugulava, N., Hadad, I., and Crofts, A. R. (2000) *Biochemistry* **39**, 7436-7444
51. Gong, X., Yu, L., Xia, D., and Yu, C.-A. (2005) *J. Biol. Chem.* **280**, 9251-9257
52. Darrouzet, E., Valkova-Valchanova, M., Moser, C. C., Dutton, P. L., and Daldal, F. (2000) *Proc Natl Acad Sci U S A* **97**, 4567-4572
53. Covian, R., Gutierrez-Cirlos, E. B., and Trumpower, B. L. (2004) *J Biol Chem* **279**, 15040-15049
54. Cooley, J. W., Ohnishi, T., and Daldal, F. (2005) *Biochemistry* **44**, 10520-10532
55. Cooley, J. W., Roberts, A. G., Bowman, M. K., Kramer, D. M., and Daldal, F. (2004) *Biochemistry* **43**, 2217-2227
56. Zhang, L., Li, Z., Quinn, B., Yu, L., and Yu, C. A. (2002) *Biochim Biophys Acta* **1556**, 226-232
57. Crofts, A. R., Lhee, S., Crofts, S. B., Cheng, J., and Rose, S. (2006) *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **In Press, Uncorrected Proof**
58. Schmitt, M. E., and Trumpower, B. L. (1990) *J Biol Chem* **265**, 17005-17011

FOOTNOTES

*The authors wish to thank Drs. Jonathan Cape, James Hurst, Mike Kahn, and Atsuko Kanazawa for stimulating discussion. This work was supported by NIGMS Grant GM61904 (MKB), NIGMS Grant GM 20379 (BLT), and US Department of Energy DE-FG02-04ER1559 (DMK)

Part of this work was performed at the WR Wiley Environmental Molecular Sciences Laboratory, a national scientific user facility sponsored by the U.S. Department of Energy's Office of Biological and Environmental Research and located at Pacific Northwest National Laboratory, operated by Battelle for the U.S.D.O.E.

[1]The abbreviations used are: cyt, cytochrome; Q_o site, quinol oxidase site; Q_i site, quinol reductase site; SQ, semiquinone; cyt b_L, low-potential cyt *b* heme; cyt b_H, high-potential cyt *b* heme; AA, Antimycin A; SMP's, submitochondrial particles; K_m, Michaelis constant; V_{QC}, turnover for the uninhibited complex (Q-cycle); V_{SOP}, turnover number for superoxide production; E_a, Arrhenius activation energy; E_m, Equilibrium redox mid-point potential (vs. SHE); mV, millivolts; DCET, double-concerted electron transfer; ΔG, Gibbs free energy; SOP, superoxide production.

Table and Figure legends

Scheme I Three QH_2 oxidation models proposed to account for vanishing yields of superoxide production during the Q-cycle. Panel A: Unstable SQ model; Panel B: Stable SQ model; Panel C: DCET model. QH_2: quinol; SQ: semiquinone; Q: quinone; 2Fe2S: Rieske Iron-Sulfur Cluster; b_L: low potential *b*-type heme of cytochrome *b*; $O_2^{\cdot-}$: superoxide ion

Table 1 Kinetic and thermodynamic data collected for wild-type and three yeast [2Fe2S] subunit mutants (Yeast numbering used). $E_{m,7}$: Equilibrium mid-potential for the [2Fe2S] cluster (Taken from (33)); V_{QC}: Turnover number for uninhibited complex (mol cyt c^{+3} reduced·mol^{-1} cyt bc_1 complex·sec^{-1}); V_{SOP}:Turnover number for superoxide production (mol superoxide produced·mol^{-1} cyt bc_1 complex·sec^{-1}); K_m: Michaelis constant for ubiquinol in the Q_o site; E_a: Steady-state Arrhenius activation energy for uninhibited complex; $E_{a(AA)}$: Steady-state Arrhenius activation energy for Antimycin A bypass reaction

Fig. 1 Arrhenius plots for the *A,* uninhibited turnover, and *B*, superoxide production steady state turnover. Closed Squares ■: Wild-type; Open Circles ○: S183T; Closed Triangles ▲: Y185F; Open Diamonds ◊: S183A

Fig. 2 Effect of temperature on the pre-steady-state rates of reduction of cyt b and cyt c_1 in cyt bc_1 complex purified from wild-type yeast. The enzyme was reduced with variable concentrations of decylubiquinol at temperatures ranging from 10-35 ^oC. Rates of cyt b reduction are shown in (A) and of cyt c_1 reduction in (B).

Fig. 3 Arrhenius plots for pre-steady state rates of cyt b and cyt c_1 reduction. Rates of cyt b reduction are shown by the solid circles, and rates of cyt c_1 reduction are shown by the open circles.

Fig. 4 *A*, Arrhenius activation energy as a function of change in E_m from wild-type. Closed Squares ■: Uninhibited reaction; Open circles ○: Superoxide Production. *B*, Relationship between the measured Arrhenius activation energies for the steady state turnover of the uninhibited enzyme vs. AA treated enzyme.

Fig. 5 The logarithm of the room-temperature (25 ^{o}C) turnover (mol cyt c^{+3} reduced·mol^{-1} cyt bc_1 complex·sec^{-1}) for superoxide production as a function of the driving force for electron transfer

Table 1-1

Mutant	**$E_{m,7}$[1]**	**V_{QC}**	**V_{SOP}**	**K_M**	**E_a (kJ·mol^{-1}) (no inhibitors)**	**E_a (kJ·mol^{-1}) (Antimycin A Bypass)**
WT	+285 mV	185	2.7	4.5 ± 0.5 μM	57.0 ± 3.1	59.5 ± 5.1
S183T	+269 mV	124	2.5	12.4 ± 1.1 μM	60.4 ± 2.0	62.8 ± 4.1
Y185F	+217 mV	59	0.9	9.2 ± 3.6 μM	62.3 ± 0.8	66.6 ± 3.0
S183A	+165 mV	23	0.15	4.3 ± 1.2 μM	68.6 ± 1.3	73.4 ± 5.3

[1]Data from (25)

Figure 2-1

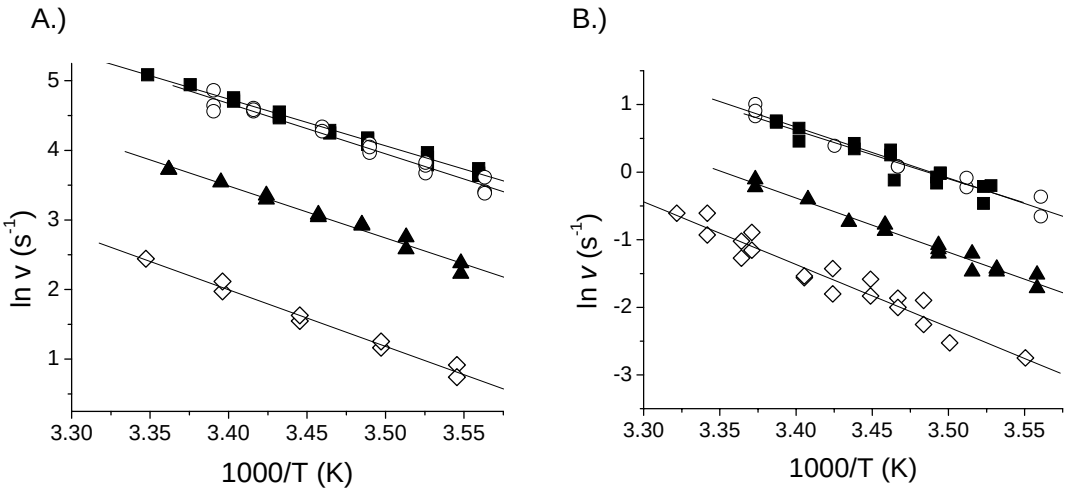


Figure 2-2

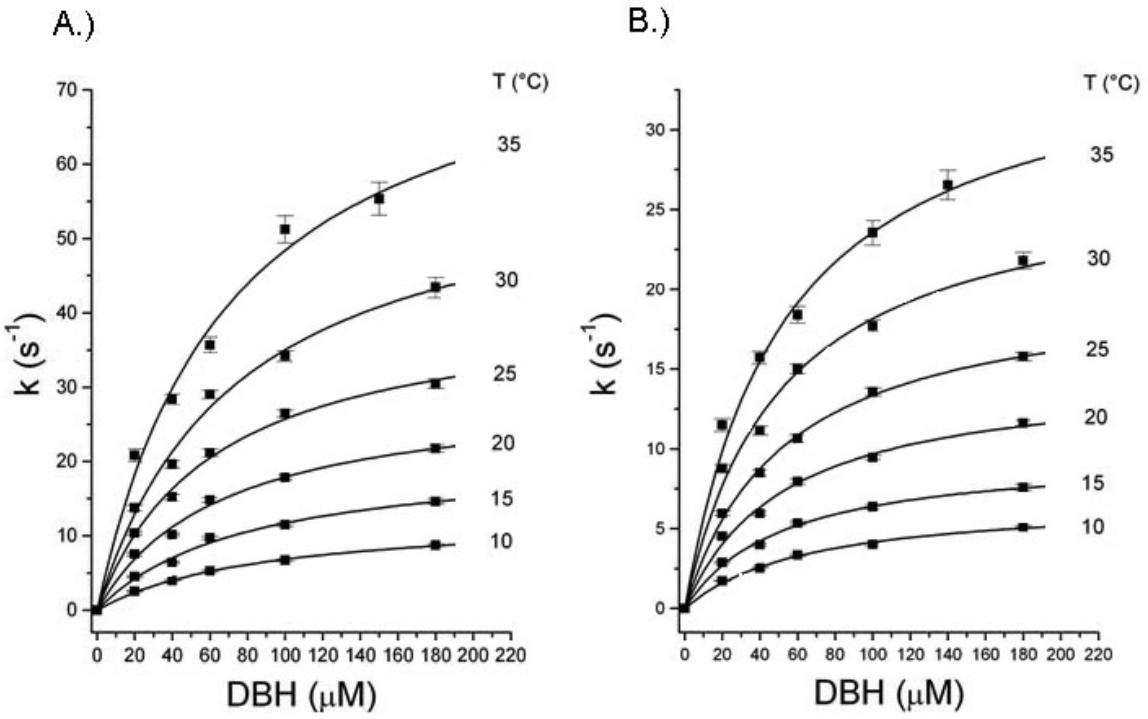


Figure 2-3

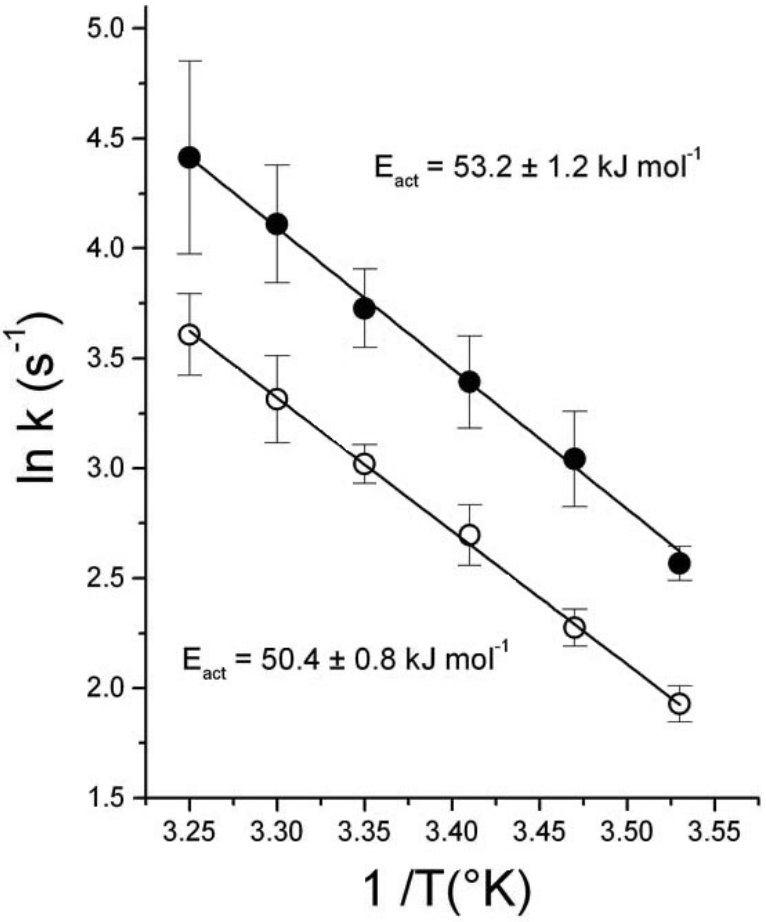
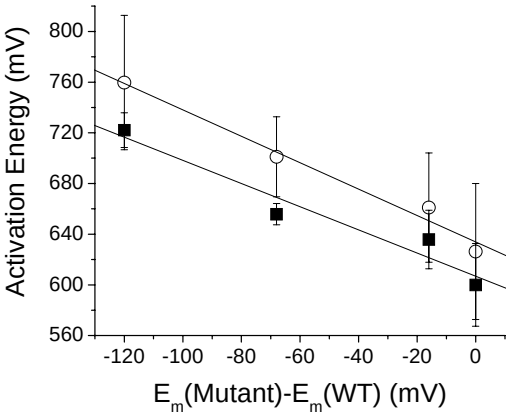

5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
ln k (s-1)
3.25
3.30
3.35
3.40
3.45
3.50
3.55
1 /T(°K)
Eact = 53.2 ± 1.2 kJ mol-1
Eact = 50.4 ± 0.8 kJ mol-1

Figure 2-4

A.)


Activation Energy (mV)
800
760
720
680
640
600
560
-120
-100
-80
-60
-40
-20
0
E_m(Mutant)-E_m(WT) (mV)

B.)

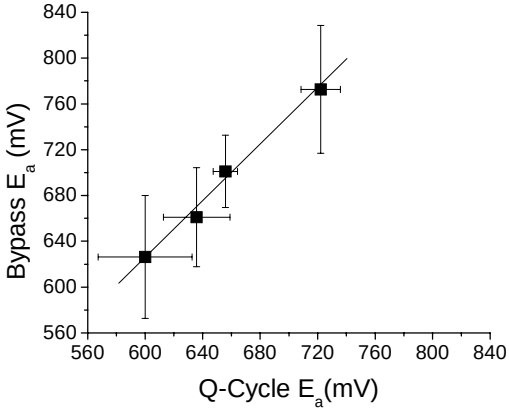
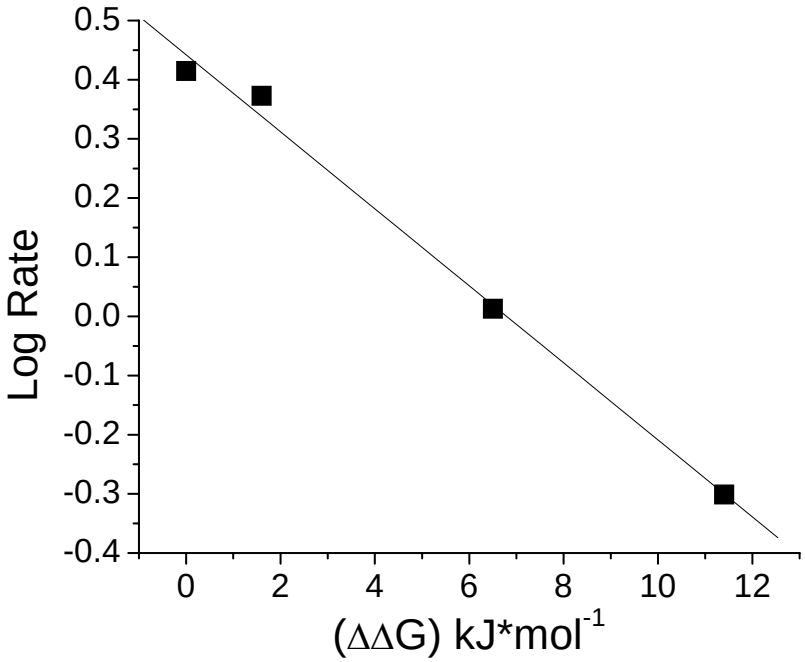

Bypass E_a (mV)
840
800
760
720
680
640
600
560
560
600
640
680
720
760
800
840
Q-Cycle E_a(mV)

Figure 2-5



Chapter 3

Alkoxyacridones with Anti-malarial Properties Target the Q_o site of the Cytochrome *bc*$_1$ Complex in *Plasmodium* spp.

Isaac Forquer[*], Rolf Winters[#], Martin Smilkstein[#], Jane Kelly[#], Mike Riscoe[#], David M. Kramer[*]

[*]Institute of Biological Chemistry, Washington State University, Pullman, WA 99163
[#]Medical Research Service, Department of Veterans Affairs Medical Center and Department of Chemistry, Portland State University, Portland, OR

Abstract

We investigated the mechanism of action of a series of acridones that inhibit oxygen consumption in and retards growth of the parasite *Plasmodium spp.* in culture. Previously, it was shown that certain acridones with alkyl tails in the 3-position of the tricyclic acridone system inhibits growth at lower concentrations than similar compounds with the alkyl tail in the 2- and 4-position. In order to define the target of these compounds, we tested the hypothesis that 3-(6,6,6-trifluorohexoxyl)-9-acridone inhibits the cytochrome *bc*$_1$ complex at the quinol oxidase site of the enzyme. We show that when the C-3 position of the tricyclic acridone system is derivatized with an alkoxy chain, the acridone targets the cytochrome *bc*$_1$ complex at the quinol oxidase site of the enzyme.

Introduction

The quinol oxidase (Q_o) site of the cytochrome (cyt) *bc*$_1$ complex (*bc*$_1$ complex) in *Plasmodium* spp. is inhibited by atovaquone (1-3), a common prophylaxis against malaria. However, resistance to atovaquone develops in a large number of patients (4-9), and the

development of new compounds with well defined targets is critical to the global fight against malaria (10-12). To that end, we have developed a series of acridones (representatives of which are compounds **1**-**4** in Fig. 1**)** and screened them for anti-*Plasmodium* spp. properties (13). These compounds inhibited the growth of *Plasmodium* spp. in blood culture and apparently by inhibiting O_2 consumption. This observation and the previous work by Oettmeier and coworkers (14-16) which showed some acridones are inhibitors of the cyt bc1 complex, led us to the hypothesis that compounds **1**-**4** also act at the cyt bc_1 complex.

The cyt bc_1 complex mediates electron transfer from the ubiquinol in the mitochondrial electron transport chain to a c-type cyt (22,23). The modified Q-cycle (17-21) is now generally accepted to explain the catalytic mechanism of the cyt bc_1 complex. In the Q-cycle, quinol (QH_2) is oxidized to quinone (Q) at the Q_o site in a stepwise fashion, with the first electron traveling through the high potential chain, consisting of the Rieske iron sulfur protein, cyt c_1, and finally to cyt *c*, which dissociates from the complex to provide the reducing equivalent to cyt *c* oxidase. The one-electron oxidation of QH_2 results in the formation of a semiquinone (SQ) in the Q_o site that is oxidized by the low potential chain, which consists of two hemes, cyt b_L and b_H. Cyt b_H then reduces a bound Q in the quinone reductase site (Q_i) forming a stable SQ. Once the entire cycle is repeated, the semiquinone in the Q_i site is reduced to a QH_2. The net yield of the Q-cycle is two protons transferred across the mitochondrial inner-membrane for every one electron transferred to cyt *c*. The proton translocation activity of the cyt bc_1 complex contributes to the proton motive force which is ultimately conserved by the generation of ATP by ATP synthase, though new data suggests that ATP formation does not take place in mitochondria from *Plasmodium* spp. (22).

Several derivatives of each of the compounds in Fig 1. were presented in Winter et al. (13); compounds with alkoxy tails on 3-position of the tricyclic ring routinely showed lower IC_{50}'s against *Plasmodium falciparum* than compounds with tails in the 2- and 4-position of the tricyclic ring. We aim here to test if acridones modified with an alkoxy tail on the 3-position of the tricyclic acridone ring are cyt bc_1 complex inhibitors. Further, we will determine if the relative concentration dependence on cyt bc_1 complex inhibition can explain the observed discrepancies in the IC_{50}'s between the compounds **2** and **4** and compounds **1** and **3**. Compound **4** is included in this report to test if the length of the alkyl chain has a measurable effect on the inhibition of cyt bc_1 complex, as reported by Oettmeier and coworkers (15,16,23).

Materials and Methods

Preparation of mitochondria from Plasmodium yoelli

Frozen parasites were obtained using standard procedures (24) from outbred female CF-1 mice infected with *P. yoelli.* The parasites remained frozen at -80^{o}C until needed for mitochondrial isolation.

The thawed parasites were broken by suspending them in "Lysis buffer", containing 75 mM Sucrose, 225 mM mannitol, 5 mM $MgCl_2$, 5 mM KH_2PO_4, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM phenylmethanesulphonylfluoride (PMSF), 5 mM (N-[2-hydroxyethyl) piperazine-N'-]2-ethanesulfonic acid) (HEPES), at pH 7.4. The cells were then broken by homogenization in a glass homogenizer. The cell debris was removed by centrifugation at 800 X G for 5 minutes. Mitochondrial particles were then harvested at 20,000 X G for 10 minutes.

The pellet was resuspended in lysis buffer, and mixed to a final concentration of 30% glycerol. The aliquots of mitochondrial particles were frozen at -70^{o}C until needed.

Rat heart tissue extraction:

Male Sprague-Dawley rat subjects (Harlan) (from unrelated work in another lab) were decapitated and the hearts immediately removed using standard procedures. (only control animals were used, receiving only saline in addition to normal dietary requirements). The animals were grown and handled in accordance with animal use policies at Washington State University.

Once the rats were decapitated, the hearts (three total) were promptly immersed in cold extraction buffer: 100 mM bis-Tris-propane, 0.5 mM EDTA, 1 mM potassium ferricyanide, 1 mM PMSF, pH = 8.8. The heart was minced and put in a cold waring blender and the material was processed with a quick pulse. The resulting slurry was then homogenized in a glass homogenizer (2 passes) and passed through 3 layers of cheese cloth. The mitochondria were then suspended in 35 ml of buffer: 20 mM Tris·HCl, 0.2 mM EDTA, 1 mg/ml BSA. The mitochondria were pelleted at 10,000XG and then washed twice in the same buffer. The mitochondria were then resuspended in a minimum amount of the same buffer and made 30% v/v glycerol and stored for later use at -80^{o}C.

Test of enzymatic activity and inhibition in mitochondria isolated from Plasmodium yoelli:

Cyt bc_1 complex activity was measured by monitoring the reduction of horse heart cyt *c* (Fisher) spectrophotometrically using the wavelength pair 550-542 nm (ε=17·mM^{-1}·cm^{-1}) in reaction buffer containg 50 mM N-Tris(hydroxymethyl)methylglycine (Tricine), 100 mM KCl, 2 mM KCN, 50μM decylubiquinol, 50 μM cyt *c* at pH = 8.0. Separate stocks of 3-(6,6,6-trifluorohexoxyl)-9-acridone were prepared so that the same volume of the delivery solvent

(dimethylsulfoxide (DMSO)) was added during each measurement at different final concentrations of the compound.

Measurements were initiated by addition of decylubiquinol, and a baseline was collected for approximately 20 seconds to account for the non-enzymatic reduction of cyt *c* by decylubiquinol. Once the baseline collection was complete, enzyme was added to a final concentration of 15 $\mu g \cdot ml^{-1}$.

Test of enzymatic activity and inhibition in rat heart mitochondria:

Mitochondria isolated from rat hearts were used because of the very limited availability of mitochondria from the parasite. At the enzymatic level, compounds **2** and **4** were equally effective at inhibiting the cyt bc_1 complex from both rat hearts and the parasite.

To prepare the thawed mitochondria for activity measurements, the mitochondria were first diluted to a suitable concentration (about 1-2 μM in cyt bc1 complex) for reliable steady state activity measurements. The resulting suspension was then made 6 mg/ml dodecyl maltoside and allowed to incubate on ice for one hour. The solubilized material was then clarified at approximately 40,000 X G in a Beckman airfuge for 5 minutes in a cold room. The concentration of cyt bc_1 complex was then measured by taking a dithionite-ascorbate difference spectrum and using the extinction coefficient of $12.8 \cdot mM^{-1} \cdot cm^{-1}$ for the wavelength pair 562-578 (25).

Enzymatic activity was measured as it was for *P. yoelii*. Once the baseline collection was complete, enzyme was added to a final concentration of 5 nM cyt bc_1 complex. Once the kinetic trace was finished, the baseline was visually subtracted from the initial rate of enzymatic activity. The rate of cyt *c* reduction was calculated from the slope of the initial rate using an extinction

coefficient of $17 \cdot mM^{-1} \cdot cm^{-1}$. The turnover number was then calculated by dividing the rate of cytochrome *c* reduction by the concentration of the enzyme. The turnover number is reported with the units: mol cyt *c* reduced$\cdot s^{-1} \cdot$mol cyt bc_1^{-1}.

Titrations with inhibitors were done using the activity measurement described above with the addition of 10 μL of DMSO containing various concentrations of the compound, and for the control, DMSO containing no inhibitors was added.

For the competition assays (Figure 4), antimycin A and methoxyacrylate (MOA) stilbene were added at 10 μM and 7.5 μM respectively to the rat heart solubilized material as described above for the inhibitor titration experiment. Compound **2** was then added to the reaction mixture.

Results

Figure 2 shows the effects of compound **2** and **4** on the steady-state activity of cyt bc_1 complex in mitochondrial fragments isolated from *P. yoelli*. The results demonstrate that compounds **2** and **4** are strong (nanomolar) inhibitors of the cyt bc_1 complex in *P. yoelii*. In contrast, compounds **1** and **3**, with tails in the two- and four-position on the tricyclic acridone ring respectively, were tested at 1 μM and did not show inhibition (data not shown).

Figure 3 shows that compounds **2** and **4** were effective inhibitors of the rat heart cyt bc_1 complex whereas compounds **1** and **3** were not. With compound **4**, lengthening the tail by 2 carbons slightly lowered the EC_{50} (the concentration of inhibitor required to inhibit 50% of the uninhibited enzymatic rate) . Inhibition by **2** was essentially complete upon addition of saturating concentrations, with less than 0.5% of uninhibited activity remaining in the presence of saturating concentrations of compound **2**.

Antimycin A and MOA stilbene inhibit the cyt bc_1 complex (from rat heart) at the Q_i and Q_o site respectively. However, both are partial inhibitors such that when they are at saturating concentrations, QH_2 oxidation still proceeds but at a slower (1-5% of uninhibited) rate. Figure 4 shows that compound **2** inhibited the antimycin A, but not the MOA stilbene residual activity. Both antimycin A and MOA stilbene would have outcompeted compound **2** for binding at their respective binding sites because they were added at concentrations well above their respective EC_{50}'s, (26,27), whereas the acridone was only added to concentration equaling it's measured EC_{50}. None of the activity represented in Figure 4 is attributable to residual activity from the acridone, as the titration shown in Figure 3 extrapolates to complete inhibition.

Discussion

Of the four acridone derivatives discussed in here **2** and **4** were by far the most effective agents in preventing growth in cultured *P. yoelli* cells (Table 1) as well as the tightest inhibitor of the cyt bc_1 isolated from *P. yoelli* and rat heart. We thus conclude that the position of the alkoxy tail is critical for function.

The trend in IC_{50}'s measured for compound **4** suggests that lengthening the alkoxy tail by 2 carbons apparently makes the acridone a more potent inhibitor of the cyt bc_1 complex. This result is consistent with reports by Oettmeier and coworkers (14-16,23,28), but we suggest that the observed phenomenon may actually be due to slightly different partition coefficients of the compounds, with the hexyloxy derivative having a higher lipid:aqueous phase partition coefficient. Inhibitors of the Q_o site would require access to the lipid phase of the mitochondrial inner-membrane bilayer, and favorable partitioning into the membrane would result in a lower apparent IC_{50}.

Compounds **1** and **3** did not inhibit the cyt bc_1 complex at up to 3 uM, but did kill *Plasmodium* spp. albeit at a much higher concentration than compound **2** (Table 1). These results also suggest that compound **2** and **4** acts *in vivo* as a cyt bc_1 inhibitor, but that **1** and **3** may act elsewhere, perhaps as proposed by Oettmeier and coworkers (29), as protonophores, uncoupling the oxidative phosphorylation from ATP synthesis. Though *Plasmodium* spp. apparently does not depend on oxidative phosphorylation for ATP production (30), the proton motive force generated by the mitochondrial electron transfer chain may still be important in mitochondrial protein import (2,31).

It is interesting that compound **2** is a good inhibitor of the cyt bc_1 complex from both *Plasmodium* spp. and the mammalian material (rat heart), but in *vivo*, the compound is apparently not toxic to mammalian cells (13). Obviously, the intact mammalian cell somehow prevents the compound from inhibiting the cyt bc_1 complex.

The most obvious possibility is that the mammalian cells can exclude the acridones with one of their many ATP-binding cassette (ABC) pumps (38), as has been demonstrated for anti-parasite and cancer drugs (39,40). This sort of activity would would make it appear that the compound is not active against fully coupled cells, while still allowing for potent inhibition on isolated mitochondria. Similar phenomenon have been described by Souid and coworkers (41), though they do not propose an exact mechanism, where the inhibitory effects of certain cannabinoid compounds inhibit respiration in isolated mitochondria from sperm, but in plasma the sperm are relatively resistant to the compounds.

Future studies will certainly have to test the hypothesis that the acridones are being partitioned away from the mitochondria, either within the cell, or outside the cell as would be

expected if an ABC pump is acting on these compounds andr if PSACs are allowing the selective entry of the acridones into cells infected with *Plasmodium* spp.

Figure 4 shows that compound **2**, when added at saturating levels, can completely inhibit the cyt bc_1 complex. This is consistent with the action of certain Q_o site inhibitors like stigmatellin, which bind to the Q_o site and do not allow entry of QH_2. In contrast, compound **2** does not behave like a typical Q_i site inhibitor, such as antimycin A, which allows the Q_o site to bind and oxidize QH_2, albeit at a slower rate than the uninhibited complex (42,43). Certain Q_o site inhibitors, such as MOA stilbene, also allow QH_2 binding and oxidation, though at slower rates than the uninhibited complex (42,43). Since the low potential chain cannot operate in the presence of AA or MOA stilbene, the residual activity results in so-called 'Q-cycle bypass reactions', including especially superoxide production (42-45). We did not observe significant superoxide production by the cyt bc_1 inhibited by **2** (data not shown). We thus propose that Acridone **2** binds directly at the Q_o site thus preventing QH_2 oxidation illustrated by the inhibition of the Antimycin a inhibited enzyme. It is also possible that the acridone is acting non-competitively at the Q_o site, for instance by causing an allosteric effect that prevents QH_2 from binding(43,45), though no inhibitors for the cyt bc_1 complex have ever been discovered with this activity.

Ward and coworkers (46) tested a series of acridinediones as inhibitors of the cyt bc_1 complex. Similar to our results, the potent compound in their study (compound **5**, Figure 1) also had moieties attached to C-3 of the saturated ring of the acridine, which has strong geometric similarities to acridones. The parallel observation that a hydrophobic moiety placed on the respective C-3 positions of the tricyclic ring systems of both acridinediones and acridones is

consistent with the observation that compounds like acridones require a hydrophobic 'tail' in the C-3 position of the tricyclic ring system to target the a given compound to the Q_o site.

While they propose a very similar inhibitor binding site (the Q_o site), they propose that inhibition of the cyt bc_1 complex results in death to the parasite because of the collapse in the proton motive force and subsequent loss of ATP production at ATP synthase. While this would be a satisfactory explanation for most mitochondria-containing organisms, recent work suggests that *Plasmodium* spp. does not rely on mitochondrial electron and proton transfer for ATP (10,22,47,48). Vaidya and coworkers (22) suggest that the sole reason for the respiratory chain in the parasite is to act as an electron sink for dihydroorotate oxidation. Mutations in the Q_o site which confer resistance to the anti-malarial drug Atovaquone result in an enzyme with much diminished (10% of WT) catalytic activity (2), which would have the same net effect on the contribution of the enzyme to the proton motive force as inhibiting the enzyme.

In conclusion acridones appear to be effective inhibitors of the Q_o site of the cyt bc_1 complex in *Plasmodium* spp. A general rule has emerged suggesting that substitution of a hydrophobic 'tail' at the three position of the tricyclic acridone ring give the best activity. This key different should enable future structural studies to reveal the mode of acridone binding to the Q_o site.

Abbreviations

Q_o site, quinol oxidase site; cyt, cytochrome; QH_2, quinol; Q, quinone; SQ, semiquinone; Q_i site, quinone reductase site; IC_{50}, inhibitor concentration at which 50 % of growth is inhibited; EDTA, ethylenediamine tetraacetic acid; PMSF, phenylmethanesulphonylfluoride; HEPES, N-[2-hydroxyethyl) piperazine-N'-]2-ethanesulfonic acid; TRIS, tris(hydroxymethyl)-aminomethane; TRICINE, N-Tris(hydroxymethyl)methylglycine; DMSO, dimethylsulfoxide; MOA stilbene, methoxyacrylate stilbene; PSAC, plasmodial surface anion channel; RBC, red blood cell; EC_{50}

References

1. Krungkrai, J., Krungkrai, S. R., Suraveratum, N., and Prapunwattana, P. (1997) *Biochem Mol Biol Int* **42**, 1007-1014
2. Mather, M. W., Darrouzet, E., Valkova-Valchanova, M., Cooley, J. W., McIntosh, M. T., Daldal, F., and Vaidya, A. B. (2005) *J. Biol. Chem.* **280**, 27458-27465
3. Srivastava, I. K., and Vaidya, A. B. (1999) *Antimicrob. Agents Chemother.* **43**, 1334-1339
4. Syafruddin, D., Siregar, J. E., and Marzuki, S. (1999) *Mol Biochem Parasitol* **104**, 185-194
5. Musset, L., Pradines, B., Parzy, D., Durand, R., Bigot, P., and Le Bras, J. (2005) *J. Antimicrob. Chemother.*, dki420
6. Musset, L., Le Bras, J., and Clain, J. (2007) *Mol Biol Evol* **24**, 1582-1585
7. Kessl, J. J., Lange, B. B., Merbitz-Zahradnik, T., Zwicker, K., Hill, P., Meunier, B., Palsdottir, H., Hunte, C., Meshnick, S., and Trumpower, B. L. (2003) *J. Biol. Chem.* **278**, 31312-31318
8. Kessl, J. J., Ha, K. H., Merritt, A. K., Lange, B. B., Hill, P., Meunier, B., Meshnick, S. R., and Trumpower, B. L. (2005) *J. Biol. Chem.* **280**, 17142-17148
9. Gassis, S., and Rathod, P. K. (1996) *Antimicrob Agents Chemother* **40**, 914-919
10. Vaidya, A. B., Painter, H. J., Morrisey, J. M., and Mather, M. W. (2008) *Trends in Parasitology* **24**, 8-9
11. Smilkstein, M. J., Forquer, I., Kanazawa, A., Kelly, J. X., Winter, R. W., Hinrichs, D. J., Kramer, D. M., and Riscoe, M. K. (2008) *Mol Biochem Parasitol*
12. Vivas, L., Easton, A., Kendrick, H., Cameron, A., Lavandera, J.-L., Barros, D., de las Heras, F. G., Brady, R. L., and Croft, S. L. (2005) *Experimental Parasitology* **111**, 105-114
13. Winter, R. W., Kelly, J. X., Smilkstein, M. J., Dodean, R., Bagby, G. C., Rathbun, R. K., Levin, J. I., Hinrichs, D., and Riscoe, M. K. (2006) *Exp Parasitol* **114**, 47-56
14. Oettmeier, W., Masson, K., and Soll, M. (1992) *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1099**, 262-266
15. Oettmeier, W., Masson, K., and Soll, M. (1994) *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1188**, 125-130
16. Oettmeier, W., Masson, K., Soll, M., and Reil, E. (1994) *Biochem Soc Trans* **22**, 213-216
17. Cape, J. L., Bowman, M. K., and Kramer, D. M. (2007) *Proc Natl Acad Sci U S A* **104**, 7887-7892
18. Cape, J. L., Bowman, M. K., and Kramer, D. M. (2006) *Trends Plant Sci* **11**, 46-55
19. Crofts, A. R. (2004) *Annu Rev Physiol* **66**, 689-733
20. Crofts, A. R., Lhee, S., Crofts, S. B., Cheng, J., and Rose, S. (2006) *Biochimica et Biophysica Acta (BBA) - Bioenergetics*
Proton Transfer Reactions in Biological Systems **1757**, 1019-1034
21. Forquer, I., Covian, R., Bowman, M. K., Trumpower, B. L., and Kramer, D. M. (2006) *J. Biol. Chem.* **281**, 38459-38465
22. Painter, H. J., Morrisey, J. M., Mather, M. W., and Vaidya, A. B. (2007) **446**, 88-91
23. Hoefnagel, M. H., Wiskich, J. T., Madgwick, S. A., Patterson, Z., Oettmeier, W., and Rich, P. R. (1995) *Eur J Biochem* **233**, 531-537
24. Fry, M., and Beesley, J. E. (1991) *Parasitology* **102 Pt 1**, 17-26

25. Ljungdahl, P. O., Pennoyer, J. D., Robertson, D. E., and Trumpower, B. L. (1987) *Biochim Biophys Acta* **891**, 227-241
26. Gutierrez-Cirlos, E. B., Merbitz-Zahradnik, T., and Trumpower, B. L. (2004) *J Biol Chem* **279**, 8708-8714
27. Brandt, U., and Djafarzadeh-Andabili, R. (1997) *Biochim Biophys Acta* **1321**, 238-242
28. Reil, E., Soll, M., Masson, K., and Oettmeier, W. (1994) *Biochem Soc Trans* **22**, 62S
29. Horvath, G., Droppa, M., Fodorpataki, L., Istokovics, A., Garab, G., and Oettmeier, W. (1996) *Proc Natl Acad Sci U S A* **93**, 3876-3880
30. Lang-Unnasch, N., and Murphy, A. D. (1998) *Annual Review of Microbiology* **52**, 561-590
31. Rawls, J., Knecht, W., Diekert, K., Lill, R., and Loffler, M. (2000) *European Journal of Biochemistry* **267**, 2079-2087
32. Kelso, G. F., Porteous, C. M., Coulter, C. V., Hughes, G., Porteous, W. K., Ledgerwood, E. C., Smith, R. A. J., and Murphy, M. P. (2001) *J. Biol. Chem.* **276**, 4588-4596
33. James, A. M., Cocheme, H. M., Smith, R. A. J., and Murphy, M. P. (2005) *J. Biol. Chem.* **280**, 21295-21312
34. James, A. M., Sharpley, M. S., Manas, A.-R. B., Frerman, F. E., Hirst, J., Smith, R. A. J., and Murphy, M. P. (2007) *J. Biol. Chem.* **282**, 14708-14718
35. Lisk, G., and Desai, S. A. (2005) *Eukaryot Cell* **4**, 2153-2159
36. Alkhalil, A., Hill, D. A., and Desai, S. A. (2007) *Cell Microbiol* **9**, 851-860
37. Lisk, G., Scott, S., Solomon, T., Pillai, A. D., and Desai, S. A. (2007) *Mol Pharmacol* **71**, 1241-1250
38. Sharom, F. J. (2008) *Pharmacogenomics* **9**, 105-127
39. Oude Elferink, R. P., and de Waart, R. (2007) *J Physiol Biochem* **63**, 75-81
40. Girardin, F. (2006) *Dialogues Clin Neurosci* **8**, 311-321
41. Badawy, Z. S., Chohan, K. R., Whyte, D. A., Penefsky, H. S., Brown, O. M., and Souid, A. K. (2008) *Fertil Steril*
42. Muller, F., Roberts AG, Bowman, MK and Kramer, DM. (2003) *Biochemistry* **42**, 6493 - 6499
43. Muller, F. L., Crofts A.R., Kramer,D. M. (2002) *Biochemistry* **41**, 7866 -7874
44. Mitchell, P., Mitchell, R., Moody, A. J., West, I. C., Baum, H., and Wrigglesworth, J. M. (1985) *FEBS Lett* **188**, 1-7
45. Kramer, D. M., Roberts, A. G., Muller, F., Cape, J., and Bowman, M. K. (2004) *Methods Enzymol* **382**, 21-45
46. Biagini, G. A., Fisher, N., Berry, N., Stocks, P. A., Meunier, B., Williams, D. P., Bonar-Law, R., Bray, P. G., Owen, A., O'Neill, P. M., and Ward, S. A. (2008) *Mol Pharmacol* **73**, 1347-1355
47. Mather, M. W., Henry, K. W., and Vaidya, A. B. (2007) *Curr Drug Targets* **8**, 49-60
48. Krungkrai, J. (2004) *Parasitology* **129**, 511-524

Table and Figure Legends

Table 1 IC_{50} and EC_{50} values measured *in situ* and *in vitro*. [1]*In situ* measurements were determined by the given compounds effect on living parasites grown in red blood cells. [2]*In vitro* measurents were by directly assaying the cyt bc_1 complex in mitochondria isolated from rat tissue as described in materials and methods.

Fig. 1 The "General Acridone" is shown for carbon numbering reference. Compound **1**, 2-(6,6,6-trifluorohexoxyl)-9-acridone; Compound **2**, 3-(6,6,6-trifluorohexoxyl)-9-acridone; Compound **3**, 4-(6,6,6-trifluorohexoxyl)-9-acridone; Compound **4,** 6-Cl-3-(6,6,6-n-alkyloxyl)-9-acridone; Compound **5**, WR 249685 (S enantiomer)

Fig. 2 Compound **2** titration using mitochondrial fragments from *P. yoelli*. Activity is defined as μmol cyt *c* reduced·s^{-1}·mg $protein^{-1}$.

Fig. 3 Inhibition of the cyt bc_1 complex by the acridones.
Y-axis is relative steady state cytochrome c^{3+} reduction (compared to the uninhibited enzyme). ■, 2-(6,6,6-trifluorohexoxyl)-9-acridone; ○,3-(6,6,6-trifluorohexoxyl)-9-acridone ; Δ,4-(6,6,6-trifluorohexoxyl)-9-acridone

Fig. 4 Competition of 3-(6,6,6-trifluorohexoxyl)acridone (ACRID) with Antimycin a (AA) and MOA stilbene (MOA). Each condition was measured with a saturating concentration of ubiquinol (50 μM). 10 nM Antimycin a and MOA stilbene were used. The IC_{50} concentration (10 nM) of the acridone was used.

Table 3-1

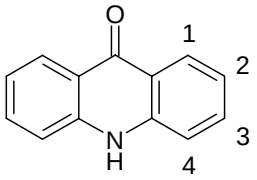
Compound	***in vivo*[1] IC_{50} (nM)**	***in vitro*[2] EC_{50} (nM)**
2-(6,6,6-trifluorohexoxyl)acridone	**36**[*]	**> 10,000**
3-(6,6,6-trifluorohexoxyl)acridone	**0.4**[*]	**10**
6-Cl-3-(4,4,4-trifluorobutyloxyl)acridone	**1.0**[*]	**50**[#]
6-Cl-3-(5,5,5-trifluoropentyloxyl)acridone	**0.3**[*]	**10**[#]
6-Cl-3-(6,6,6-trifluorohexoxyl)acridone	**0.2**[*]	**1**[#]
4-(6,6,6-trifluorohexoxyl)acridone	**446**[*]	**> 10,000**

[*] data from: **Winter, R. W., Kelly, J. X., Smilkstein, M. J., Dodean, R., Bagby, G. C., Rathbun, R. K., Levin, J. I., Hinrichs, D., and Riscoe, M. K. (2006) *Exp Parasitol* 114, 47-56**

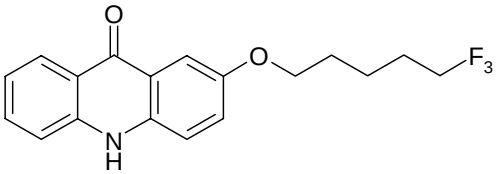
[#] estimate based on single point titration obtained during drug screen

Figure 3-1

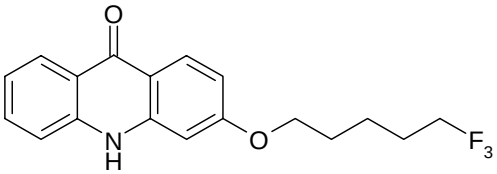
General Acridone



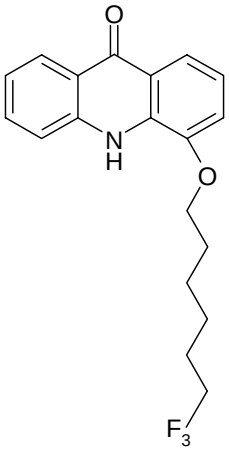
1



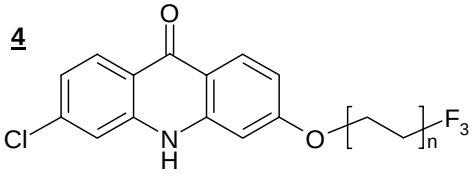
2



3



4



5

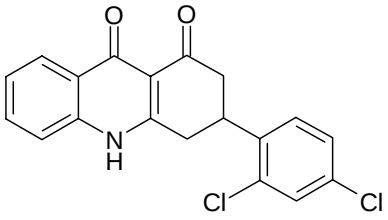


Figure 3-2

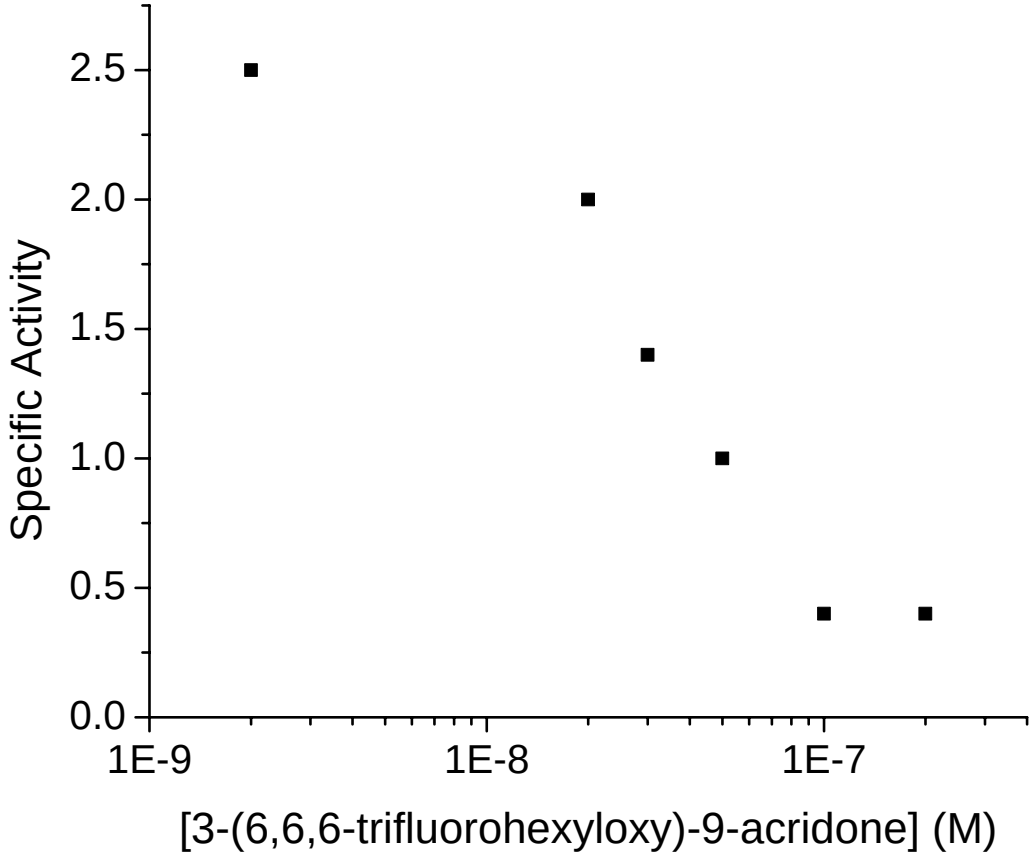

Specific Activity
2.5
2.0
1.5
1.0
0.5
0.0
1E-9
1E-8
1E-7
[3-(6,6,6-trifluorohexyloxy)-9-acridone] (M)

Figure 3-3

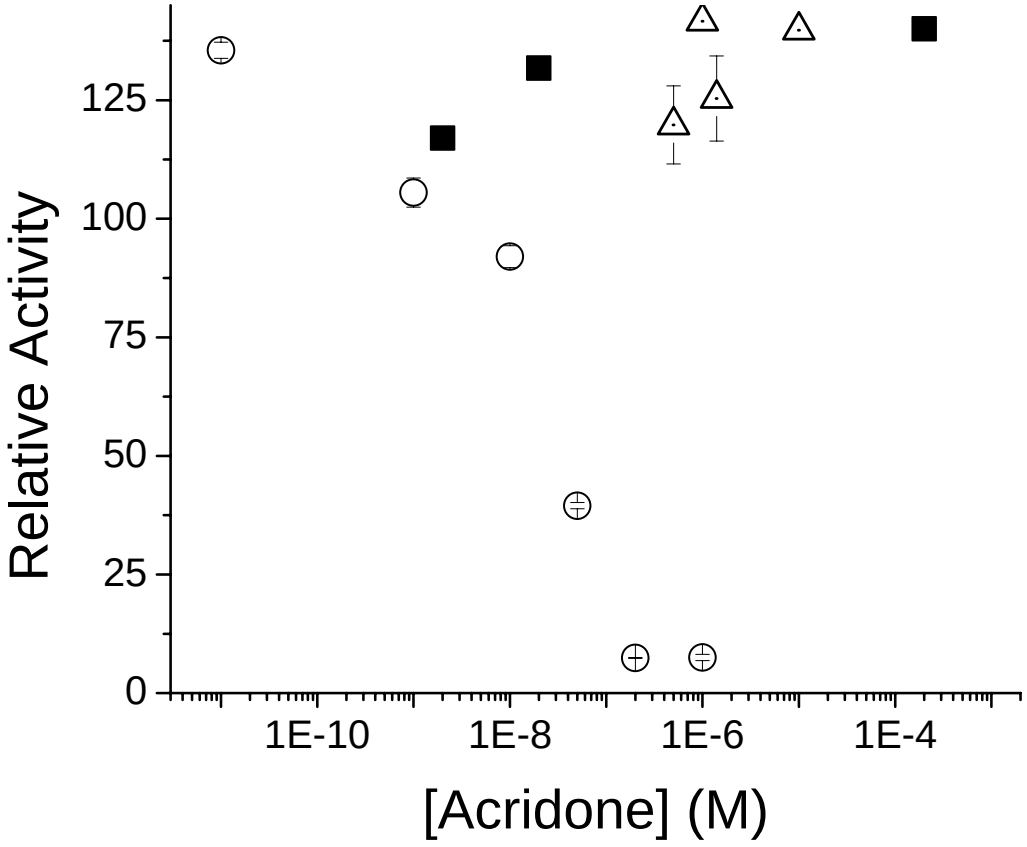
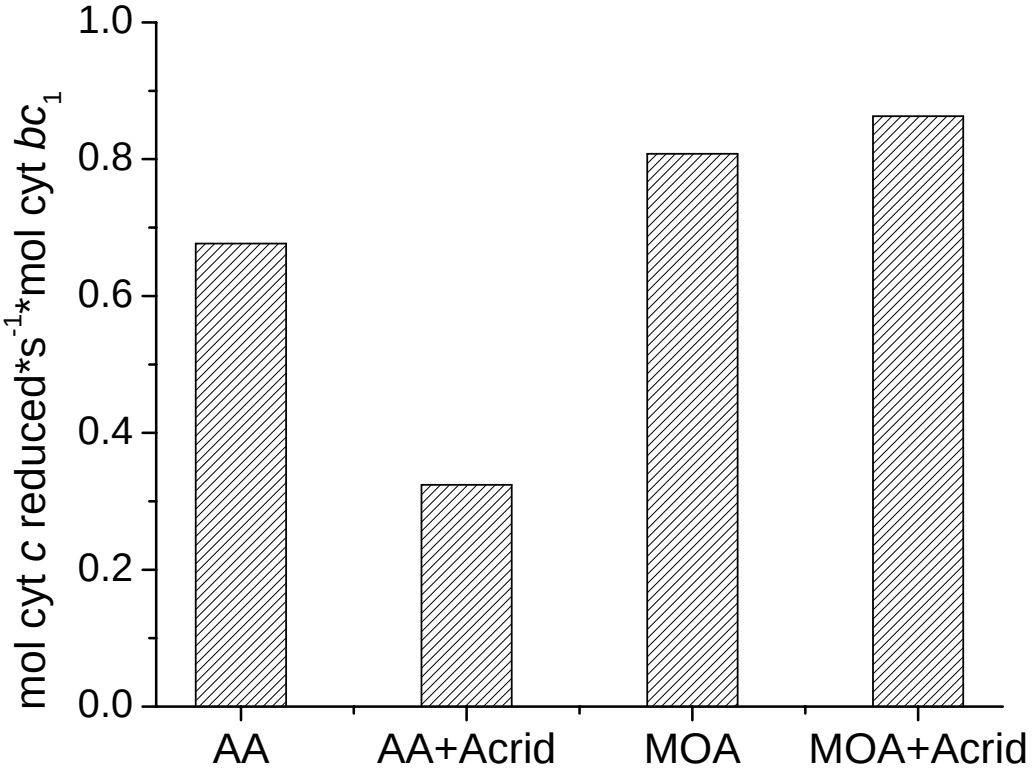

Relative Activity
0
25
50
75
100
125
1E-10
1E-8
1E-6
1E-4
[Acridone] (M)

Figure 3-4


mol cyt c reduced*s-1*mol cyt bc1
1.0
0.8
0.6
0.4
0.2
0.0
AA
AA+Acrid
MOA
MOA+Acrid

Chapter 4

Dihydroorotate dehydrogenase Acts as an Alternative Oxidase in *Plasmodium yoelli*

Isaac Forquer[*], Martin Smilkstein[#], Caleb Martin[*], Michael Riscoe[#] and David M. Kramer[*]

[*]Institute of Biological Chemistry, Washington State University, Pullman, WA 99163
[#]Medical Research Service, Department of Veterans Affairs Medical Center and Department of Chemistry, Portland State University, Portland, OR

Abstract

The electron transfer chain of the *Plasmodium* spp. mitochondrion is required for oxidation of dihydroorotate and has been the target for anti-malarial drug development. Unfortunately, the parasite has rapidly developed resistance to the drugs against mitochondrial targets. One possible mechanism for resistance to drugs targeting respiration is the activation of alternate oxidation pathways. Early work suggested that *Plasmodium* spp. possessed an 'alternative oxidase' that could compensate for inhibition of the cytochrome bc_1 complex (complex III) and cytochrome oxidase (complex IV). However, analysis of the genome of several species of *Plasmodium* revealed no classical alternative oxidases. Here we demonstrate that direct reduction of O_2 by mitochondrial (type II) dihydroorotate dehydrogenase can completely account for the observed alterative oxidase activity, and constitutes a new pathway for orotate synthesis that *Plasmodium* spp. can harness to evade drugs. Based on this finding, we propose an alternative strategy for targeting drugs in *Plasmodium* spp.

Introduction

The unique properties of the *Plasmodium* spp. mitochondrion makes it an interesting target for antimalarial drugs (1-9). Indeed, Atovaquone is a well-known antimalarial agent which binds tightly to the quinol oxidase (Q_o) site of mitochondrial complex III in *Plasmodium* spp, but not as tightly to that of the human host (2,10,11). However, in order to assess the usefulness of such approaches and the likelihood of various forms of emerging drug resistance, it is critical to understand the role and function of the mitochondria in *Plasmodium* spp. If, for example, certain mitochondrial pathways are redundant, resistance to drugs targeting these functions will likely emerge rapidly.

Land-Unnasch and coworkers (12) suggested that *Plasmodium* spp. does not rely on mitochondria for ATP production, but rather employs fermentation (12). Instead, the mitochondrion may be retained because it serves other functions. In particular, *Plasmodium* spp. lacks enzymes for pyrimidine salvage and is thus completely reliant on its mitochondrial dihydroorotate dehydrogenase (DHODH) for pyrimidine biosynthesis (13). Most provocatively, it has recently been proposed that DHODH oxidation is the sole reason for *Plasmodium* spp. to retain its mitochondrial electron transfer chain; it is no longer needed if a cytoplasmic DHODH oxidation pathway is introduced (1).

A further complication in understanding mitochondrial function in *Plasmodium* spp. is the observation that electron transfer from dihydroorotate (DHO) to O_2 is not completely inhibited by saturating levels of KCN (4,14), suggesting the operation of an alternative (cyanide-resistant) quinol oxidase, similar to reported cases of plant mitochondria and several other Eukaryotes (15-18). This apparent redundancy in oxidative pathways is important for drug development, since targeting complexes III or IV by themselves could simply lead to up-regulation of the alternative pathway (see Scheme I).

Surprisingly, none of the *Plasmodium* spp. genomes contain sequences for any 'classical' alternative (or quinol) oxidase (19). We thus attempt to identify the source of the activity previously attributed to alternative oxidase in order to narrow the viable synergistic drug targets on the respiratory chain of *Plasmodium* spp.

Materials and Methods

Growth and Plasmodium strains:

P. yoelii – Outbred female CF-1 mice were infected by injection of 10^7 parasitized red blood cells (RBCs) from infected donor. When parasitemia reached ~40% (by microscopy of Giemsa-stained blood smears), anesthetized mice were exsanguinated by cardiac puncture, and blood was ACD anticoagulated, pooled, kept on ice, and maintained at this temperature during processing. Leukocytes were removed by serial centrifugation and removal of buffy coat and uppermost cells, followed by iced PBS wash. Absence or near-absence of white blood cells was confirmed by microscopy.

Preparation of mitochondrial fragments:

Mitochondrial fragments were prepared by a procedure modified from (5,20). RBC's were thawed and mixed 1:1 in 125 ml each of RPMI 1640 medium and RPMI 1640 medium containing 0.15% saponin. The mixture was incubated for 20 minutes at 37^{o}C. The parasites were then harvested by centrifugation at 8,000 X G for 10 minutes. The intact parasites were then washed four times in phosphate buffered saline (PBS) and 1 mM PMSF 4 times in and centrifuged at 8000 X G for 10 minutes. Once the parasites were harvested, they were frozen and stored until needed for preparation of mitochondrial fragments. The thawed parasites were broken by suspending them in "Lysis buffer", containing 75 mM Sucrose, 225 mM mannitol, 5 mM $MgCl_2$, 5 mM KH_2PO_4, 1 mM EDTA, 1 mM PMSF, 5 mM HEPES, at pH 7.4. The cells

were then broken by homogenization in a glass homogenizer. The cell debris was removed by centrifugation at 800 X G for 5 minutes. Mitochondrial particles were then harvested at 20,000 X G for 10 minutes.

The pellet was resuspended in lysis buffer, and mixed to a final concentration of 30% glycerol. The aliquots of mitochondrial particles were frozen at -70^{o}C until needed.

Recombinant DHODH preparation:

Professor Joseph Clardy, Harvard University, kindly provided a DNA construct ligated into a pRSET plasmid containing the truncated (but functional) gene for DHODH from *P. falciparum*. Transformation of *E. coli*, induction, over-expression and isolation of the protein were performed as described in (21).

Enzyme assays:

Recombinant DHODH activity was measured essentially as described previously (22) in buffer containing 100 μM L-dihydroorotate, 50 mM Tricine, 100 mM KCl at pH = 8.0. We chose to use the UV wavelength pair 283-300 to measure dihydroorotate oxidation directly. Superoxide production was measured in the same reaction mixture, with addition of 10 μM Amplex Red (Molecular Probes, Eugene, OR), 5 units of horseradish peroxidase and 100 units of Mn-containing superoxide dismutase (Sigma). The sample was pulsed with a green LED with λ_{max}= 525 nm at 2.3 KHz. Fluorescence excitation was measured by an in-house built fluorimeter with a 5 nm bandwidth filter centered at 605 nm covering the photodiode (Hamamatsu 1223). The signal output was amplified with an in-house built amplifier and processed with a lock-in amplifier (Thor Labs Inc. model LIA100). The system was calibrated by titrating H_2O_2 to quantitate the signal amplitude to known amounts of product (resorufin).

Superoxide production in detergent solubilized mitochondrial fragments from *P. yoelli*i were done as described above, by adding 20 mg/ml total protein to the cuvette. Alternatively, superoxide production was measured by Mn-SOD sensitive cyt c^{+3} reduction (23) using the wavelength pair 550-542 ($\varepsilon = 17 \cdot mM^{-1}$).

Oxygen consumption by the mitochondrial fragments was measured in the same reaction mixture in a Rank Bros. 1ml Perspex oxygen electrode. The signal was calibrated by taking the air saturated buffer to be full scale (250 μM O_2) against the dithionite saturated sample (assumed to have negligible amounts of O_2).

Under each of the conditions used, the concentration dependence of superoxide production on the concentration of dihydroorotate was measured to ensure that signals we assign to the activity of dihydroorotate dehydrogenase are not artifacts. Indeed, in all cases the signal saturated at around 10 μM dihydroorotate, suggesting that we are observing the activity of the same enzyme under all conditions (see table 1).

Results

Isolated DHODH can reduce O_2 or exogenous cyt c in competition with UQ

Figure 1 shows that the activity of isolated (truncated) DHODH is strongly dependent on the concentration of the electron acceptor, decyl-ubiquinone, with a K_m of approximately 3 uM, consistent with previous observations that UQ is the natural substrate for DHODH (21). In the complete absence of added UQ, however, a small (approximately 25% of maximal) residual rate of DHO oxidation by DHODH remained. This residual activity could be attributed to a direct reduction of oxygen by DHODH, resulting in superoxide production as originally described in (24). In this case, O_2 acts as an alternative electron acceptor for DHODH, albeit at a slower rate than occurs with saturating concentrations of UQ. Superoxide production by DHODH is

inhibited by addition of UQ with a concentration dependence that mirrors the increase is DHO oxidation, i.e. the apparent K_M for ubiquinone is approximately equal to the [UQ] which inhibits the rate of the superoxide production by half. Addition of 50 μM of the decylubiquinol (the reduced form of UQ)(QH_2) had little effect on either superoxide production or DHO oxidation (data not shown) implying that the the effects of UQ on superoxide production occur at the level of catalysis.

These results strongly suggest that ubiquinone and oxygen compete for electrons from DHODH, probably at the reduced flavin (25). The reduction of O_2 by isolated DHODH in the absence of UQ, was abolished by complete removal of oxygen from the sample (Figure 2). However, addition of 50 uM horse heart cytochrome c under these conditions reestablished 40% of the activity (Figure 2). Since DHO was unable to directly reduce cyt c in the absence of DHODH (data not shown), we conclude that a reduced intermediate within DHODH can directly deliver electrons to exogenous cyt c.

Plasticity of electron transfer in P. yoelli mfitochondrial membranes

As expected for mitochondria from most many species (26), both the UQH_2 and DHO were oxidized by *P. yoelli* mitochondrial particles with the reduction of oxygen (Figure 3). In contrast with most species studied thus far, however, oxidation of either QH_2 or DHO in the absence of inhibitors (23,27,28) resulted in significant superoxide production. With UQH2 and DHO as substrates, superoxide production represented approximately 11% and 29% respectively of maximal electron transfer to oxygen (Figure 4). When DHO was used as a substrate, addition of stigmatellin (shown if Figure 4), antimycin or myxothiazol which block complex III, or KCN which blocks complex IV, decreased oxygen consumption but increased the rate of superoxide production. By contrast, when UQH_2 was used as a substrate, oxygen consumption decreased

and the rate of superoxide production decreased (Table 1). This is consistent with previous observations (4,14) that oxygen consumption by *Plasmodium* spp. mitochondria in the presence of cyanide only inhibits O_2 consumption by about 75% (Table 1). However, in contrast to what would be expected if the O_2 was reduced by an alternative oxidase, we observed that cyanide-insensitive oxygen consumption produced near stoichiometric amounts of superoxide (Table 1).

Discussion

In many bacteria and mitchondria, electron transfer in oxidative phosphorylation branches at the UQ pool, with one pathway leading to O_2 through complexes III and IV, while the other passes through a so-called 'alternative oxidase.' Classically, the activity of an alternative oxidase has been revealed by the presence of KCN-resistant respiration, i.e. that remains after KCN inhibits complex IV, as illustrated in Scheme I. A similar situation was thought to occur in *Plasmodium* since treatment with KCN or other Complex III inhibitors only inhibited electron flow by 75% (4,14). On the other hand, full length genomic sequences showed that no 'classical' alternative oxidases were present in Plasmodium (19), suggesting a different origin for the 'alternative oxidase' activity.

Here, we confirm the existence of O_2 uptake in *P yoelli* membranes when electron transport to cytochrome *c* oxidase is inhibited, at about 25% of the uninhibited rate (Fig. 2, Table 1). However, the observed stigmatellin-resistant oxidation was only observed with DHO, and not with UQH_2, as electron donors, i. e. electrons can be fed into the 'alternative oxidase' from DHO but not from UQH2. In contrast, 'classical' alternative oxidases use UQH_2 as their natural substrate, and thus should be accessible to both exogenous UQH_2 and DHO, which feeds electrons into UQ. Thus, our results imply that the alternative oxidase step occurs between DHO and the UQ pool, i.e. that DHODH itself acts as an oxidase. Indeed, (24,29-31) showed that, in

the absence of its 'native' oxidant, UQ, isolated DHODH can reduce O_2 to superoxide at a relatively rapid rate. Our results (Figs. 1-4) broadly support this observation, both in isolated DHODH and in mitochondrial membranes from Plasmodium. leading us to conclude that both UQ and O2 may act as 'natural' oxidants for DHODH. The idea that DHODH is, effectively, an alternative oxidase is further supported by two observations. First, essentially all stigmatellin- or KCN-insensitive O_2 reduction resulted in superoxide production. This result is not consistent with operation of a classical alternative oxidase, which is expected to reduce O_2 to water, but consistent with the O_2-reduction side reaction described for DHODH (24,29-31). Second, we found that UQ and O_2 compete at DHODH for electrons from DHO (Fig. 1). Taken together, our results lead us to propose that Plasmodium DHODH may operate naturally as a DHO:UQ-oxidoreductase:oxygenase, with UQ as the 'preferred' substrate, but that when downstream electron transfer is blocked, the UQ pool becomes fully reduced, allowing highly reducing species to build up within DHODH, triggering O_2 reduction, accounting for the previously observed 'aternative oxidase' behavior.

In vivo, when electron transfer is rapid the UQ/QH_2 pool will be predominantly oxidized and UQ would be the predominant oxidant at DHODH. However, when electron transfer is hindered, e.g. by high proton gradient or the presence of inhibitors, the UQ pool will become progressively more reduced, allowing O_2 to compete for DHO electrons. This behavior is seen in Fig. 1; upon lowering the concentration of decylubiquinone, the rate of superoxide production increases, indicative of increased electron transfer to O_2.

Recently, Painter et al. (1) showed that transformants of *P. falciparum* expressing a cytoplasmic (Type I) DHODH, were insensitive to inhibitors of mitochondrial electron transport, i.e. mitochondrial electron transfer appears to be superfluous if an alternative DHO oxidation

pathway is present. Based on these results, Painter et al. suggested that as *Plasmodium* spp. adapted to a parasitic lifestyle, energy production by the mitochondrion became superfluous, and mitochondrial functions were progressively lost. Presently, DHO oxidation for pyrimidine biosynthesis may be the only remaining requirement for maintaining the *Plasmodium* spp. mitochondrial electron transfer and even that role may be obviated by complementing with a type I DHODH.

We demonstrate here that the mitochondrial DHODH, in native membranes, can directly transfer electrons to O_2, bypassing the downstream electron transfer chain. Given the results of Painter et al (1), we suggest that, under certain conditions, this process could allow *Plasmodium* spp. to survive even in the absence of functional complexes III and IV, e.g. in the presence of drugs such as atovaquone or stigmatellin.

It is useful to consider the possibility that direct O_2 reduction by DHODH could sustain *Plasmodium* spp. in the absence of classical respiration. Certainly, the kinetics of direct reduction of O_2 by DHODH in mitochondrial membranes in the presence of inhibitors was maximally only a fraction (29%) of normal turnover. However, it is unclear if DHO oxidation is rate-limiting for cell growth. It is also conceivable that mutations within DHODH could render it an even better O_2 reductant.

However, one may reasonably argue that superoxide that results from direct reduction of O_2 at DHODH could be deleterious (32). This possibility suggests that antimalarials that target respiration, e.g. atovaquone, at leat partly affect cells via increased ROS producction. On the other hand, some organisms can deal with quite high intracellular rates of superoxide production. In particular, photosynthetic organisms (from which Plasmodium (33)) produce extremely high levels off superoxide via its photosynthetic electron transfer chain, but possess an adequate

detoxification system to prevent damage (34). One can thus envision a case where adaptation could allow Plasmodium to rapidly detoxify DHODH-generated superoxide, thus allowing survival without functional complexes III or IV. We suggest that such adaptations may have already been isolated. The A6 strain of *P. falciparum* (35) is resistant to a large range of Complex III inhibitors *in vivo*, despite having no mutations in its cytochrome b protein. Moreover, complex III is fully sensitive to these inhibitors *in vitro* while its multi-drug resistance pumps appear to be unaffected by mutations. Since A6 has retained sensitivity to 5-fluoroorotate, it appears that it still requires activity of DHODH but not downstream electron transfer, i.e. A6 can oxidize DHO without functional Complexes III and IV. A simple explanation is that the 'alternative oxidase' activity of DHODH is fulfilling its proposed role using oxygen rather than ubiquinone as an electron acceptor in the presence of Q_o site inhibitors. In preliminary results (not shown), we found that DHODH in A6 membranes had very similar properties as wild type, suggesting that the difference is not in the rate of direct O_2 reduction, but rather on downstream processes. We thus suggest that A6 may have developed a mechanism of coping with an additional load of superoxide production due to 'dihydroorotate oxidase' activity.

References

1. Painter, H. J., Morrisey, J. M., Mather, M. W., and Vaidya, A. B. (2007) **446**, 88-91
2. Meshnick, S. R., and Trumpower, B. (2005) *J Infect Dis* **191**, 822; author reply 822-823
3. Baldwin, J., Michnoff, C. H., Malmquist, N. A., White, J., Roth, M. G., Rathod, P. K., and Phillips, M. A. (2005) *J. Biol. Chem.* **280**, 21847-21853
4. Suzuki, T., Hashimoto, T., Yabu, Y., Kido, Y., Sakamoto, K., Nihei, C.-i., Hato, M., Suzuki, S.-i., Amano, Y., and Nagai, K. (2004) *Biochemical and Biophysical Research Communications* **313**, 1044-1052
5. Krungkrai, J. (2004) *Parasitology* **129**, 511-524
6. Vaidya, A. B., and Mather, M. W. (2005) *Curr Top Microbiol Immunol* **295**, 233-250

7. Mi-Ichi, F., Takeo, S., Takashima, E., Kobayashi, T., Kim, H., Wataya, Y., Matsuda, A., Torii, M,. Tsuboi, T. and Kita, K. (2003) in *Tropical Diseases From Molecule to Bedside* (Marzui, V. a. S., ed) Vol. 531, pp. 117-133, Kluwer Academic/ Plenum Publishers, New York
8. Krungkrai, J., Kanchanarithisak, R., Krungkrai, S. R., and Rochanakij, S. (2002) *Experimental Parasitology* **100**, 54-61
9. Learngaramkul, P., Petmitr, S., Krungkrai, S. R., Prapunwattana, P., and Krungkrai, J. (1999) *Mol Cell Biol Res Commun* **2**, 15-20
10. Mather, M. W., Darrouzet, E., Valkova-Valchanova, M., Cooley, J. W., McIntosh, M. T., Daldal, F., and Vaidya, A. B. (2005) *J. Biol. Chem.* **280**, 27458-27465
11. Kessl, J. J., Ha, K. H., Merritt, A. K., Lange, B. B., Hill, P., Meunier, B., Meshnick, S. R., and Trumpower, B. L. (2005) *J. Biol. Chem.* **280**, 17142-17148
12. Lang-Unnasch, N., and Murphy, A. D. (1998) *Annual Review of Microbiology* **52**, 561-590
13. Cordeiro, A. T., Feliciano, P. R., and Nonato, M. C. (2006) *Acta Crystallographica Section F* **62**, 1049-1051
14. Murphy, A. D., and Lang-Unnasch, N. (1999) *Antimicrob Agents Chemother* **43**, 651-654
15. Castro-Guerrero, N. A., Krab, K., and Moreno-SÃ¡nchez, R. (2004) *Journal of Bioenergetics and Biomembranes* **36**, 459-469
16. Grabelâ€™nykh, O., Kolesnichenko, A., Pobezhimova, T., Zykova, V., and Voinikov, V. (2006) *Russian Journal of Plant Physiology* **53**, 418-429
17. Juarez, O., Guerra, G., Velazquez, I., Flores-Herrera, O., Rivera-Perez, R. E., and Pardo, J. P. (2006) *FEBS Journal* **273**, 4603-4615
18. Juszczuk, I. M., and Rychter, A. M. (2003) *Acta Biochim Pol* **50**, 1257-1271
19. Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W., Carlton, J. M., Pain, A., Nelson, K. E., Bowman, S., Paulsen, I. T., James, K., Eisen, J. A., Rutherford, K., Salzberg, S. L., Craig, A., Kyes, S., Chan, M.-S., Nene, V., Shallom, S. J., Suh, B., Peterson, J., Angiuoli, S., Pertea, M., Allen, J., Selengut, J., Haft, D., Mather, M. W., Vaidya, A. B., Martin, D. M. A., Fairlamb, A. H., Fraunholz, M. J., Roos, D. S., Ralph, S. A., McFadden, G. I., Cummings, L. M., Subramanian, G. M., Mungall, C., Venter, J. C., Carucci, D. J., Hoffman, S. L., Newbold, C., Davis, R. W., Fraser, C. M., and Barrell, B. (2002) *Nature* **419**, 498-511
20. Krungkrai, J., Krungkrai, S. R., Suraveratum, N., and Prapunwattana, P. (1997) *Biochem Mol Biol Int* **42**, 1007-1014
21. Hurt, D. E., Widom, J., and Clardy, J. (2006) *Acta Crystallographica Section D* **62**, 312-323
22. Ullrich, A., Knecht, W., Fries, M., and Loffler, M. (2001) *European Journal of Biochemistry* **268**, 1861-1868
23. Kramer, D. M., Roberts, A. G., Muller, F., Cape, J., and Bowman, M. K. (2004) *Methods Enzymol* **382**, 21-45
24. Forman, J. H., and Kennedy, J. (1975) *J. Biol. Chem.* **250**, 4322-4326
25. Kussmaul, L., and Hirst, J. (2006) *Proc Natl Acad Sci U S A* **103**, 7607-7612
26. Rasmusson, A. G., Geisler, D. A., and Møller, I. M. (2008) *Mitochondrion Unique aspects of plant mitochondria* **8**, 47-60
27. Muller, F. L., Crofts A.R., Kramer,D. M. (2002) *Biochemistry* **41**, 7866 -7874

28. Muller, F., Roberts AG, Bowman, MK and Kramer, DM. (2003) *Biochemistry* **42**, 6493 - 6499
29. Hines, V., and Johnston, M. (1989) *Biochemistry* **28**, 1222-1226
30. Knecht, W., Henseling, J., and Loffler, M. (2000) *Chemico-Biological Interactions* **124**, 61-76
31. SÃ¸rensen, P., and Dandanell, G. (2002) *Extremophiles* **V6**, 245-251
32. Hoye, A. T., Davoren, J. E., Wipf, P., Fink, M. P., and Kagan, V. E. (2008) *Accounts of Chemical Research* **41**, 87-97
33. Moore, R. B., Obornik, M., Janouskovec, J., Chrudimsky, T., Vancova, M., Green, D. H., Wright, S. W., Davies, N. W., Bolch, C. J. S., Heimann, K., Slapeta, J., Hoegh-Guldberg, O., Logsdon, J. M., and Carter, D. A. (2008) **451**, 959-963
34. Asada, K. (2006) *Plant Physiol* **141**, 391-396
35. Smilkstein, M. J., Forquer, I., Kanazawa, A., Kelly, J. X., Winter, R. W., Hinrichs, D. J., Kramer, D. M., and Riscoe, M. K. (2008) *Mol Biochem Parasitol*

Table and Figure Legends

Table 1 Rate of oxygen consumption or superoxide production in the presence of either dihydroorotate or decylubiquinol as substrate

Scheme I AO, Alternative Oxidase; DH, Dihydroorotate Dehydrogenase; III, Complex III; IV, Complex IV; QH_2, ubiquinol; Q, ubiquinone; DHO, dihydroorotate; ORO, orotate; C_{RED}, reduced cytochrome *c*; $C_{OX,}$ oxidized cytochrome *c*; KCN, cyanide A. Generalized model of major electron transport reactions in *Plasmodium* spp. Arrows indicate enzymatic oxidations and reductions. In the general scheme, Q is reduced to QH_2 by DH via the $2e^-$ flavin, and QH_2 is oxidized by *either* AO (putatively 25% of oxygen consumption or III (apparently 75% of oxygen consumption). B. If electron flow through III and IV is disrupted by for instance cyanide, the AO serves as the only sink for electrons, and could account for cyanide resistant oxygen consumption. C. If, as the genome data supports, no such AO exists, then disruption of electron flow through III and IV will force DH to use an alternative electron acceptor, such as oxygen, when the electron transport chain becomes fully reduced, which also accounts for cyanide resistant oxygen consumption.

Fig. 1 Saturation properties of dihydroorotate oxidation by recombinant DHODH as a function of UQ concentration. □: UQ reductase activity measured by reduction of UQ; ●, superoxide production

Fig. 2 Superoxide production by the recombinant DHODH. Dihydroorotate was the only susbstrate added. No exogenous electron acceptors were included. Upon the addition of dihydroorotate (100 μM), the reduction of cytochrome *c* commenced (first column), and the activity is 35% sensitive to the removal of oxygen. In the second column, superoxide production is monitored by amplex red (see text), and removal of oxygen virtually eliminates activity.

Fig. 3 Oxygen Consumption in the presence of dihydroorotate (DHO) or decylubiquinol (QH2) as substrates with or without stigmatellin (STIG).

Fig. 4 Cytochrome *c* reduction with dihydroorotate as substrate in the presence of stigmatellin (STIG). SOD, superoxide dismutase. The third bar represents the difference of Stig-(Stig+SOD) which indicates the rate of superoxide production. The fourth bar is rate of superoxide production measured using the amplex red system.

Table 5-1

Substrate	Oxygen Consumption[1]	Oxygen Consumption +STIG[1]	Superoxide Production[2]	Superoxide Production +STIG[2]
Dihydroorotate	**0.60±0.04**	**0.17±0.01**	**0.02±0.03**	**0.13±0.05**
Decylubiquinol	**0.40±0.01**	**0.05±0.01**	**0.06±0.01**	**0.01±0.01**

[1] **µMol O_2 consumed·mg protein^{-1}·second^{-1} with or without STIG, stigmatellin**
[2] **µMol superoxide produced·mg protein^{-1}·second^{-1} with or without STIG, stigmatellin**

Scheme I:

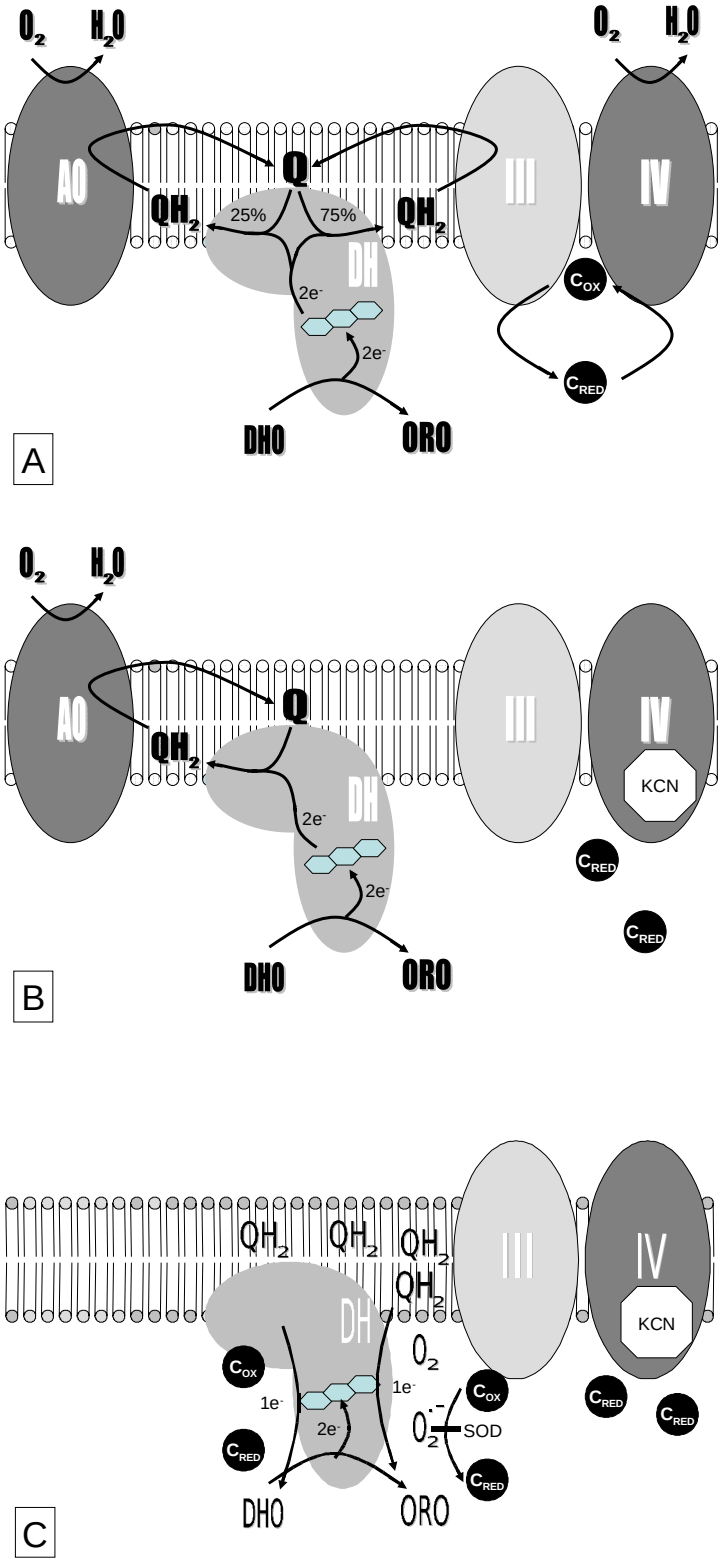

O2
H2O
AO
Q
QH2
25%
75%
DH
2e-
DHO
ORO
III
IV
COX
CRED
A
KCN
B
1e-
O2
O2.-
SOD
C

Figure 4-1

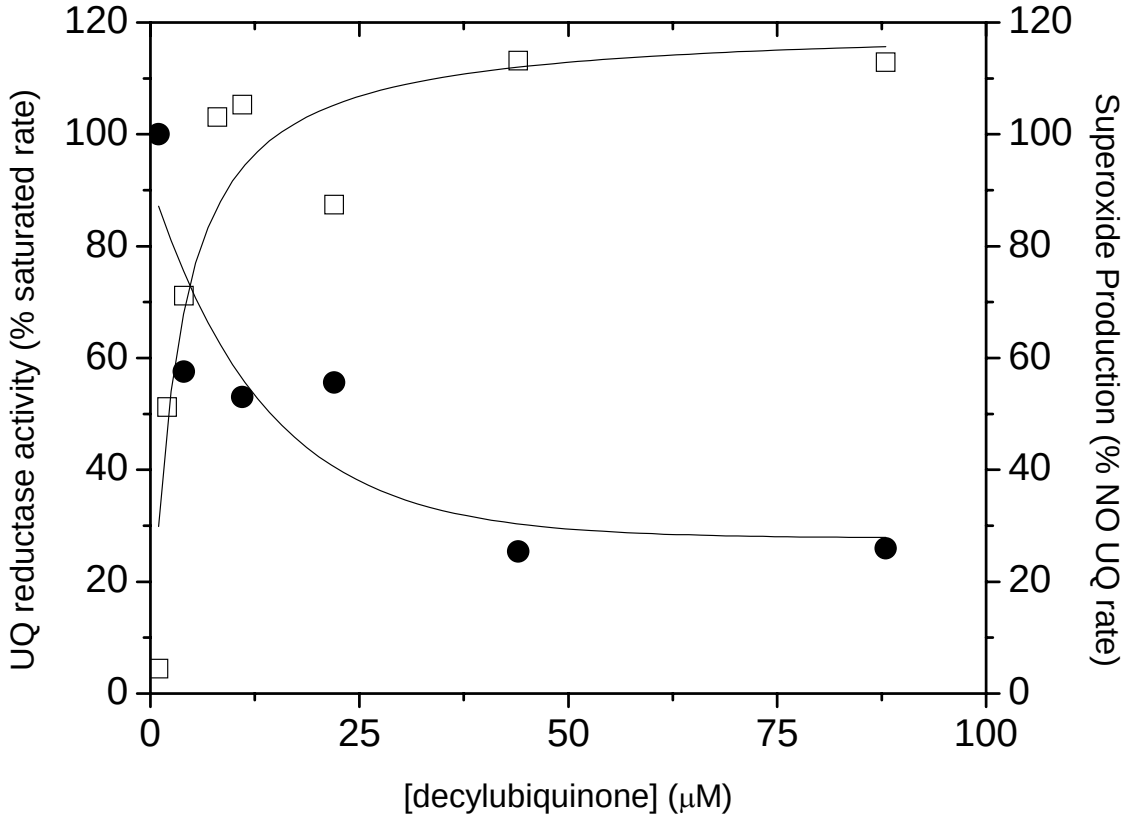

UQ reductase activity (% saturated rate)
Superoxide Production (% NO UQ rate)
[decylubiquinone] (μM)
0
20
40
60
80
100
120
0
25
50
75
100

Figure 4-2

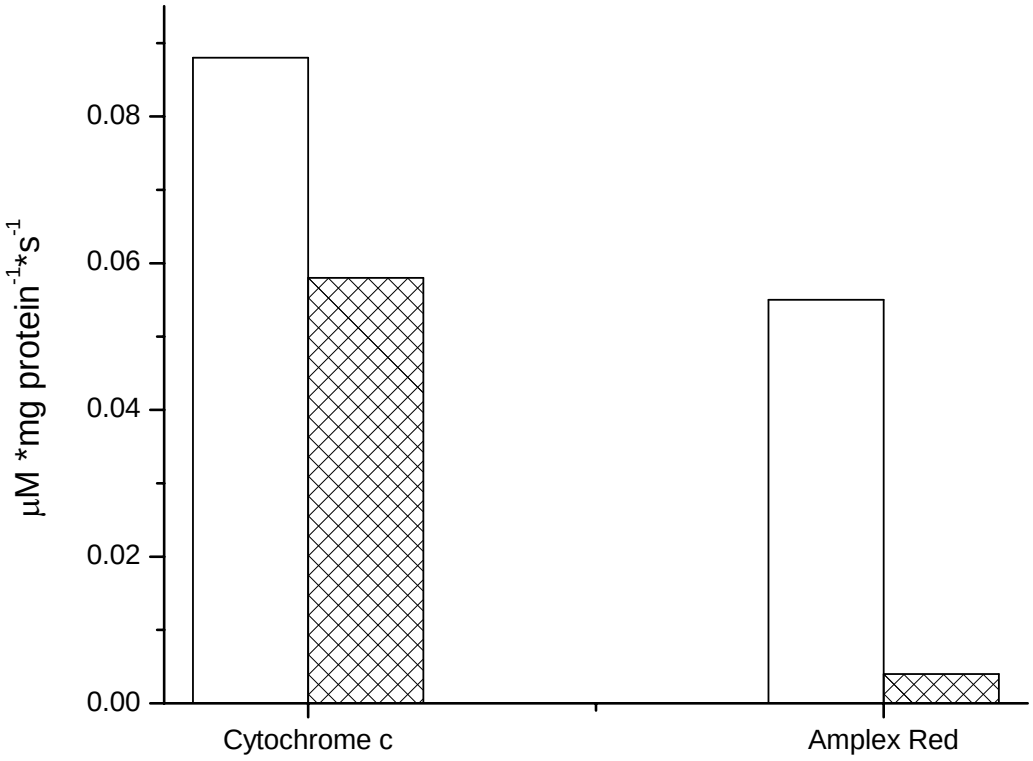


Figure 4-3

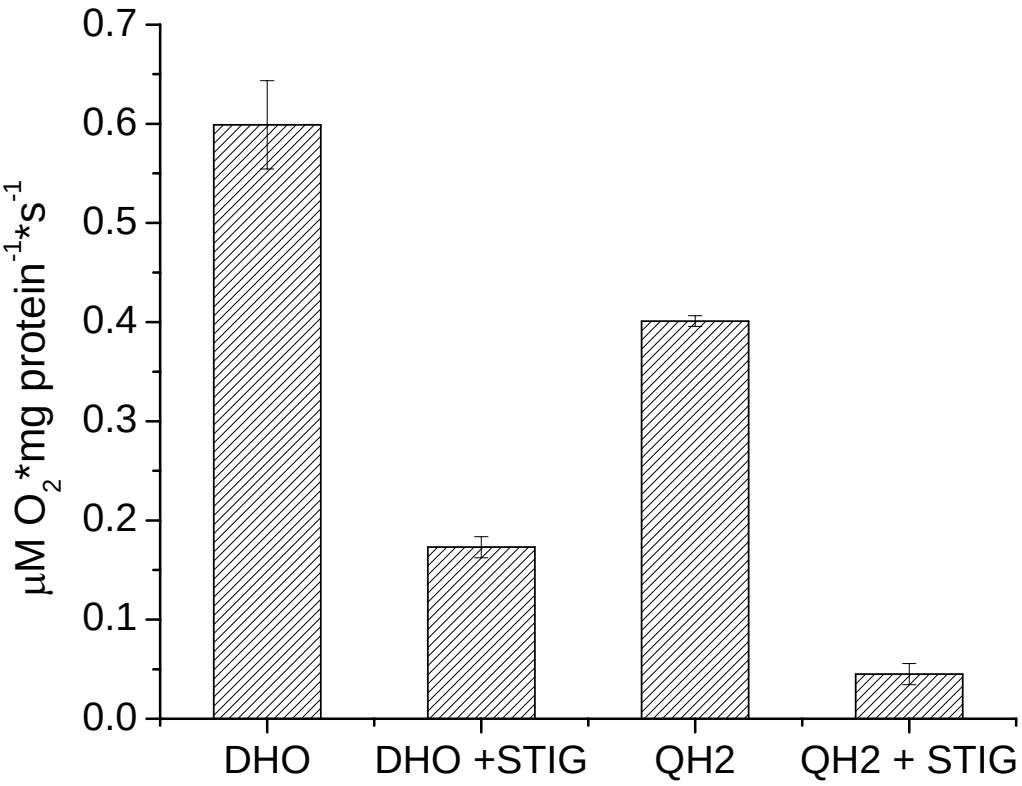
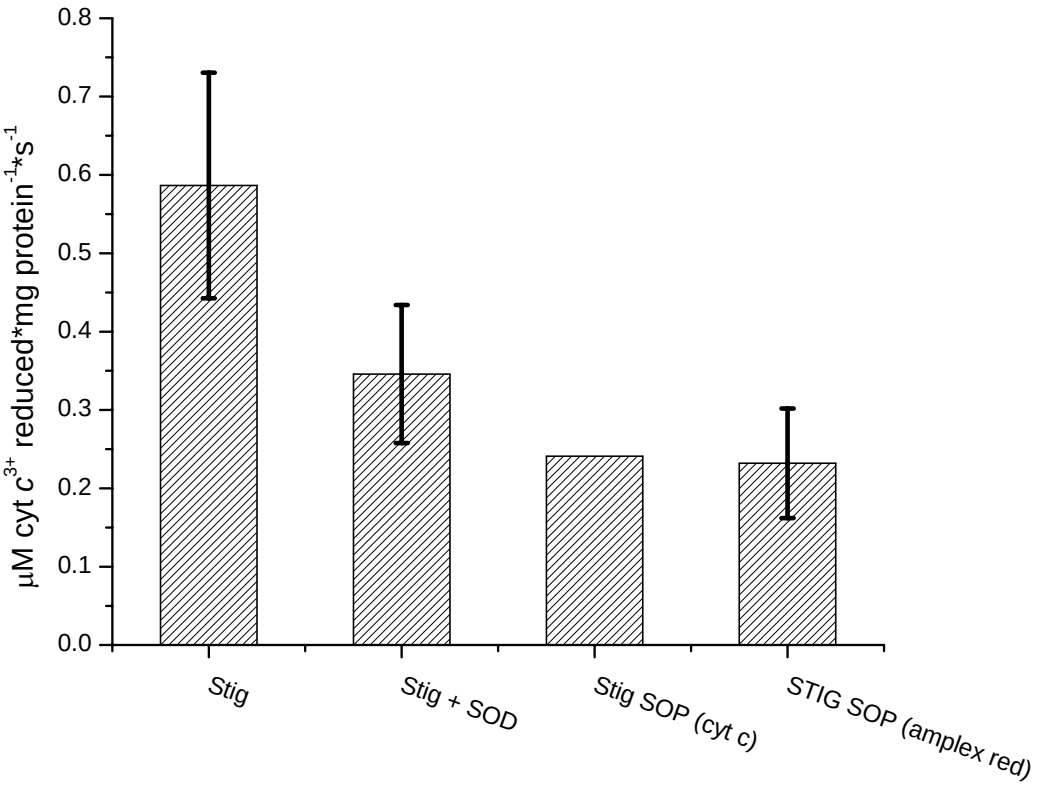

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
µM O2*mg protein-1*s-1
DHO
DHO +STIG
QH2
QH2 + STIG

Figure 4-4


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
µM cyt c3+ reduced*mg protein-1*s-1
Stig
Stig + SOD
Stig SOP (cyt c)
STIG SOP (amplex red)

Chapter 5

Conclusion

Isaac Forquer

The main focus of the first part of this dissertation was to test the hypothesis that a semiquinone intermediate is formed during normal Q-cycle catalysis, and that this same intermediate is the direct reductant during superoxide production. The hypothesis was clearly tested in the first chapter of this dissertation, and there is evidence to support rejecting the hypothesis. The data allow for the rejection of the "Double Concerted Electron Transfer" model, in which there would most certainly have to be two distinct reaction intermediates during catalysis. Some key points await further experimentation.

One exciting possibility is the exploitation of the cyt *bc* complex in *Bacillus* spp. Interestingly, species within this genus invariably utilize menaquinone, instead of ubiquinone as its primary pool quinone in the electron transport chain. What is interesting is that the redox potential of all of the cofactors in it's cyt *bc* complex are adjusted downward in a linear fashion with the lower potential quinone. Reading textbooks alone, one would reasonably ask why the potential of the 2Fe2S center is lowered. We have all learned that enzymes were designed to lower the activation barrier and increase the forward rate of the enzyme.

Interestingly, a sequence alignment (Figure 1) of the Rieske protein in the immediate vicinity of the 2Fe2S center reveals a striking detail, the same amino acid that was substituted in the S183A mutant of Chapter 3 in this document to generate the 2Fe2S redox potential of +165 mV vs. SHE (wt is +285 mV, or a ΔG of about 11 $kJ \cdot mol^{-1}$) is conservatively substituted in all

species of *Bacillus* spp. studied to date. What makes this so striking is that in (1), the redox potential of the Rieske was measured to be +165 mV vs. SHE. What is so exciting about this is that raises the possibility that a mutant could be developed to actually *increase* the driving force for the first electron transfer without having to use artificial substrates. One could then test the hypothesis that raising the redox potential of the 2Fe2S center will increase the probability of measuring bypass reactions without the addition of superoxide elicitors. Testing this hypothesis could help answer the bigger question of determining whether the cyt *bc* complexes in general optimize, rather than maximize the amount of semiquinone formed during steady state catalysis, and do so by actually raising the activation barrier of the very reaction it 'catalyzes'.

The last part of this dissertation originally set out as a drug screening exercise, in which the main hypothesis to be tested was that certain acridones and other novel small molecules were inhibitors of the cyt bc_1 complex in *Plasmodium* spp. While this certainly proved to be a worthwhile project, the spin-off has far greater implications. While attempting to determine if these novel inhibitors were inhibiting another enzyme (DHODH) in the mitochondrial electron transport chain, it was discovered that some of the redox active compounds (quinones themselves!) were actually acting as substrates. It actually turns out that the electron acceptor binding pocket (for which ubiquinone is the physiological ligand) is quite promiscuous. As outlined in chapter 5, it actually turns out that DHODH can utilize a host of artificial electron acceptors… including oxygen.

On a side project associated with Chapters 4 & 5 of this dissertation, we tested the inhibitor sensitivity of a strain of *Plasmodium falciparum* (hereafter A6) that was VERY insensitive to every cyt bc_1 inhibitor it was challenged with, both known classical inhibitors such as antimycin and novel inhibitors related to those presented in Chapter 4. We showed

conclusively that the compound (6-NH_2-3-(6,6,6-trifluorhexyloxy)-9H-acridone – see compound **B** in Chapter 4) the strain was raised against to generate resistance, in addition to stigmatellin and antimycin, were able to exert normal, wild-type like inhibitory effects on the enzyme during *in vitro* enzyme assays. What is interesting is that these compounds are nearly refractory against the organism *in vivo*. The current idea is that dihrdroorotate is still getting oxidized (A6 is still sensitive to DHODH donor side inhibitors), but something else, such as oxygen, is acting as the electron acceptor.

The next step in this project will clearly involve taking a step back from the details of the electron transport chain. The simplest hypothesis at this point is that *P. falciparum* (strain A6) has developed an expression strategy to deal with an increased load of reactive oxygen species generation to accommodate dihydroorotate oxidation. It stands then that the next round of experiments should center around answering two questions: 1. Is A6 resistant to compounds such as paraquat or small doses of superoxide/hydrogen peroxide? And 2. Are there differences in the expression profiles of A6 vs. WT, or perhaps A6 in standard growth conditions vs. growth conditions containing oxidative agents such as paraquat. This strategy has been employed to study the reaction of bacteria to oxidative stress in (2).

In any event, it is interesting that once the selective pressure to maintain a proton motive force is removed from the mitochondrial electron transport chain, it opens up new possibilities and allows great flexibility to the organism to deal with new selective challenges.

References:

1. Liebl, U. P., Stephane; Riede, Astrid; Kellner, Eva; Nitschke, Wolfgang. (1992) *The Journal of Biological Chemistry* **267**, 14068-14072
2. Bruno-Barcena, J. M., Andrus, J. M., Libby, S. L., Klaenhammer, T. R., and Hassan, H. M. (2004) *Appl Environ Microbiol* **70**, 4702-4710

Figure 1 Legend: Sequence alignment of residues in the vicinity of the 2Fe2S center of the Rieske protein from numerous taxonomic sources. The top six organisms utilize ubiquinone as their primary pool quinone, whereas the bottom two use menaquinone. The residue highlighted is the equivalent residue mutated in the S183A mutant from Chapter 3.

Figure 6-1

```
UCRI_RHOSH   -EAGEWLVMWGVCTHLGCVPIGGV-SGDFGGWFCPCHGSHYDSA--GRIRKGPAPEN-LPIPLAKFIDETTIQLG-----
UCRI_RHOCA   -NTGEWLVMLGVCTHLGCVPMGDK-SGDFGGWFCPCHGSHYDSA--GRIRKGPAPRN-LDIPVAAFVDETTIKLG-----
UCRI_PARDE   -EAGEWLVMIGVCTHLGCVPIGDG-AGDFGGWFCPCHGSHYDTS--GRIRRGPAPQN-LHIPVAEFLDDTTIKLG-----
UCRI_HUMAN   ---PEWVILIGVCTHLGCVPIAN--AGDFGGYYCPCHGSHYDAS--GRIRLGPAPLN-LEVPTYEFTSDDMVIVG-----
UCRI_YEAST   ---PQWLIMLGICTHLGCVPIGE--AGDFGGWFCPCHGSHYDIS--GRIRKGPAPLN-LEIPAYEFD-GDKVIVG-----
UCRI_BRAJA   --HEQWLVVIGICTHLGCIPIAH--EGNYDGFFCPCHGSQYDSS--GRIRQGPAPAN-LPVPPYQFVSDTKIQIG-----
QCRA_BACTC   --KGDIIALSPVCKHLGCTVDWNTDKNNPNHFFCPCHYGLYTKD--GTNVPGTPPTAPLDRYEFEVK-DGKLYLGKAKPR
QCRA_BACSU   --GDEIVALSPICKHLGCTVNWNSDPKNPNKFFCPCHYGLYEKD--GTNVPGTPPLAPLDHYEQEVK-DGFLYLGKAKPK
```

Appendix I

Roles of Individual Authors:

Chapter 2:

Isaac Forquer, Primary Author, conducted all experiments on mutant yeast lines

Raul Covian, conducted experiments using pre-steady state activation parameters

Michael K. Bowman, contributed significant intellectual support

Bernard L Trumpower, contributed significant intellectual support, supervised RC

David M. Kramer, Principal Investigator

Chapter 3:

Isaac Forquer, Primary Author, conducted all kinetic experiments

Rolf Winter, synthesized compounds

Martin Smilkstein, provided material for experiments on the *Plasmodium* system.

Jane Kelly, contributed significant intellectual support

Michael Riscoe, contributed significant intellectual support, supervised RW, MS and JK

David M. Kramer, Principal Investigator

Chapter 4:

Isaac Forquer, Primary Author, conducted all kinetic experiments

Martin Smilkstein, contributed significant intellectual support, provided material for experiments on the *Plasmodium* system

Caleb Martin, assisted with kinetic experiments, and preparation of assays. Contributed intellectual support

Michael Riscoe, contributed significant intellectual support, supervised MS

David M. Kramer, Principal Investigator