PROBING THE PHYSIOLOGICAL ROLE OF CHLOROBIUMQUINONE IN *CHLOROBACULUM TEPIDUM* USING A NOVEL SYNTHETIC ANALOG

by

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“If we are uncritical we shall always find what we want: we shall look for, and find, confirmations, and we shall look away from, and not see, whatever might be dangerous to our pet theories. In this way it is only too easy to obtain what appears to be overwhelming evidence in favor of a theory which, if approached critically, would have been refuted." -Karl Popper

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KEY TO ABBREVIATIONS

BChl:	Bacteriochlorophyll
BHI:	Brain Heart Infusion
BLAST:	Basic Local Alignment Search Tool
CAN:	Cerium (IV) Ammonium Nitrate
CK:	Chlorobiumquinone
CKA:	Chlorobiumquinone Analog
CP:	Chlorobium Plating
DCM:	Dichloromethane
DMK:	Demethylmenaquinone
GBS:	Green Sulfur Bacteria
HCL:	Hydrochloric Acid
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC:	High Pressure Liquid Chromatography
IMG:	Integrated Microbial Genomes Database
MK:	Menaquinone
MOON:	2-methyl-3-(1′-oxooctadecyl)-1,4-napthoquinone
MTBE:	Methyl Tert-Butyl Ether
NMR:	Nuclear Magnetic Resonance
PBS:	Phosphate Buffered Solution
PCC:	Pyridinium Chlorochromate
PE:	Petroleum Ether

Q-RT-PCR:	Quantitative Real-Time Polymerase Chain Reaction
RBF:	Round Bottom Flask
R_f :	Retention Factor
SQR:	Sulfide Quinone Oxidoreductase
TLC:	Thin Layer Chromatography
THF:	Tetrahydrofuran
UQ:	Ubiquinone

ABSTRACT

Chlorobaculum (*Cba.*) *tepidum* is a model organism for studying the anoxic autotrophic oxidation of reduced sulfur compounds. Understanding the cellular mechanisms in which these compounds are oxidized is necessary for developing bioremediation technologies that mitigate the release of toxic sulfur compounds, such as hydrogen sulfide, into the environment. Sulfide: quinone oxidoreductases (SQRs) oxidize sulfide to persulfide and donates the resulting electrons to the quinone pool. The two major quinones in *Cba. tepidum* are menaquinone-7 (MK-7) and chlorobiumquinone (CK). This thesis investigates the role of CK in sulfide metabolism by performing physiological studies using a novel chlorobiumquinone analog (CKA) that was chemically synthesized. Growth studies involving CKA determined that the analog inhibits growth in CK producing organisms through bacteriostatic means. Cyclic voltammetry studies showed that addition of CKA to cells has no significant impact on the ability of *Cba*. *tepidum* to uptake sulfide. However, fluorescent measurements revealed that CKA is an efficient quencher of cellular fluorescence. These results support prior work in the literature suggesting that CK is involved in sequestering energy transfer from BChl *c* to the reaction center complex under oxidizing conditions in order to prevent the production of highly toxic oxygen radicals. Additionally, the gene CT1509 whose gene product may be involved in the biosynthesis of CK was identified though a precomputed bi-directional BLASTP genome comparison between *Cba*. *tepidum* and *Leishmania major* strain Friedlin (a eukaryote that produces CK) . Future work involving the silencing of CT1509 gene

expression and physiological studies of the resultant mutant will further contribute to our understanding of the biosynthesis and physiological relevance of chlorobiumquinone.

Chapter 1

INTRODUCTION

1.1 Statement of problems and significance

The global sulfur cycle has profound implications on the stability of local economies and ecosystems worldwide. Anaerobic sulfur oxidation is the most poorly understood part of the global sulfur cycle. The cycling of inorganic sulfur compounds in the environment is mainly driven by microbial activity (Fig. 1.1; Hanson, unpublished). Anaerobic sulfur oxidizers have been observed to detoxify sulfidic coastal waters (32), play an essential role in deep sea hydrothermal vent communities (4), can be used in mining operations to recover metal from low grade ore deposits (7), and have been utilized in technologies to remove sulfide from industrial waste (29, 52).

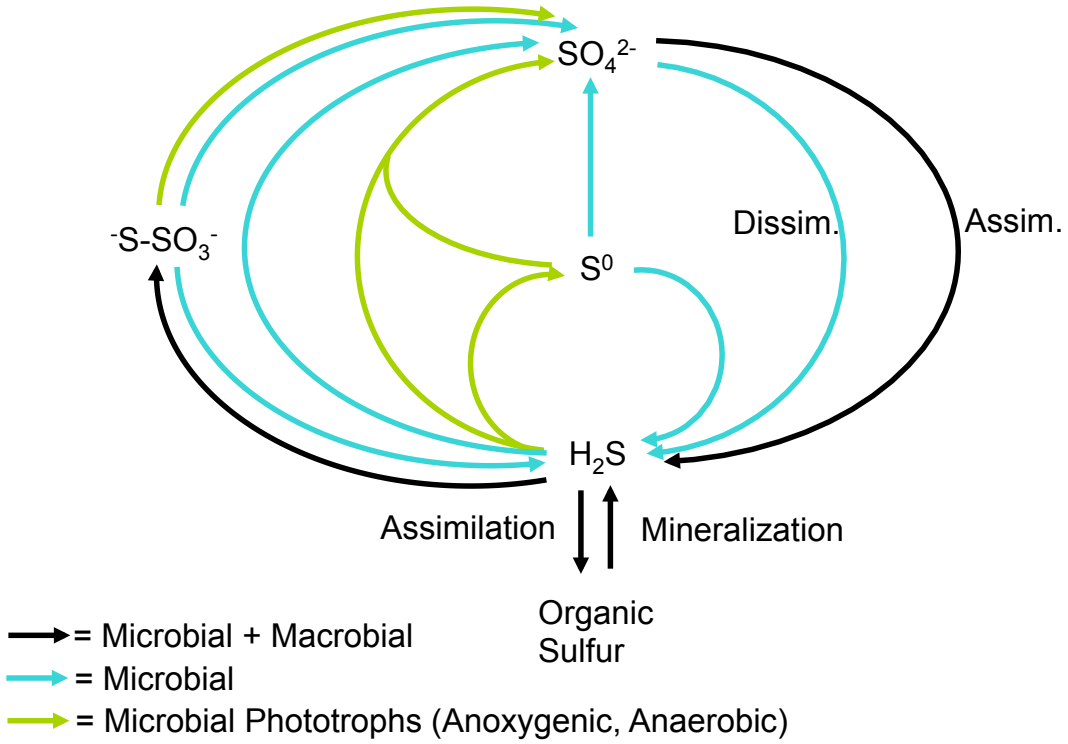


Figure 1.1 The biogeochemical sulfur cycle. Colored arrows specify the types of organisms involved in each sulfur species redox-reaction.

Chlorobaculum (formerly *Chlorobium*) *tepidum* is an anaerobic thermophilic green sulfur bacterium that has been recognized as a model organism for studying microbial sulfur oxidation (13, 50). *Cba. tepidum* is able to oxidize a variety of sulfur species, which it uses as electron donors in order to produce reducing equivalents for biomass generation in the presence of CO_2 and light (15, 38, 45). Each reduced sulfur species is oxidized by a different enzyme or enzyme system. Sulfide: quinone oxidoreductases (SQR's) oxidize sulfide to elemental sulfur and donate the resulting electrons to the quinone pool (9, 46). The two major quinones in *Cba. tepidum* are MK-7 and CK (17, 18). It has been hypothesized that CK is involved in quenching

cellular fluorescence under oxidizing conditions (6, 17, 18). However, it has not been firmly established which of these quinones is involved in accepting electrons from SQR and transferring them down the electron transport chain. Moreover, the genes involved in the biosynthesis of chlorobiumquinone have not been identified.

This thesis investigates the function of chlorobiumquinone by performing physiological studies using a novel chlorobiumquinone analog (CKA). The data presented here indicate that when *Cba. tepidum* encounters oxidizing conditions chlorobiumquinone becomes an efficient quencher of energy transfer from Bchl *c* to the reaction center complex. The gene product of CT1509 may also be involved in the biosynthesis of CK.

1.2 Green Sulfur Bacteria

The green sulfur bacteria (GSB; the *Chlorobiacea*) are photosynthetic bacteria that use special types of chlorophylls (bacteriochlorophylls) as their light-harvesting pigment (22, 44). The antenna complex in GSBs is called the chlorosome (48). It is a rod or cigar shaped structure that contains tens of thousands of bacteriochlorophylls (Bchl *c* in *Cba. tepidum*) and carotenoids enclosed by a unilamellar membrane (14). Because GSBs are the lowest light adapted of all phototrophic organisms known, the chlorosome is thought to be evolutionarily optimized for photon capture in extremely low light environments (14, 15, 34, 41). The chlorosome is connected to a baseplate of Bchl *a* molecules (Fig. 1.2). When light-energy is harvested, it is transferred from the Bchl *c* molecules inside the chlorosomes through the Bchl *a* baseplate where they are transferred to the reaction center complex (16).

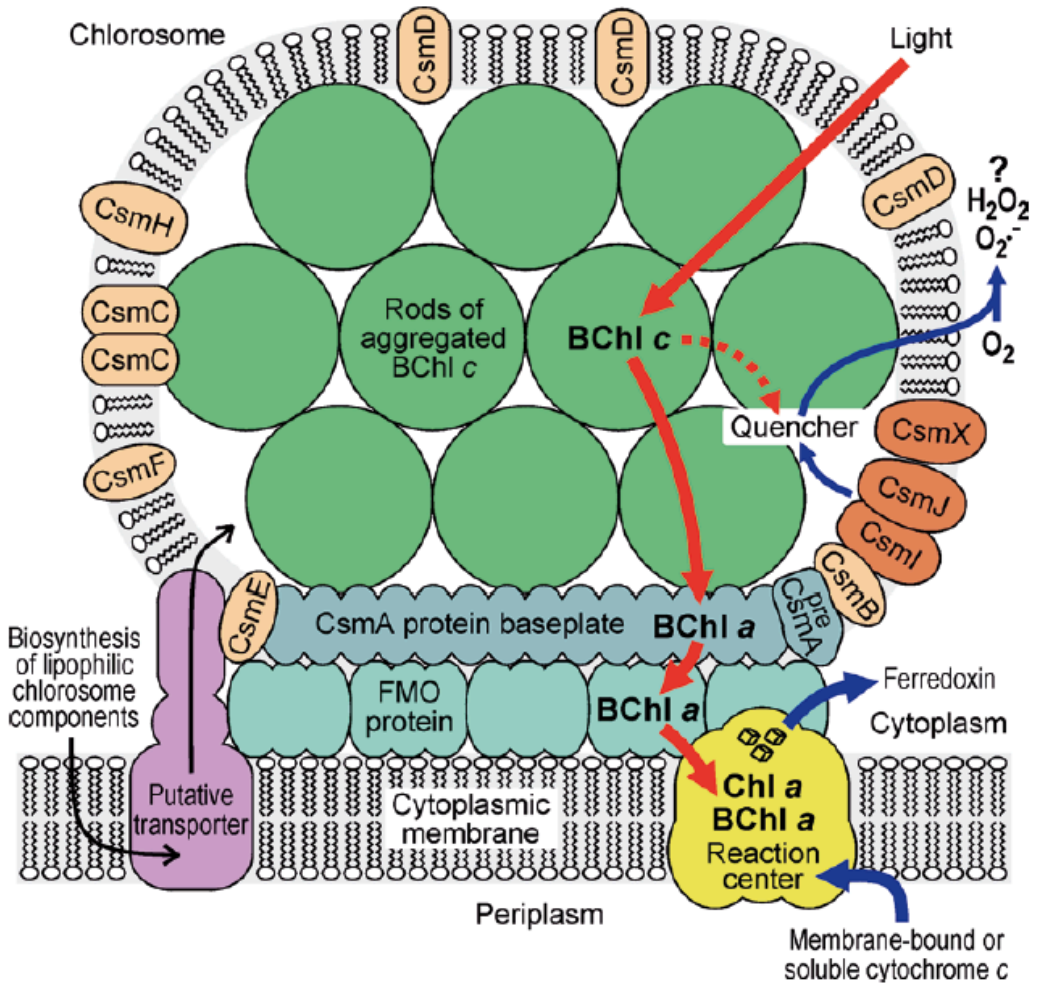


Figure 1.2 Simplified model of the arrangement of the light-harvesting and electron transferring components of the photosynthetic apparatus in *C. tepidum*. Figure taken from Frigaard and Bryant (2004) (14). Red arrows show excitation transfer. Blue arrows show electron transfer. Under reducing conditions excitation energy is transferred via the solid red pathway. Under oxidizing conditions, it is hypothesized that a quencher is activated that absorbs most of the excitation energy and thereby inhibits photosynthetic energy transfer as shown by the broken red arrow.

1.3 Proposed role of chlorobiumquinone in *Cba. tepidum*

CK was first isolated from *Chlorobium thiosulphatophilum* (22); however, the correct structure was not solved until 1968 (Fig. 1.3) (43). Quinone concentrations in *C. thiosuphatophilum* grown in a sulfide medium and *Chloropseudomanas (Cps.) ethylicum* grown on ethanol were determined in order to shed light on the function of the newly discovered quinone (44). It was found that concentrations of chlorobiumquinone and a polar menaquinone were much greater in *C. thiosulphatophilum* than in *Cps. ethylicum* but that the concentration of MK-7 was essentially identical between the two species (44). However, when *Cps. ethylicum* was exposed to the sulfide medium, chlorobiumquinone content was found to increase by an order of magnitude (51.8 mmol (mol chlorophyll)$^{-1}$ vs. 6.0 mmol (mol chlorophyll)$^{-1}$); giving rise to the hypothesis that chlorobiumquinone is involved in the photo-oxidation of sulfide (44).

Over the years, models of the chlorosome structure and function were refined. It was found that energy transfer efficiency in chlorosome antennas of GSB were highly sensitive to the redox potential of the medium suspension (51). Adding oxidants or oxidized compounds quenched cellular fluorescence, while adding reductants or reduced compounds allowed for efficient energy transfer to the reaction center complex to occur (51). *Chloroflexus* (*Cfx.*) *aurantiacus* possesses chlorosomes similar to the *chlorobi* but lack a redox-dependent quenching mechanism (19). When oxidized exogenous quinones were added to the green filamentous bacterium, it was shown that energy transfer to the reaction center was inhibited (19). This inhibition is unique since it is not observed when untreated cells were exposed to O_2 (19). Therefore, the addition of oxidized quinones causes *Cfx. aurantiacus* to display a chlorosomal energy-transfer sequestration similar to that observed in GSB and that the

ability of a quinone to quench fluorescence is dependent mostly on a high midpoint potential and high hydrophobicity (19).

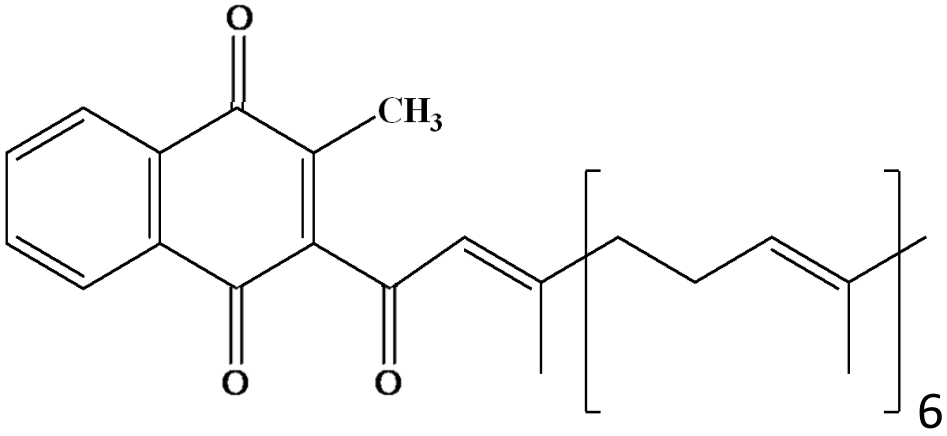


Figure 1.3 Chemical structure of chlorobiumquinone.

1.4 Specific Aims

The preceding review of the literature identified two competing hypotheses for the physiological role of chlorobiumquinone in *Cba. tepidum*: electron acceptor for sulfide oxidation vs. quenching of excited Bchl molecules. Furthermore, the biosynthesis of CK is not yet solved. In particular, how the 1′-oxo group bridge between the naphthoquinone ring and prenyl tail is incorporated is unclear in this strictly anaerobic organism. Therefore, a major goal of my thesis research is to perform experiments that can differentiate between the two possible functions and shed light on the biosynthesis of chlorobiumquinone. Overall, this study aimed to achieve the following specific goals:

1.4.1 Quantitative analysis of quinone pools under different physiological conditions

Initially it needed to be shown that the endogenous quinones MK-7 and CK from *Cba. tepidum* could be extracted and quantitatively analyzed by HPLC. The next step was to determine if a quinone analog could be resolved concurrently with the endogenous quinones via HPLC. The quantitative analysis of the endogenous and exogenous quinone pool is necessary for determining the growth conditions that yield maximum and minimum pool sizes of CK. By understanding how and when *Cba. tepidum* regulates CK synthesis, we will not only be better able to assess the function of CK but also the involvement of genes controlling the regulation and synthesis of CK in future experiments using quantitative real time polymerase chain reaction (Q-RT-PCR) and gene knock-outs.

1.4.2 Synthesis of a chlorobiumquinone analog (CKA)

It was desirable to obtain a chlorobiumquinone analog for use in physiological studies utilizing *Cba. tepidum* in order to probe the possible function of chlorobiumquinone. Synthesis of the chlorobiumquinone analog 2-methyl-3-(1′-oxooctadecyl)-1,4-napthoquinone (MOON) was reported and utilized to study what kinds of quinones play a role in the electron transport system of *Leishmania donavani* promastigote since it had been found that CK is the major isoprenoid quinone in this parasitic protozoa (5). However, the synthesis of MOON was found not to be feasible after multiple attempts. Upon reviewing the literature further, a possible route for production of a novel chlorobiumquinone analog, 2-methyl-3-(1'-oxo-n-pentyl)-1, 4-napthoquinone, (CKA) was outlined and achieved (Section 2.3).

1.4.3 Characterization of physiological responses of CKA on *Cba. tepidum, L. tarentolae*, and *E. coli*.

The physiological effect of CKA on *Cba. tepidum* was determined using growth studies, fluorescence, and voltammetry techniques (Sections 2.6-2.9). The ability of the analog to inhibit growth was also tested on *Escherichia* (*E*.) *coli* and *Leishmania* (*L*.) *tarentolae* (Sections 2.11-2.12). CK is not found in *E. coli* but is expected to be found in *L. tarentolae* (a *Leishmania* species that is fatal to geckoes but not dangerous to humans). CK had previously been implicated in the GSB as quencher of fluorescence (16-18); however, this hypothesis has not been firmly established. By analyzing the physiological effect of CKA on *Cba. tepidum* the data here are most consistent with the function of CK acting as an energy quencher when cells encounter oxidizing conditions.

Chapter 2

MATERIALS AND METHODS

2.1 Bacterial growth conditions and media

Cba. tepidum wild-type strain WT2321 was grown in Pf-7 medium buffered to pH 6.95 with 1,3-bis(tris(hydroxymethyl)methylamino)propane (BTP, MP Biomedicals, Solon, OH), a mineral medium with no supplements, or on Chlorobium Platting (CP) solid medium as previously described (10, 50). All cultures were maintained anaerobically and pressurized to 10 psi with O_2 scrubbed 5% CO_2/95% N_2. *Cba. tepidum* cultures were grown at 47 $^{\circ}$C with 20 μmol photons m^{-2} s^{-1} of irradiance supplied by neodymium full-spectrum bulbs (Lumiram Electric Corp., Larchmont, NY) in tubes (20 mL) or bottles (100 mL) in temperature controlled aquaria. Culture density was measured by quantifying total cellular protein using the Bradford assay (39).

2.2 Determination of CK and MK-7 content per gram of biomass under three different sulfide concentrations

Quinones were extracted from a stationary phase culture with hexanes (25) and analyzed by HPLC (23). A Bradford assay (39) was done immediately before harvest to calculate total protein in the culture. The content of MK-7 per unit protein was calculated by dividing the number of moles of MK-7 extracted by total amount of protein in the culture. CK was unable to be quantified since its isolation was unable to be confirmed and no standards were available (Section 3.1).

2.2.1 Culture conditions for analyzing quinones

Cba. tepidum was grown anaerobically in Pf-7 medium in 100-mL bottles as previously described (10, 50). Twenty hours after inoculation sulfide was added to cultures (6 mM or 2 mM final conc.) or not at all. Two hours after the addition of sulfide, cells were harvested by centrifugation and washed once with phosphate buffer solution (PBS). Each culture condition was done in triplicate and samples for quantifying cellular protein by Bradford assay (39) taken immediately before harvest.

2.2.2 Quinone extraction and analysis

Cell pellets were suspended in acetone/methanol (1:1, v/v). This suspension was extracted three times with hexanes. The hexane fractions were then combined and washed three times with methanol/water (9:1, v/v) in a separatory funnel in order to extract out excess Bchl (25). Running a stream of air over the solvent evaporated hexanes and the resulting residue was dissolved in 3 mL of ethanol and filtered through a 0.2-μm nylon membrane (Pall Life Sciences, Ann Harbor, MI).

Quinone extracts in ethanol were analyzed by reverse HPLC on a Shimadzu Class VP system equipped with a column oven, UV/visible detector, and a fraction collector using a Prevail C18 analytical 5 μm particle size column (150 mm length, 4.6 mm diameter). A mobile phase of methanol/isopropyl ether (83:17, v/v) was pumped at a flow rate of 1 mL min^{-1} and absorbance taken at 270 nm (23). Authentic MK-7 (Wako Pure Chemicals Industries Inc., Osaka, Japan) was used to generate a standard curve for quantifying native MK-7.

2.3 Experimental synthesis of a CK analog: 2-methyl-3-(1′-oxo-n-pentyl)-1,4-naphthoquinone

2.3.1 General experimental procedures and synthetic route:

Nuclear Magnetic Resonance (NMR) spectra of ^{1}H and ^{13}C were recorded as solutions in deuteriochloroform ($CDCl_3$) at 400 MHz and 101 MHz, respectively. ^{13}C multiplicities were determined with the aid of a JVERT pulse sequence, differentiating the signals for methyl and methane carbons as "down" from methylene and quaternary carbons as "up". Retention factor (R_f) values refer to thin layer chromatography (TLC) on 2.5 x 10 cm, 250 μm Analtech analytical plates coated with silica gel GF and developed in solvent system of methyl tert-butyl ether (MTBE) and petroleum ether (PE) as indicated. All glassware was oven dried and rinsed with dry solvent before use. THF and diethyl ether were distilled from sodium metal/benzophenone ketyl under dry N_2.

Synthesis of 2-methyl-3-(1′-oxo-n-pentyl)-1,4-napthoquinone used menadione (2-methyl-1,4-napthoquinone) as the starting material and required five reaction steps to obtain the desired chlorobiumquinone analog. To the best of our knowledge this specific analog has not been reported in the literature. Initially a one-pot ether synthesis was performed (42) followed by a Friedel-Crafts formylation at the 3-position (28). The resulting 1,4-dimethoxy-2-methyl-3-naphthaldehyde was then converted to 1,4-dimethoxy-2-methyl-3-(1′-pentanol)-1,4-napthoquinone by nucleophilic anionic addition to the carbonyl using n-butyl-lithium (40). Oxidation of the methoxy-groups was then accomplished using cerium (IV) ammonium nitrate (CAN) (30). Finally, the alcohol was oxidized to the ketone using pyrimidine chlorochromate (PCC) (11) to yield 2-methyl-3-(1′-oxo-n-pentyl)-1,4-napthoquinone. See Fig. 3.3 for reaction scheme.

2.3.2 Synthesis of 1, 4-dimethoxy-2-methylnapthalene (2) through 2-methylnaphthalene-1, 4-diol intermediate

In a 125-mL Erlenmeyer flask containing 35 mL of THF the following components were added in order: menadione (3.0 g, 17.4 mmol), sodium hydrosulfite ($Na_2S_2O_4$) (9 g, 0.0522 mol), and the phase-transfer catalyst tetrabutylammonium bromide (Bu_4NBr) (1.8 g, 5.60 mmol). The solution became a blackish/ dark purple upon addition of the reducing reagent but turned back to yellow upon addition of 35 mL of H_2O. The solution was then stirred vigorously for 1 hour, followed by the addition of the alkylating agent dimethyl sulfate (8.5 mL, 90 mmol) and aqueous sodium hydroxide (8.9 g, 222. mmol, in 17.3 mL of H_2O), respectively. The solution turned dark purple/ black again but returned to yellow after 3 min. The solution was stirred vigorously for 30 min, 21 g of ice was added after 3 min to keep reaction at ambient temperature, and the evolution of compound 2 was followed by TLC. The organic layer was then isolated and diluted with diethyl ether (35 mL). The solution was then washed with H_2O (3 x 20 mL) and saturated NaCl (10 mL); dried over Na_2SO_4; and filtered with Whatman #2 filter paper. The solvents were then removed via rotary evaporation, and the residue dissolved in petroleum ether (50 mL) and put at -20 $^{\circ}$C. Upon formation of crystals, the solvent was decanted and drying under high vacuum resulted in crude 2-methylnaphthalene-1,4-diol (2) as pinkish/white crystals that were used for the next step. TLC and NMR were used to identify the production of compound (2). Compound (2) was produced in a 53.7% yield and displayed an R_f value of 0.64 in 10% MTBE/PE on TLC plates.

2.3.3 Synthesis of 1, 4-dimethoxy-2-methyl-3-naphthaldehyde (3)

A small round bottom flask (RBF) was charged with compound (2) (367.4 mg, 1.82 mmol) and dissolved into 2 mL of distilled dichloromethane (DCM) under dry N_2

atmosphere. The temperature was reduced to -40 °C with an acetone and ice bath, after which 220 μL of 1 M $TiCl_4$ was added drop wise. When the temperature reached -20 °C, 182 μL of dichloro(methoxy)methane (Cl_2CHOMe) was added and the mixture was stirred for 2 hours at 0 °C followed by quenching with water (6 mL). The mixture was extracted with DCM (3 x 14 mL). Extracts were combined and washed with H_2O (3 x 8 mL). The organic phase was then dried with Na_2SO_4, evaporated under reduced pressure, and the resulting compound purified by flash column chromatography. The desired compound was concentrated under reduced pressure and analyzed by TLC and NMR. Compound (3) was produced in a 28.7% yield and displayed an R_f value of 0.35 in 10% MTBE/PE on TLC plates.

2.3.4 Synthesis of 1,4-dimethoxy-2-methyl-3-(1′-pentanol)-naphthalene (4)

A small RBF was charged with compound (3) (120.0 mg, 0.52 mmol) followed by 3 mL of anhydrous THF under inert atmosphere. The temperature was reduced to -70 °C using an acetone and dry ice bath after which 2 M n-butyl-lithium (650 μL) was added dropwise. The mixture turned a dark reddish color. The reaction was quenched with H_20 and the reaction turned yellow. Saturated ammonium chloride (5 mL) was added to ensure the alcohol was protonated. The mixture was extracted with diethyl ether (3 x 10 mL). Extracts were combined and washed with 1 M HCl (3 x 10 mL). The organic phase was then dried with Na_2SO_4, evaporated under reduced pressure, and product (4) was purified by flash column chromatography and analyzed by TLC and NMR. This resulted in a 60.2% yield. R_f value was 0.12 in 10% MTBE/PE.

2.3.5 Synthesis of 2-methyl-3-(1′-pentanol)-1,4-napthoquinone (5)

A small RBF was charged with compound (4) (90.0 mg, 0.31 mmol) and dissolved in 2 mL of acetonitrile. The temperature was reduced to 0 °C. CAN was added in excess (600.0 mg). The reaction was stirred for 30 min and then quenched with water. The mixture was extracted twice with diethyl ether, extracts combined, and then extracts washed with water (2x) and brine. The organic phase was then dried with $NaSO_4$, evaporated under reduced pressure, and compound (5) purified by flash column chromatography and analyzed by TLC and NMR. This resulted in a 74.4% yield. R_f value in 30% MTBE/PE was 0.52.

2.3.6 Synthesis of 2-methyl-3-(1′-oxo-n-pentyl)-1,4-napthoquinone (6)

A small RBF was charged with compound (5) (93.0 mg, 0.36 mmol) and dissolved in 5 mL DCM. To this, 100 mg of Molecular Sives (4A Powder, < 50 μm), 100 mg of sodium acetate, and 100 mg of PCC were added to the flask, respectively. The reaction was stirred for 30 min at room temperature after which the mixture was filtered though a silica-plug using diethyl ether in order to filter out non-dissolved masses. The diethyl ether was then evaporated and the crude material was purified though flash column chromatography and analyzed by TLC and NMR. This resulted in a 55.8% yield. R_f value in 10% MTBE/PE was 0.29. NMR was taken to confirm synthesis of product (6) that will now be referred to as CKA.

2.4 Reduced/oxidized spectrum of CKA

All ultraviolet-visible (UV/Vis) spectrophotometry measurements were made with a DU 600 Series spectrophotometer (Beckman Coulter, U.S.A.). CKA had previously been purified by liquid chromatography in the fully oxidized state as shown by NMR (Figs. 3.4 & 3.5) and dissolved in absolute ethanol. Reduction of

menaquinones was achieved by adding 5 μL of a freshly prepared aqueous solution of $NaBH_4$ (5 mg mL^{-1}) to 1 mL of the ethanolic CKA solution containing 5 μL of 0.5 M sodium acetate buffer, pH 5.2 (8, 31).

2.5 Polarography of CKA

The midpoint potential for the two redox-reactive keto-groups on the naphthoquinone ring of CKA were determined using a three-electrode voltammetry setup very similar to that depicted in Figure 2.1. The electrode system consisted of a potentiostat (600D Series Electrochemical Analyzer, CH Instruments, Austin, Texas) connected to a glass/carbon working electrode (W), an $Ag/AgNO_3$ reference electrode (R), and a Pt counter electrode. In an anaerobic glove box (Innovative Technology, Pure Lab, Amesbury, MA) CKA and tetrabutylammonium hexafluorophosphate were added to a final concentration of 1 mM and 100 mM, respectively. Anoxic acetonitrile was used as the solvent. Two overlapping scans were taken with a scan rate of 100 mV s^{-1} from -0.4 V to -2.1 V.

2.6 Growth inhibition of *Cba. tepidum* by CKA

In order to determine the effect of CKA on freshly inoculated cultures of *Cba. tepidum*, cultures were inoculated to 4 μg mL^{-1} of protein in Pf-7 media (2 mM sulfide plus thiosulfate or thiosulfate only) and incubated at 47 °C with 20 μmol photons m^{-2} s^{-1} of light intensity. CKA was injected into the medium prior to inoculation. Culture protein was analyzed after 24 hours of incubation. Inhibition constants (K_i) were determined by plotting the inverse of percent growth yield versus the inverse of CKA concentration. A log-scale was used for both axes and a trendline was fitted using a power function. In order to determine the inverse concentration of CKA that elicited

50% inhibition, the functions were set equal to 2 and solved. The inverse of this result gave the concentration of CKA where percent growth relative to the control was 50%: also known as K_i.

The effect of CKA on already growing cultures of *Cba. tepidum* was then determined. Cultures were inoculated to 4 μg mL^{-1} of protein in Pf-7 media (2 mM sulfide plus thiosulfate) and incubated at 47 °C with 20 μmol photons m^{-2} s^{-1} of light intensity. Cultures reached an average protein concentration of 62 μg mL^{-1} after 12 hours at which time CKA was added to a final concentration of 5, 10, 20, 40, or 60 μM. Culture protein was analyzed 12 hours post-CKA addition and the percent growth after CKA addition was calculated. The K_i from the percent growth curve was calculated as described for the freshly inoculated cultures treated with CKA except the baseline used was 62 μg mL^{-1} instead of 4 μg mL^{-1}.

2.7 CKA toxicity

The following experiment was conducted to determine if CKA exhibited bacteriostatic or bactericidal properties. Cultures were inoculated to 4 μg mL^{-1} of protein in Pf-7 media and incubated at 47 °C with 20 μmol photons m^{-2} s^{-1} of light intensity for 12 hours. At this time, cultures were either treated with CKA (60 μM) or with an equivalent volume of ethanol. Both culture conditions were performed in triplicate. Cultures were then incubated for another 12 hours. Cell viability counts were made by serial dilutions on CP plates (10, 26, 49). Plates were incubated at 47 °C with 20 μmol photons m^{-2} s^{-1} of light intensity until colonies formed (~ 3-4 days).

2.8 Fluorescence of whole cells exposed to CKA

Cba. tepidum cultures were inoculated to 4 μg mL^{-1} in Pf-7 media and incubated at 47 °C with 20 μmol photons m^{-2} s^{-1} of light intensity until cells reached mid-log phase (~16-18 hours). CKA (60 μM) was added to cultures and an equivalent volume of ethanol was added to the control cultures. Both culture conditions were performed in triplicate. After a 2-hour incubation time, the BChl *c* concentration of each culture was determined. In an anaerobic chamber (Coy Laboratories Inc., Grass Lake, MI) whole cell samples from each culture were standardized to a BChl *c* concentration of 5 μg mL^{-1} with sulfide-free Pf-7 and anaerobically transferred to screw capped fluorometer cuvettes (Sterna). Cells were kept in the dark for one-hour immediately prior to fluorescence measurement. The voltage was set to 905 Volts and the bandpass set to 4 nm with a scan rate of 2 nm sec^{-1} (Aminco Bowman Series 2 Luminescence Spectrometer). The excitation wavelength was set to 450 nm and an emission scan was taken from 700 nm to 860 nm (38).

2.9 Rate of sulfide uptake of CKA treated and untreated whole cells

Cells were cultured as previously described (10, 50) for 16 hours and harvested in mid-log phase by centrifugation (10 min, 10,000 x g, 4 °C). Cells were then washed (3 x 10 mL) and suspended in 20 mL anoxic 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4 inside an Anaerobic Chamber (Coy Laboratories Inc., Grass Lake, MI). Rates of sulfide uptake by whole cells were measured using voltammetry. The electrode system consisted of a potentiostat (DLK-60, Analytical Instrument Systems, Inc., Flemington N.J.) connected to an Hg-plated Au working electrode (W), an Ag/AgCl reference electrode (R), and a Pt counter electrode (C) (Fig. 2.1). A DAQCard-1200 (National Instruments) connected the

potentiostat to the computer (function generator). The jacketed electrochemical cell was heated to 44 $^{\circ}$C and the amount of light entering the cell was calibrated to 5 μmol photons m^{-2} s^{-1} of light intensity. The electrochemical cell was filled with 20 mL of 100 mM HEPES pH 7.4 and bubbled with argon for 10 min to ensure anoxic conditions. Sulfide was added to a final concentration of 50 μM. Washed whole cells were immediately added to a final protein concentration of 5 mg L^{-1}. Cyclic voltammetry scans were taken every 9 sec for 8 min with a scan rate of 2000 mV s^{-1} from -0.1 V to -1.8 V with continuous slow stirring (33). Scans were analyzed using the DLK-60 analysis software provided. For testing the effect of CKA on sulfide uptake, CKA was added at a concentration of 4.7 μM immediately before the introduction of whole cells.

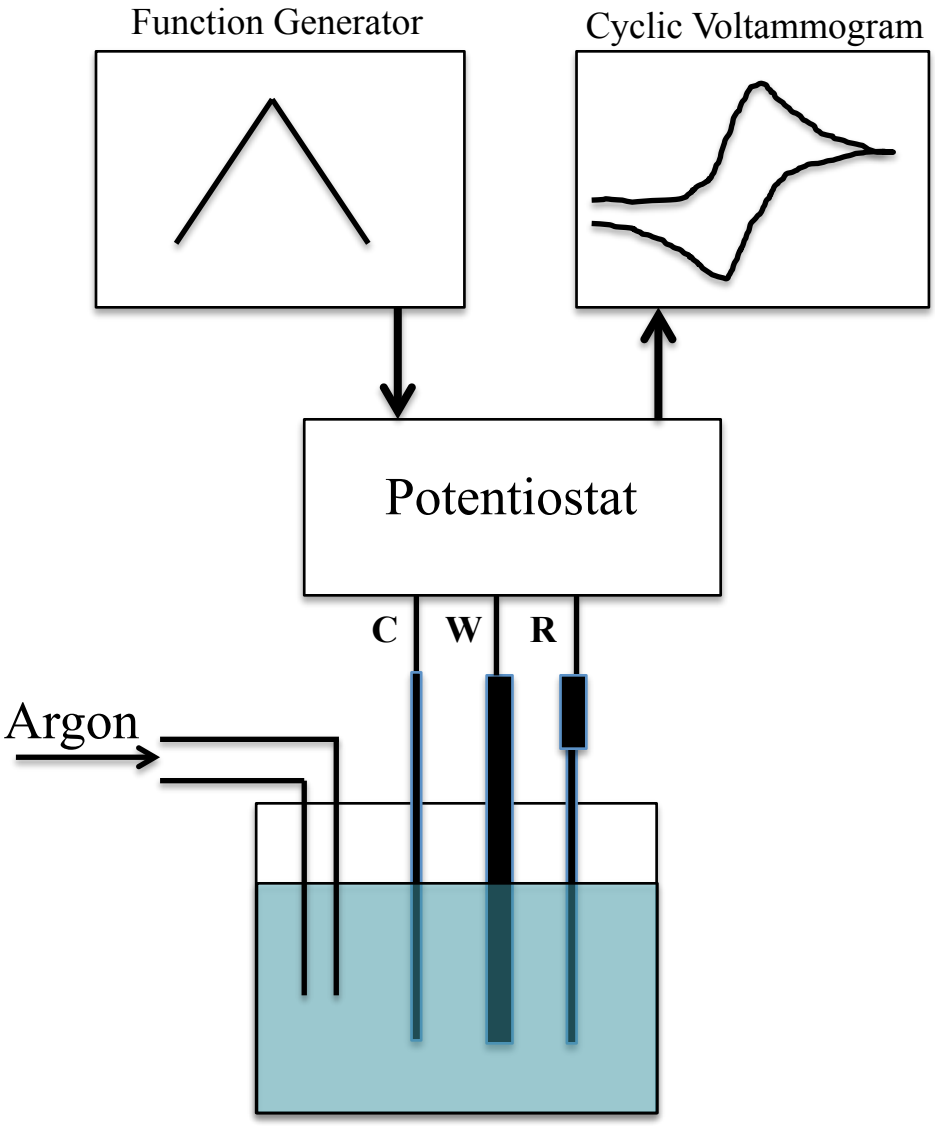


Figure 2.1 Diagram of electrode system used for cyclic voltammetry measurements.

2.10 Localization of CKA within *Cba. tepidum* cells

Cells were cultured as previously described (10, 50) in 100 mL bottles for 16 hours at which point CKA was added to a final concentration of 100 μM. An equivalent volume of absolute ethanol (200 proof, Fisher Scientific) was added to

control cultures. Cultures were incubated for an additional two hours before being harvested in mid-log phase by centrifugation (30 min, 10,000 x g, 4 °C) in 20 mL and 80 mL fractions with Nalgene 30-mL Oak Ridge centrifuge tubes (Thermo Scientific, Rochester, NY) or Nalgene 250-mL Beckman centrifuge tubes. The spent media from the 20 mL culture sample was kept at 4 °C for future analysis. Pellets from both volume fractions were washed with 20 mL of 100 mM HEPES pH 7.4. Pellets from the 20 mL volume fractions were stored at 4 °C for future analysis. Pellets from the 80-mL fractions were suspended in 5 mL of 100 mM HEPES pH 7.4, transferred to 15-mL conical falcon tubes, and sonicated (5x, output setting 5, 50% duty cycle, 1-2 min intervals) with cooling on ice between runs. Following sonication, suspensions were transferred to 30-mL Oak Ridge centrifuge tubes and cell debris was removed by centrifugation (10 min, 8,000 x g, 4 °C). Crude lysate (600 μL) was extracted with diethyl ether (1x, 10 mL; 4x, 5 mL) for CKA analysis.

The remaining crude lysate was separated into cytosol (cytoplasm and periplasm), chlorosome, and membrane fractions. Crude lysate was transferred to 1.5 mL Bechman polyallomer microcentrifuge tubes and centrifuged (1 hr, 53,000 rpm, 4 °C) using an ultracentrifuge (Optima Max, Beckman Coulter). The cytosolic fraction (supernatant) was transferred to a labeled tube. The membrane pellet was suspended in 500 μL TE Buffer (10 mM Tris, 2 M NaSCN, 5 mM EDTA, pH = 7.5) and washed via centrifugation (30 min, 53,000 rpm, 4 °C). Pellets were suspended in 1 mL of TE Buffer. Pellet was sonicated briefly (3-4 pulses) on a power setting of 1 if pellet wouldn't suspend by grinding with a small closed glass tip.

A sucrose gradient was used to separate chlorosomes from membranes (15). In a Beckman ½ x 2" UltraClear tube, 0.9 mL of each sucrose concentration was gently

added to the tube in the following order (47%, 37%, 27%, 17%, and 7%). The membrane suspension was then placed on top of the 7% sucrose layer and centrifuged (18 hrs, 215,000 x g, 4 $^{\circ}$C). The chlorosomes formed a dark band about halfway down the tube while the remaining membranes formed a hard pellet at the bottom. The chlorosome fraction was harvested in a minimal volume and the sucrose diluted with water (20 mL). Chlorosomes were centrifuged (3 hrs, 13 x g, 4 $^{\circ}$C) in order to pellet chlorosomes. The chlorosome and membrane fractions were harvested by decanting off the remaining sucrose gradient or supernatant.

Diethyl ether (10 mL) was added to the whole cell pellets from the 20 mL culture volumes and incubated for 20 min with mild vortexing to suspend pellet. The suspension was centrifuged to pellet cell debris (40 min, 13k x g, 4 $^{\circ}$C). Diethyl ether (9.5 mL) was taken and saved for future CKA analysis. Spent media and cytosolic fractions were extracted with 10 mL of diethyl ether (1x) then with 5 mL of diethyl ether (2x). Membrane and chlorosome fractions were suspended in 6 mL of diethylether/methanol (9:1, v/v) for 10 min at room temperature. In a fresh 50-mL conical tube, diethyl ether was evaporated to dryness and the residual material dissolved in 2 mL of ethanol, filtered through a 0.2-μm nylon membrane (Pall Life Sciences, Ann Harbor, MI), and run on HPLC (Section 2.2.2).

2.11 Effect of CKA on *Escherichia coli*

E. coli strain DH5 α was anaerobically cultivated in 100 mL bottles using modified M9 minimal media (35) supplemented with thiamine (10 μg mL^{-1}). Instead of using glucose, the non-fermentable substrates succinate, acetate, and malate were used to ensure that cell growth only proceeded via anaerobic respiration. All carbon source types were added to a final concentration of 0.4% by weight. Nitrate was added

as a terminal electron accepter to a final concentration of 2.12 g L^{-1}. CKA was either added to cultures at inoculation, after two hours of growth, or not at all. Protein analysis by Bradford assay (39) was used to generate a growth curve over 35 hours.

2.12 Effect of CKA on *Leishmania tarentolae*

The *L. tarentolae* strain p10 (Jena bioscience, Germany) was cultivated in 100-mL flasks with sponge stoppers using brain heart infusion (BHI) media supplemented with hemin as previously reported (21, 36). BHI (Dickinson and Company, Sparks, MD) was added to a concentration of 37 g L^{-1}. Hemin (Sigma-Aldrich, Netherlands) was added to a final concentration of 5 mg L^{-1} from a 500x stock that was prepared in 0.05 N sodium hydroxide. Ampicillin and streptomycin (Invitrogen, USA) was supplemented to 50 units mL^{-1} to deter bacterial contamination. Inoculated cultures were cultivated at 26 $^{\circ}$C in a shaker at 179 rpm. CKA was either added to cultures at inoculation, at 24 hours, or not at all. Protein analysis by Bradford assay (39) was used to generate a growth curve over 96 hours.

2.13 Discovering genes that may be involved in the CK biosynthetic pathway

A list of putative genes involved in CK synthesis was generated in the Integrated Microbial Genomes (IMG) database by searching for homologs of genes in *Cba. tepidum* in the genome of *Leishmania major* strain Friedlin (a eukaryote that has been reported to produce chlorobiumquinone) (5) and subtracting the genomes of all *E. coli* strains and *Chloroflexus aurantiacus* via pre-computed BLASTP searches (Table 1). *C. aurantiacus* is a filamentous-bacterium that contains the unique light-harvesting apparatus called the chlorosome, just like *Cba. tepidum*, but does not synthesize CK (19).

Chapter 3

RESULTS

3.1 Menaquinone-7 pool size under different sulfide concentrations

MK-7 and CK were extracted from cultures in order to determine if there was any correlation between culture sulfide concentration and cellular biosynthesis of MK-7 and CK. Previously, it had been reported that CK concentrations increase in *C. ethylicum* when exposed to sulfide containing medium (44). Extraction of quinones occurred two hours after the addition of sulfide (6 mM, 2 mM, or 0 mM) to mid-log phase cultures growing with thiosulfate as the sole electron donor. The areas under peaks in typical chromatography chromatograms were analyzed (Fig. 3.1) from three biological replicates. The results show that the size of the MK-7 pool is not statistically different under the different sulfide concentrations tested (Fig. 3.2). Originally, the peak at 4.1 min was thought to be CK. However, subsequent analysis indicated that this peak is also produced by extractions of purified biogenic elemental sulfur (data not shown). This peak increases when more sodium sulfide is added to the medium; as would expected since elemental sulfur is an extracellular intermediary metabolite in the complete oxidation of sulfide to sulfate by *Cba. tepidum*. Abiotically produced elemental sulfur was not analyzed.

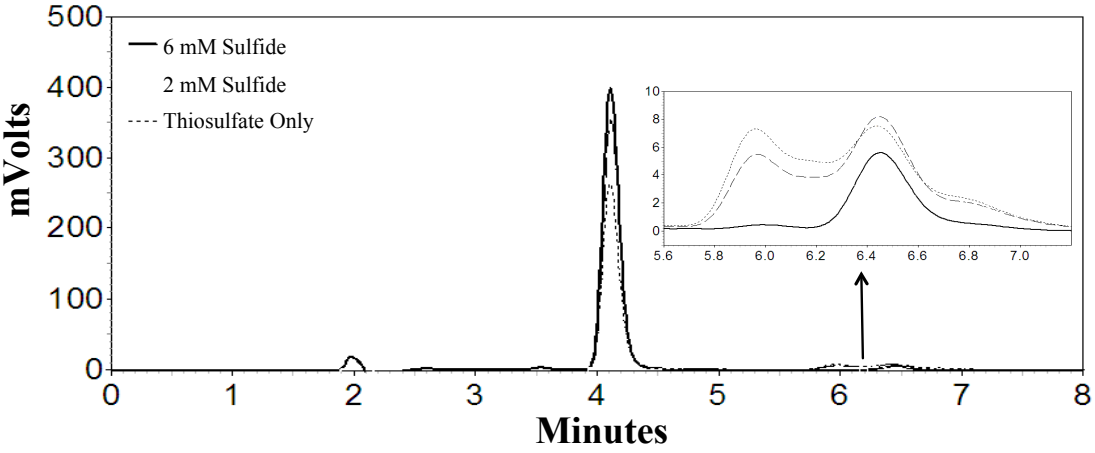


Figure 3.1 Representative HPLC chromatograms of hexane extracts from *Cba. tepidum* re-dissolved in ethanol. Sulfide was added to a final concentration of 6 mM, 2 mM, or 0 mM two hours before harvesting cells. The inset focuses on the MK-7 peak. The peak at 4.1 min was determined to be biogenic sulfur.

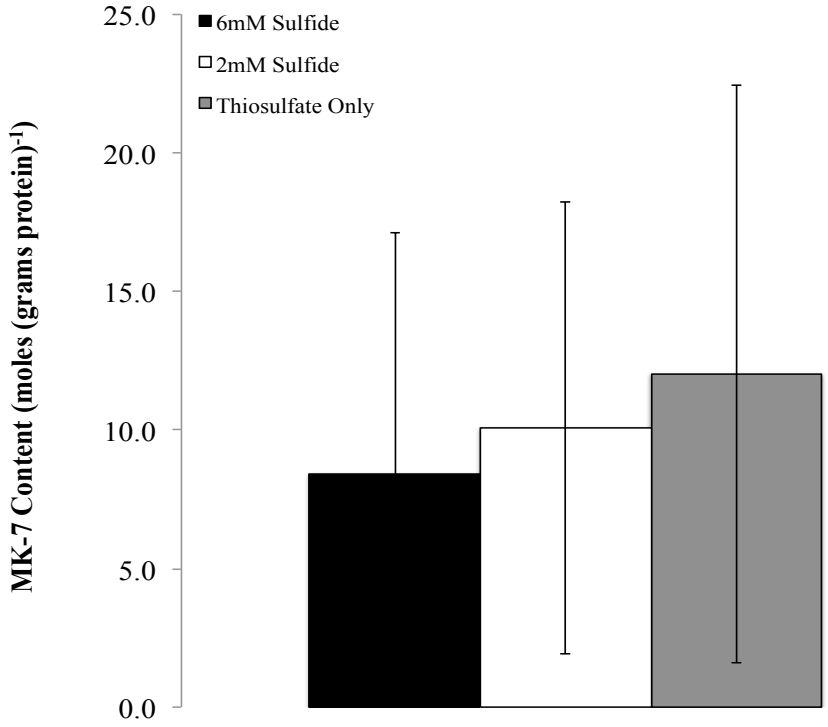


Figure 3.2 Comparison of MK-7 pools under different sulfide concentrations in wild type *Cba. tepidum*. Sulfide was added to cultures two hours before harvest and MK-7 content analyzed by HPLC.

3.2 Synthesis of a CK analog: 2-methyl-3-(1′-oxo-n-pentyl)-1,4-naphthoquinone

The original motivation for creating a CK analog was to use it for *in vitro* assays to determine if it could be used as an electron accepter for SQR. Another motivation was to possibly use it for rescuing a CK deficient mutant if this mutant strain displayed a severe growth phenotype. A CK analog (MOON) had already been reported in the literature and had been used as an electron accepter for ferricyanide reductase (5). Unfortunately, after attempting the synthesis of MOON (5) three times without success, the synthetic route described was examined in consultation with the Taber group in the Chemistry Department and deemed to be inaccurate. Therefore, synthesis of an alternative analog was planned and executed while preserving the basic structure of the 1,4-naphthoquinone and 1′-oxo group.

The 5-step synthesis (Section 2.3) gave an overall yield of 5.7%. The first reaction consisted of a 2-step one-pot reaction involving the reduction of menadione to the hydroquinone using sodium dithionite followed by alkylation of the phenol groups using dimethyl sulfate (42). The second step utilized a Friedel-Crafts formylation using dichloro(methoxy)methane as the carbonyl donating group (28). The third step consisted of a homologated addition on the formyl group through nucleophilic attack by n-butyl lithium (40). The fourth step oxidized the ether functional groups back to the ketones using cerium (IV) ammonium nitrate (CAN) (30). The final step fully oxidized the alcohol group at the 1′-position to a ketone using pyridinium chlorochromate (PCC) as the oxidant (11). See Figure 3.3 for reaction scheme. The

resultant compound 2-methyl-3-(1′-oxo-n-pentyl)-1,4-napthoquinone was confirmed by NMR (Figs. 3.4 & 3.5).

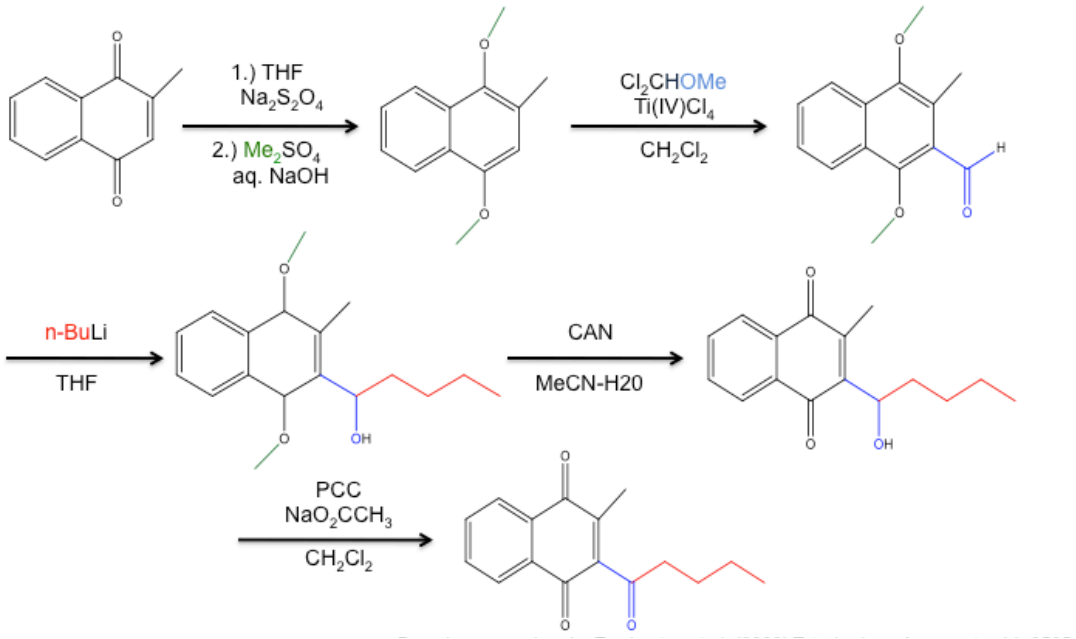


Figure 3.3 Reaction scheme for the synthesis of 2-methyl-3-(1'-oxo-n-pentyl)-1,4-napthoquinone (CKA).

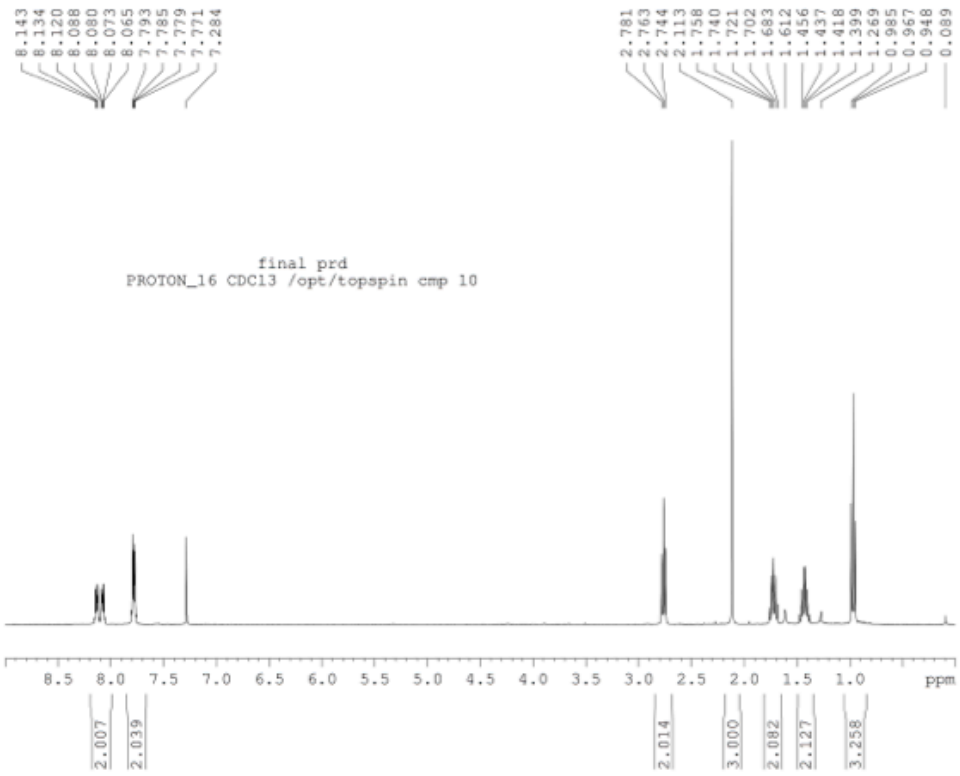


Figure 3.4 ^{1}H NMR spectrum of CKA (400 MHz, $CDCl_3$). δppm 0.95-0.98 (t, 3H), 1.40-1.46 (m, 2H), 1.68-1.76 (m, 2H), 2.11 (s, 3H), 2.74-2.78 (t, 2H), 7.77-7.79 (d, 2H), 8.06-8.14 (dd,2H)

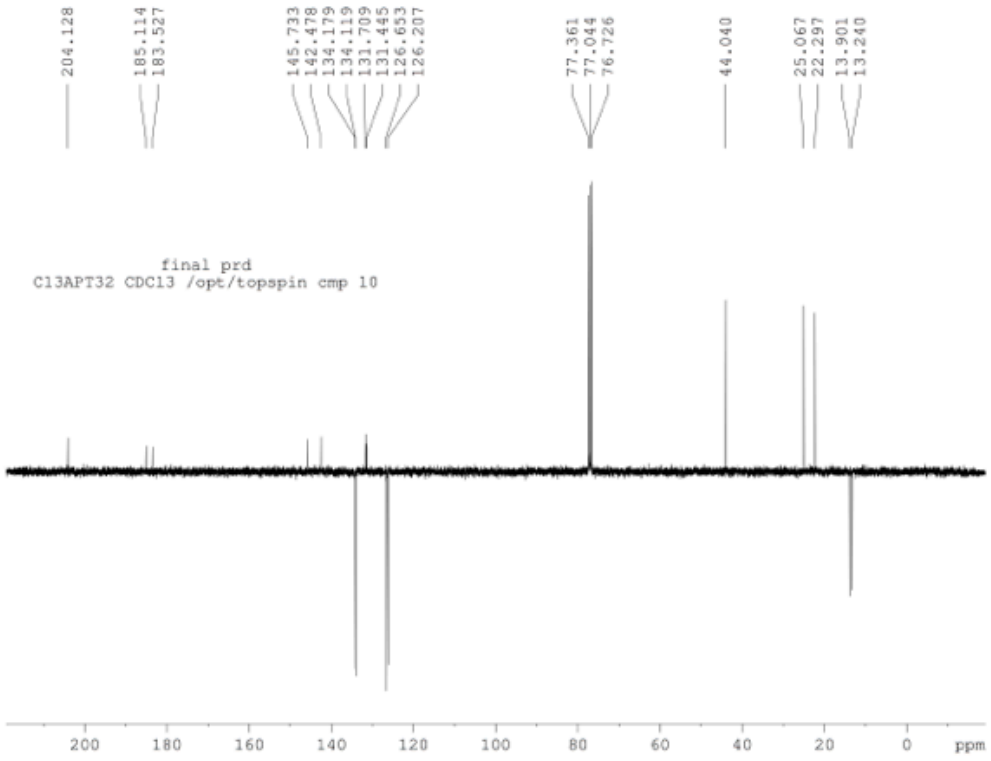


Figure 3.5 ^{13}C NMR spectrum of CKA (101 MHz, $CDCl_3$). δppm (up) 22.29, 25.06, 44.04, 131.44, 131.71, 142.47, 145.73, 183.53, 185.11, 204.13, (down) 13.24, 13.90, 126.21, 126.65, 134.12, 134.18

3.3 Reduced/oxidized spectrum of CKA

An oxidized/reduced spectrum of CKA was taken (Fig. 3.6) and was shown to have electronic properties similar to that of a "polar menaquinone" found in *C. thiosulphatophilum* and *C. ethylicum* (44), later called Quinone 1 (18), which is hypothesized to be a metabolic precursor to CK. The fully oxidized CKA had a maximal absorption at 253 nm and a lower maximum at 336 nm (Fig 3.6, solid line). The reduced state of CKA showed a maximum at 242 nm and a lower maximum at 334 nm (Fig 3.6, dashed line). The absorbances at the shorter wavelengths are due to the benzenoid rings and the absorbances at the longer wavelengths are due to the isoprenoid tail (44).

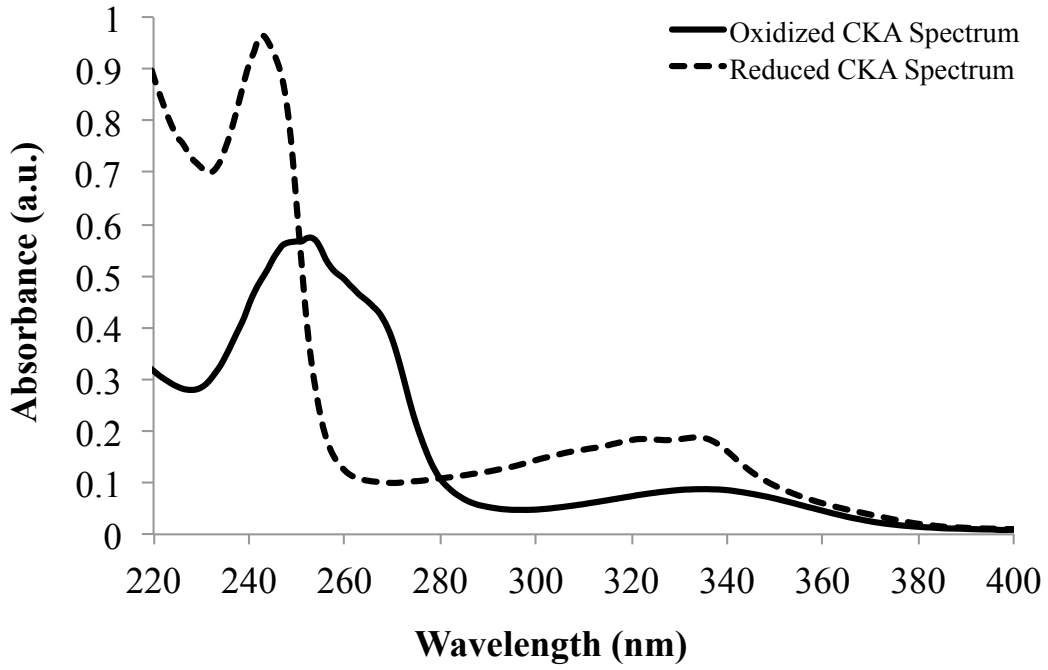


Figure 3.6 Oxidized and reduced UV-visible spectra of a buffered ethanolic solution of CKA. CKA in the oxidized form (solid line) and after reduction with $NaBH_4$ (dashed line).

3.4 CKA has a more negative midpoint potential than CK and MK-7

Cyclic voltammetry was used to obtain the oxidation-reduction midpoint potential of CKA in order to compare it to the reported value for CK. The reduction and subsequent oxidation of CKA was reversible as is observed with many quinones (37). In aqueous buffer, quinones undergo one-step two-electron reduction (24). In nonaqueous solvents, quinones undergo a two-step reduction (24). The first step is a one-electron transfer from the quinone to the semiquinone and the second step is a one-electron transfer from the semiquinone to the quinone dianion (24). The midpoint potential for the two reduction steps of CKA, one at -0.94 V and another at -1.66 V, were empirically determined utilizing an $Ag/AgNO_3$ reference electrode in acetonitrile (Fig. 3.7). The midpoint potentials for the two reduction steps of MK-7, one at -0.91 V and another at -1.62 V, were obtained for comparison with values reported in the literature (data not shown). The molar ratio between the electrolyte and analyte were 100:1, respectively.

In aqueous buffer, the one-step two-electron reduction of MK-7 has been reported to occur at -80 mV (44) in reference to the normal hydrogen electrode. The conversion constant between the $Ag/AgNO_3$ reference electrode in acetonitrile and the normal hydrogen electrode was calculated to be 1.54 V by subtracting the literature midpoint potential of MK-7 in aqueous buffer from the semiquinone/dianion midpoint potential of MK-7 determined here. Therefore, in aqueous buffer, CKA would be predicted to have a midpoint potential of -117 mV. The reported midpoint potential of CK is 39 mV (44). The result that CKA is more reducing (has a more negative midpoint potential) than MK-7 was unexpected and more experimental evidence is needed to confirm that this is indeed correct.

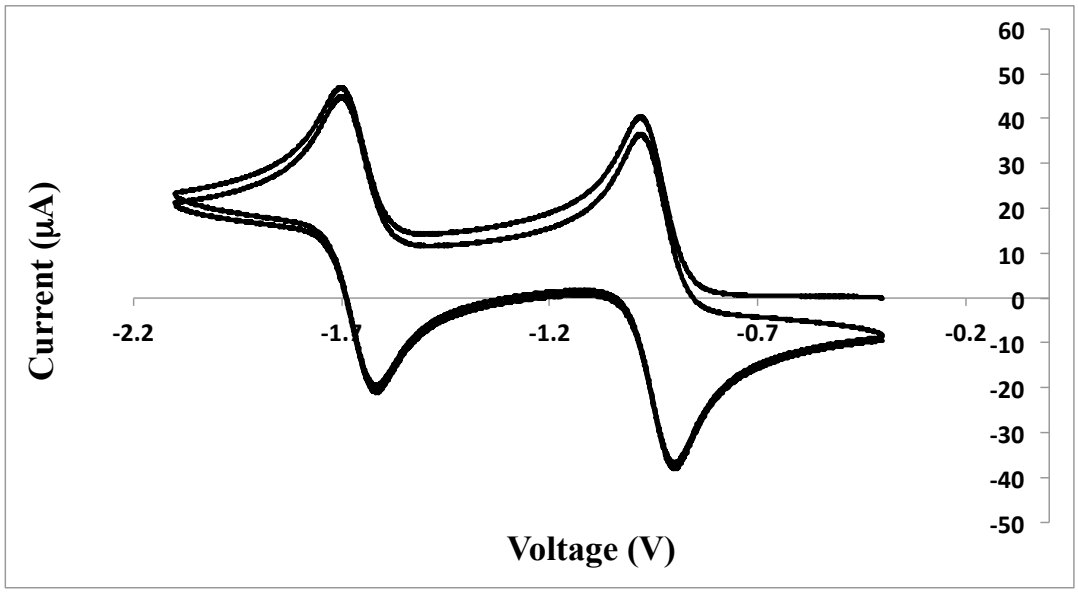


Figure 3.7 Cyclic voltammetry scan of CKA in anoxic acetonitrile.

3.5 Physiological effect of CKA on *Cba. tepidum*

Physiological effects of the newly synthesized CKA on *Cba. tepidum* were determined using growth studies, voltammetry, and fluorescence. Results from these studies are outlined below. It was hypothesized that if CKA directly competed with MK-7 for the active site of SQR that the rate of sulfide uptake might be inhibited when cells were treated with CKA. Voltammetry was used to test how the uptake of sulfide changed when CKA was added (Section 3.5.3). On the other hand, if CKA acted as a quencher during photosynthetic energy transfer it was hypothesized that cellular fluorescence would be decreased (Section 3.5.4).

3.5.1 CKA inhibits growth of *Cba. tepidum*

The effect of CKA on *Cba. tepidum* growth was tested by treating cultures with CKA either at inoculation or during mid-log phase growth (12 hours after inoculation). Cultures treated at inoculation were grown in either Pf-7 media (2 mM

sulfide and thiosulfate) or Pf-7 media without sulfide (thiosulfate only) for 24 hours. When treated at inoculation, the 2 mM sulfide and thiosulfate only cultures gave K_i values of 4.6 μM and 4.8 μM, respectively (Fig. 3.8). This is an important result because it indicates that the growth inhibition of CKA is not dependent on the use of sulfide as an electron donor for photosynthesis. Cultures treated in mid-log phase were grown for another 12 hours after the addition of CKA before being harvested. A K_i value of 12.6 μM was calculated for CKA showing that the K_i of CKA increases with increasing biomass (Fig. 3.9).

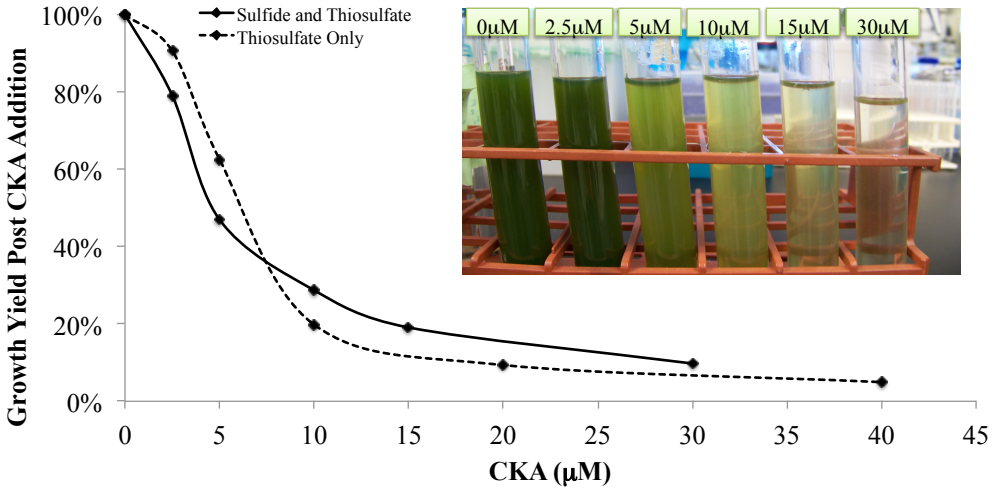


Figure 3.8 Effect of CKA on freshly inoculated cultures. Cultures with thiosulfate only (dashed line) and cultures with sulfide and thiosulfate (solid line) were inoculated into tubes containing varying concentrations of CKA. Diamonds (◆) indicate the final growth yield observed at a given CKA concentration.

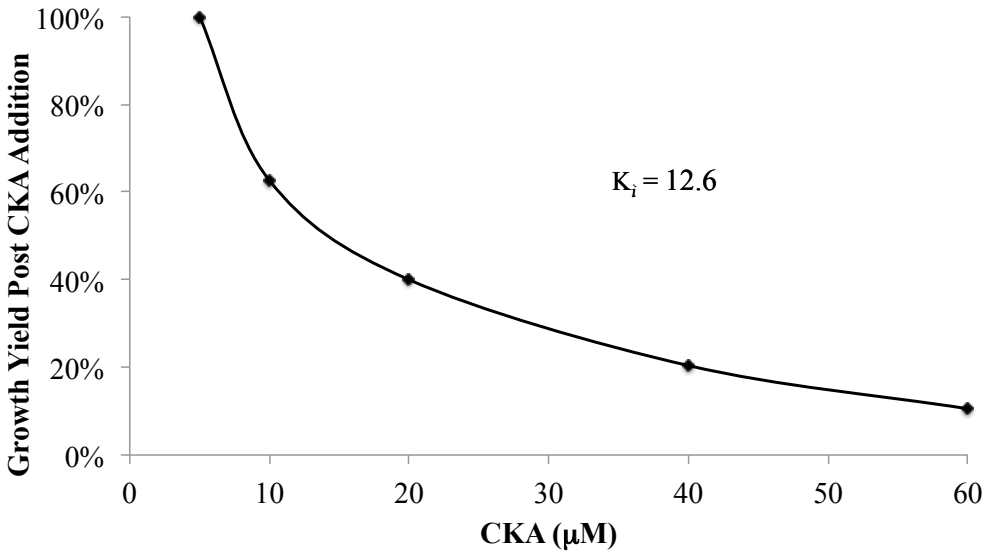


Figure 3.9 Effect of CKA on mid-log phase cultures. Diamonds (◆) indicate the final growth yield observed at a given CKA concentration added 12 hours after inoculation.

3.5.2 CKA exhibits bacteriostatic toxicity

Cell viability counts were performed after exposing cultures to CKA as described in methods (Section 2.7). Before treatment with CKA, cell viability was 1.1 x 10^8 ± 3.0 x 10^7 cells mL^{-1}. Twelve hours after CKA treatment the cell viability was 1.2 x 10^8 ± 3.0 x 10^7 cell mL^{-1}. The results show that cell viability does not change after exposure to CKA meaning that CKA exhibits bacteriostatic toxicity (Fig. 3.10).

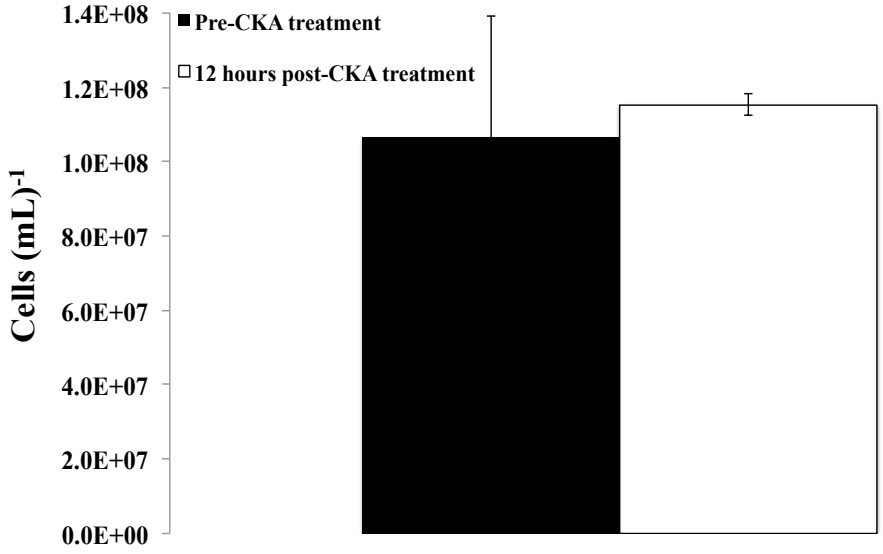


Figure 3.10 Cell viability of *Cba. tepidum* when exposed to CKA. Viable cells were counted immediately before treatment with CKA and 12 hours post-treatment. Cell counts were averaged from biological triplicates.

3.5.3 CKA does not affect rate of sulfide uptake by whole cells

Sulfide uptake by whole cells was determined using cyclic voltammetry to follow sulfide concentration over time in cell suspensions using a voltammetry setup as described in methods (Section 2.9). Cells (5 μg mL^{-1} protein) were exposed to sulfide (50 μM) in a temperature-controlled electrochemical cell and either treated with CKA (4.7 μM) or an equivalent volume of ethanol. Voltammetric scans were taken over 8 min to track sulfide concentrations in the electrochemical cell over time. The concentration of CKA used here should lead to a 50% reduction in growth yield. If this effect were due to CKA interfering with sulfide oxidation, we would expect to see a significant change in sulfide uptake rate in CKA treated cells. The average rate of sulfide uptake in the ethanol treated whole cells was 27.9 ± 6.3 mM (mg protein *

mL)$^{-1}$. The average rate of sulfide uptake in the CKA treated whole cells was 24.9 ± 6.4 mM (mg protein * mL)$^{-1}$. The decrease was found to be statistically insignificant between the CKA treated and ethanol treated samples (Fig. 3.11). This result most likely implies that CKA is not interacting with SQR and is inhibiting cellular growth by a different mechanism.

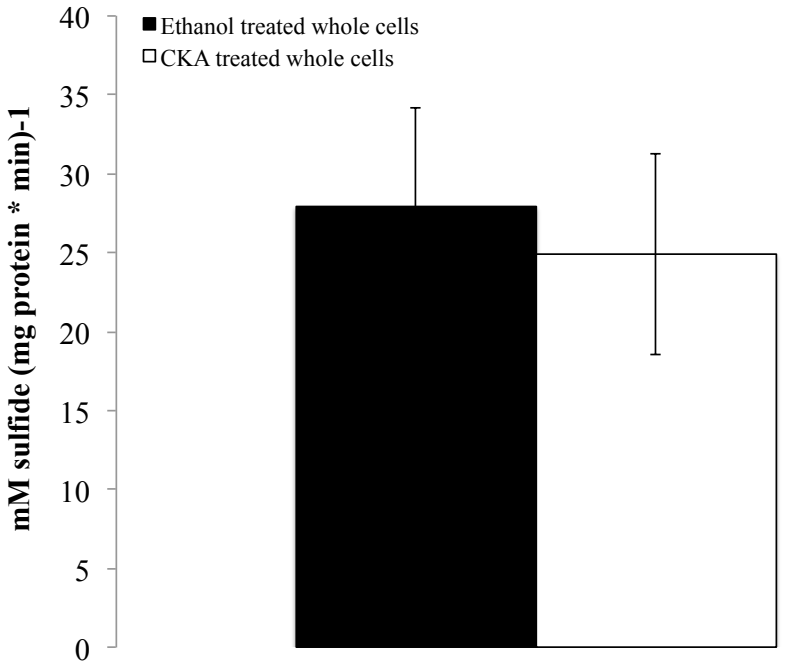


Figure 3.11 Sulfide uptake by whole cells treated with CKA or ethanol. Scans were averaged from biological triplicates.

3.5.4 CKA decreases fluorescence emitted by *Cba. tepidum* whole cells

It has been shown that oxidized quinones are able to quench fluorescence from excited bacteriochlorophylls (27). CKA is a naphthoquinone isolated in the fully oxidized state and it was hypothesized that CKA may quench fluorescence from chlorosomes when excited by light. By exposing growing cultures to CKA and

analyzing the fluorescence of the anaerobically diluted cultures, it was shown that CKA does in fact inhibit chlorosome fluorescence (Fig. 3.12). The fluorescence maxima at 771 nm and 805 nm correspond to the emission wavelengths of aggregated Bchl *c* in chlorosomes and Bchl *a* in the baseplate and FMO protein, respectively (16, 27, 38). The addition of CKA to the culture as described in the methods resulted in a 69.4% and 74.3% decrease in the fluorescence of Bchl *c* and Bchl *a*, respectively. It has been hypothesized that the decrease in fluorescence observed is the result of inhibiting energy transfer from BChl *c* to the reaction center complex (1, 16, 18, 19, 27). Therefore, since CKA was isolated as a fully oxidized quinone, it is hypothesized that CKA's mechanism of action is inhibiting energy transfer to the reaction center complex and that the resultant phenotype of growth inhibition results from this effect. This mode of interaction with the chlorosome may explain why CKA has a bacteriostatic effect on *Cba. tepidum*, since inhibiting the ability of the cells to generate reducing equivalents for biomass production would be expected to suppress cell growth and not be bactericidal.

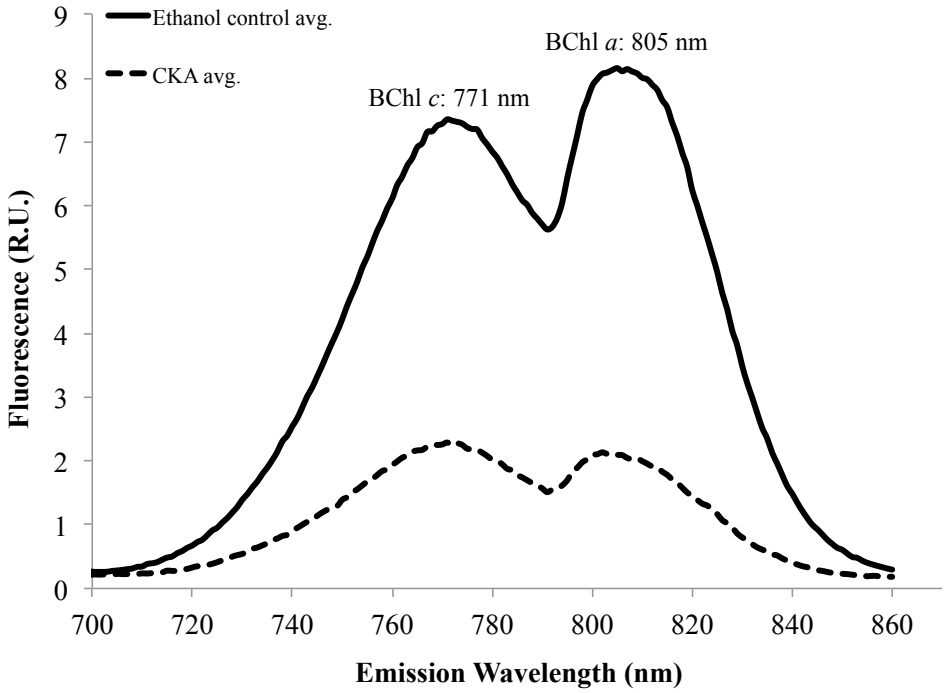


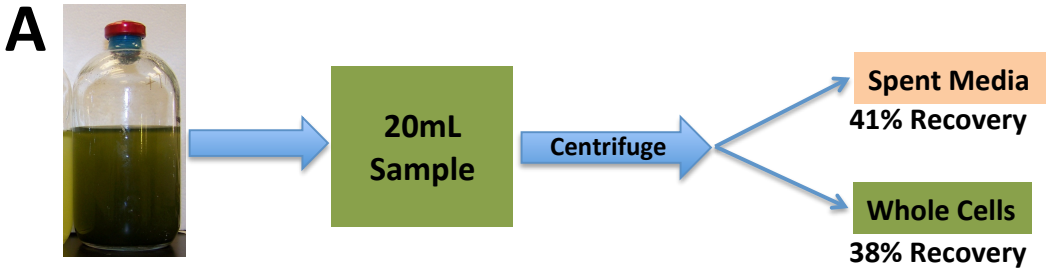
Figure 3.12 Fluorescence spectrum of cells treated with CKA or ethanol. Scans were averaged from biological triplicates.

3.6 Localization of CKA to any specific cellular fraction was inconclusive

Fractionating cultures into whole cells, spent media, crude cell lysate, cytosolic, membrane, and chlorosome fractions tested the localization of CKA (Section 2.10). Initially, 20 mL of post-CKA addition culture was centrifuged to separate whole cells from spent media. Percent recovery of CKA in these two fractions was calculated in reference to the amount of CKA initially added to the culture. From averaged biological triplicates, 38% and 41% of total CKA added two hours before harvest was found in the spent medium and whole cell fractions, respectively (Fig. 3.13A). An average of 79% recovery from cultures was obtained two hours post CKA addition.

Whole cells from an 80 mL sample of CKA treated culture were fractionated into crude cell lysate via sonication and centrifugation (Fig. 3.13B). Sonication efficiency was calculated to be near 100% from protein assays although a cell debris pellet was formed upon centrifugation of the sonicated sample. A 600 µL sample of the crude cell lysate was taken for CKA extraction. The crude cell lysate showed a 54.7% recovery of CKA in respect to the amount of CKA recovered from the extracted whole cell fraction. The crude cell lysate (3 mL) was further fractionated into cytosolic, membrane, and chlorosome fractions (Fig. 3.13B). The amount of CKA recovered from the cytosolic, membrane, and chlorosome fractions were 5.8%, 1.9%, and 0%, respectively, relative to the amount of CKA recovered from the whole cell fraction.

The result that CKA mostly localizes to the cytoplasm was unexpected. It may be possible that CKA is becoming reduced *in vivo* when it gets near the chlorosome. The absorbance of reduced CKA is 3.7 times less at the wavelength used to quantify samples (270 nm) than the fully oxidized form that was added to cells. Therefore, if CKA is being reduced inside the cells, then the percent recovery of CKA from cellular fractions would be artificially low.



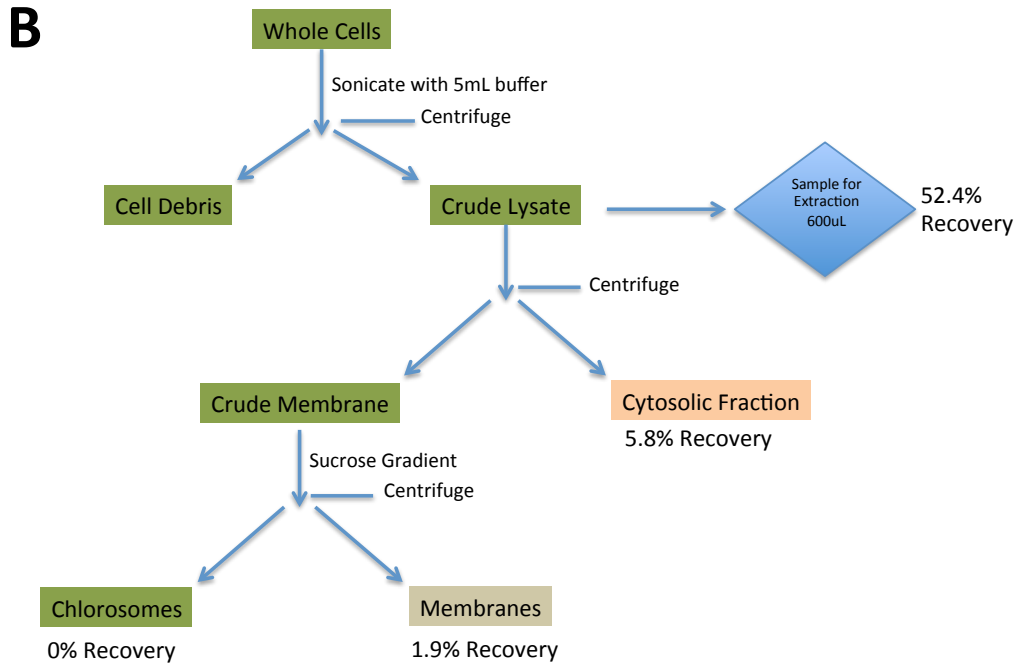


Figure 3.13 Localization and percent recovery of CKA from cell culture fractions. Percent recovery of CKA from whole cells and spent media (A). Localization and recovery of CKA upon further fractionation of whole cells (B).

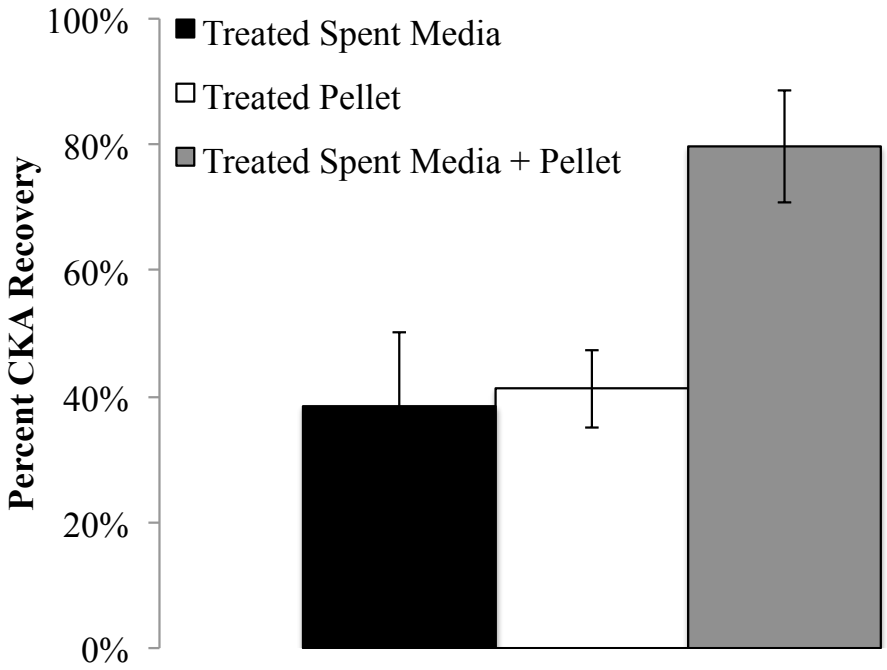


Figure 3.14 Percent recovery of CKA from spent media, whole cells, and their combined recoveries. Data was collected from biological triplicates.

3.7 CKA does not affect growth of *Escherichia coli*

The following experiment was conducted to test whether growth inhibition is restricted to organisms that possess CK. For this experiment the facultative anaerobe *E. coli* was chosen since it can perform cellular respiration under anaerobic conditions if given a non-fermentable carbon source and an electron acceptor. Cultures of *E. coli* were grown on M9 minimal media using succinate, fumarate, and malate as carbon substrates and nitrate as an electron acceptor to ensure growth could only be obtained through utilization of the electron transport chain that utilizes quinone pools for electron transfer. *E. coli* has three main quinones: ubiquinone-8 (UQ-8), menaquinone-8 (MK-8), and demethylmenaquinone-8 (DMK-8) (47). UQ-8 is used exclusively when oxygen is present for aerobic respiration. Menaquinones are utilized

when electron accepters of lower redox potential (i.e. nitrate) is all that is available (3). By providing electron acceptors under anaerobic conditions, *E. coli* is forced into utilizing menaquinones for anaerobic respiration. These quinones are very similar to chlorobiumquinone except MK-8 and DMK-8 in *E. coli* lack the 1′-oxo group. If CKA is a general inhibitor of menaquinone dependent electron transport then the addition of CKA to nitrate respiring *E. coli* should inhibit growth.

Cultures were treated with CKA to a final concentration of 40 μM either at inoculation or 2 hours after inoculation. Cultures without the addition of CKA were used as positive controls. Cultures were performed in duplicate. The untreated culture had a doubling time of 3.2 hours, while the cultures treated with CKA at inoculation and two hours after inoculation had doubling times of 3.2 hours and 3.1 hours, respectively. Thus, there was no difference in the growth rate between the treated and untreated cultures (Fig. 3.15) proving that CKA does not exhibit broad-spectrum toxicity and does not compete for the same active sites as menaquinones.

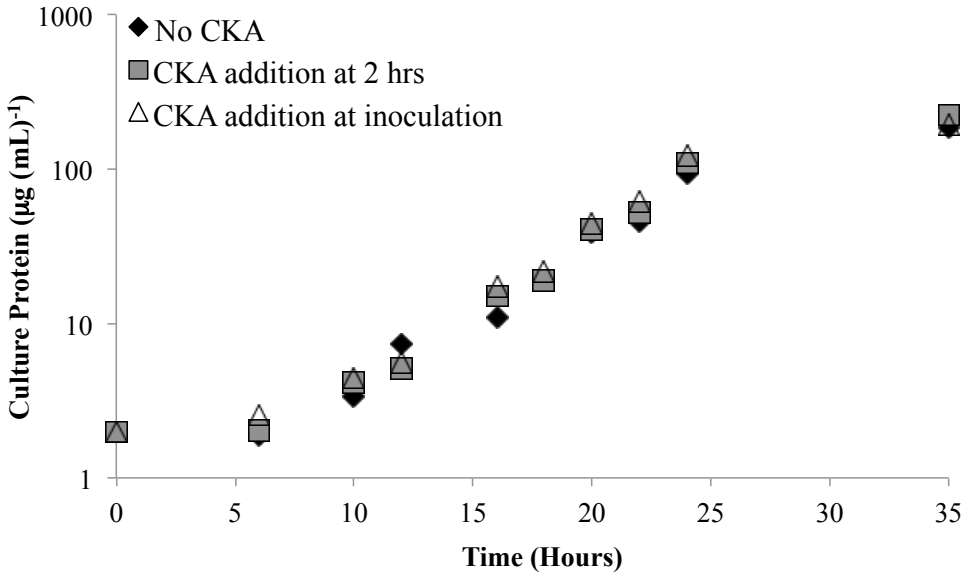


Figure 3.15 *E. coli* treated with 40 μM CKA. Cultures were treated either at inoculation, 2 hours after inoculation, or not at all. Points represent the average of biological duplicates.

3.8 CKA slightly affects growth of *Leishmania tarentolae*

It has been reported that the major isoprenoid quinone in the parasitic protozoan *L. donavani* promastigote is CK although UQ-9 is also present at a comparable amount (5). This is substantial because it is the first evidence that shows that chlorobiumquinone is not exclusively found in the phylum *Chlorobi* (5, 44). Some protozoan parasites of the genus *Leishmania* are known to cause human visceral diseases with *L. donavani* being able to cause kala-azar, a lethal form of human visceral leishmaniasis (2). Therefore, we chose to test the ability of CKA to affect growth on *L. tarentolae*: a non-human pathogenic trypanosomatid protozoan that is a parasite of the gecko *Tarentolae annularis* (21) as recommended by Dr. Colin Thorpe (University of Delaware). Cultures were treated with CKA to a final concentration of 40 μM either at inoculation or 24 hours after inoculation. Cultures without the addition

of CKA were used as positive controls. Cultures were performed in duplicate. The results show the CKA slightly inhibits the growth of *L. tarentolae* (Fig. 3.16). Control cultures of *L. tarentolae* only treated with ethanol had a doubling time of 14.7 hours. *L. tarentolae* cultures treated with CKA 24 hours after inoculation had a doubling time of 19.4 hours while cultures treated with CKA at inoculation had a doubling time of 26.6 hours. Quinone extracts from *L. tarentolae* and *Cba. tepidum* where CKA was not added were compared (Fig. 3.17). However, it was not concretely determined which of the peaks from the hexane extraction from *L. tarentolae* is CK. Therefore, it cannot be said with confidence whether or not *L. tarentolae* produces CK making the mode of action at which CKA inhibits the growth on *L. tarentolae* indeterminate at this time.

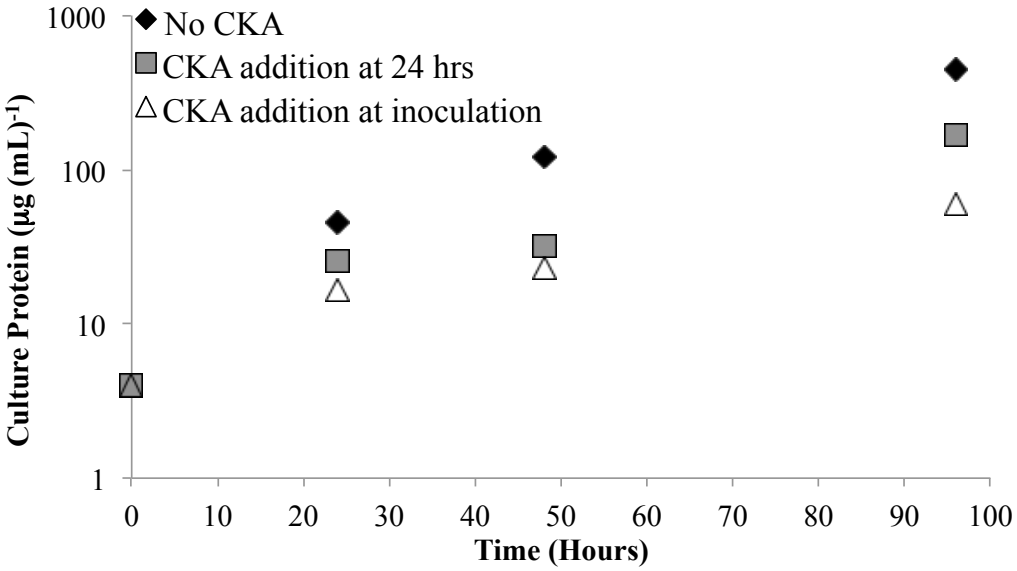


Figure 3.16 *L. tarentolae* treated with 40 µM CKA. Cultures were treated either at inoculation, 24 hours after inoculation, or not at all. Points represent the average of biological duplicates.

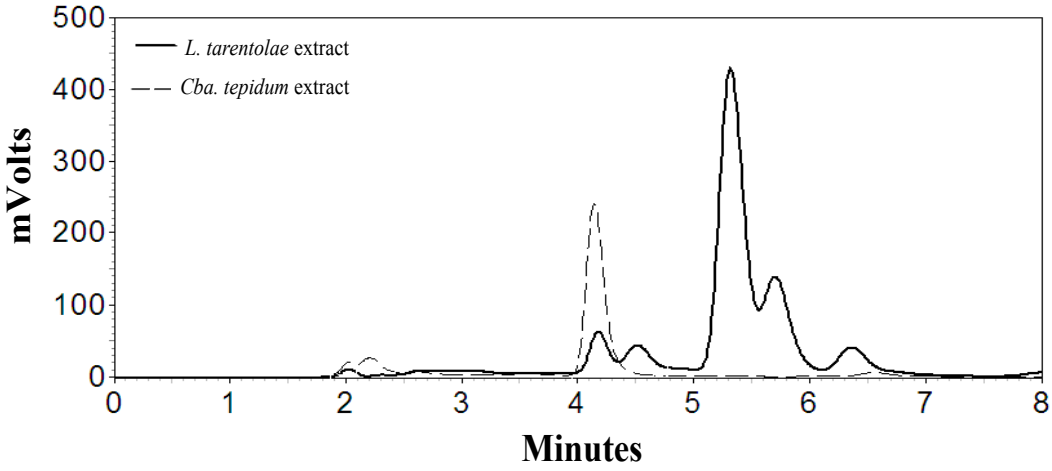


Figure 3.17 Representative chromatogram comparison of a *L. tarentolae* and a *Cba. tepidum* quinone extract.

3.9 CT1509 may encode a protein involved in the biosynthesis of CK

A list of putative genes involved in CK synthesis was generated in the Integrated Microbial Genomes (IMG) database by searching for homologs of *Cba. tepidium* in the genome of *Leishmania major* strain Friedlin (a eukaryote reported to produce chlorobiumquinone) and subtracting the genomes of all *E. coli* strains and *Chloroflexus aurantiacus* via pre-computed BLASTP searches (Table 1). *C. aurantiacus* is a filamentous-bacterium that contains the unique light-harvesting apparatus called the chlorosome, just like *Cba. tepidum*, but does not synthesize CK (19).

Upon inspecting the genetic environment in which these genes were located within the genome, it was found that CT1509 might be in the same operon as *menA* (CT1511) (Fig 3.18). The gene product of *menA* is responsible for the synthesis of 2-demethylmenaquinone, the precursor of menaquinone-7, by attaching the all-trans-octaprenyl diphosphate onto the third-position of 1,4-dihydroxy-2-naphthoate.

Therefore, by association to *menA*, it would seem likely that CT1509 might be involved in CK synthesis. Downstream of CT1509, CT1508 encodes a hypothetical protein. CT1509 is separated from *menA* by CT1510, which encodes a carbon-nitrogen hydrolase family protein. In other *Chlorobi*, this gene has been given a more specific annotation of nitrilase/cyanide hydratase and apolipoprotein N-acyltransferase. Upstream of *menA*, CT1512 encodes a putative thioredoxin reductase. The gene product of CT1509 was used as a query in BLASTP searches of all sequenced *Leishmania* genomes. It was found that orthologs of CT1509 are a universal feature of the *Leishmanias* (data not shown).

Table 1 List of five putative genes involved in chlorobiumquinone synthesis. Table was generated by inter-organismal comparison between *L. major* and *Cba. tepidum* using the IMG database.

Locus tag	Gene name	Protein length
CT0370	hypothetical protein	168aa
CT0968	hypothetical protein	333aa
CT1509	CAAX prenyl protease	415aa
CT1526	Rab family protein	1102aa
CT1932	peptidase, M16 family	442aa

Figure 3.18 Physical map of the putative *menA* operon in *Cba. tepidum*. CT1509, a gene that may be involved in chlorobiumquinone synthesis, is shaded red. *menA* is shaded green.

Chapter 4

CONCLUSIONS AND FUTURE DIRECTIONS

The original goal of my thesis research was to quantify the regulation of quinone pools in *Chlorobaculum tepidum* under different sulfide concentrations, synthesize a chlorobiumquinone (CK) analog, and generate evidence pertaining to the function of CK through physiological studies utilizing the novel analog. It was hypothesized that CK was either functioning as a quencher of energy transfer from Bchl or that it was the electron acceptor for sulfide: quinone oxidoreductase.

Data presented in Section 3.1 shows that the MK-7 pool size is statistically static in respect to biomass when *Cba*. *tepidum* is grown on different concentrations of sulfide (Fig. 3.2). This is in contrast to previous findings that *Cps*. *ethylicum* exhibits a decrease in MK-7 content and an increase in CK content when exposed to sulfide (44). Initially it was thought that the peak at 4.1 min in the chromatogram of quinone extracts was CK. However, it was later determined that this peak was biotically produced elemental sulfur (Fig. 3.1). There is also a peak at 6 min that runs very close to the MK-7 standard whose identity has not been determined. In future experiments it may be possible to purify the compound generating this peak at 6 min and obtain its structural data in order to determine if it is CK. However, a more complicated solvent system may be needed to improve quinone separation (17). Moreover, since CK was unable to be obtained, it was desirable to synthesize a CK analog to be utilized for physiological studies.

First attempts at synthesizing a CK analog using the method of Biswas *et al*. (5) were unsuccessful. With consultation from the Tabor Group at the University of

Delaware, the method was deemed inaccurate. Section 3.2 describes a synthesis route for a novel CK analog. The new method required 5 reaction steps and produced an overall yield of 5.7% relative to the number of moles used in the initial reaction. The final product ensured the integrity of the 1′-oxo group that makes CK structurally unique among quinones. The newly synthesized analog of CK, named CKA, was produced in its fully oxidized state (Figs. 3.3, 3.4, & 3.5). It was found to have a reduced/oxidized spectrum (Fig. 3.6) extremely similar to an isolated quinone previously reported in the literature (44).

Cyclic voltammetry has clearly shown that the quinone CKA goes through a reversible two-electron two-step reduction in the nonaqueous solvent acetonitrile when being electrochemically reduced (Fig. 3.7). In comparison to the literature reported values of CK (39 mV) and MK-7 (-80 mV) (44), the midpoint potential of CKA was calculated to be -117 mV in reference to a normal hydrogen electrode. Because of the ability of CKA to quench cellular fluorescence (Fig. 3.12) the result that CKA has more reducing potential than MK-7 when in the fully reduced form was unexpected. In order to better understand this unanticipated result, CK would need to be purified from *Cba. tepidum* and its midpoint potential determined using cyclic voltammetry in acetonitrile (Section 3.4).

Addition of CKA to cultures of *Cba*. *tepidum* caused severe growth inhibition (Figs. 3.8 & 3.9). Upon further investigation, the effect of CKA was found to be fundamentally bacteriostatic (Fig. 310). Addition of CKA to cultures of *E*. *coli* had no effect on growth (Fig. 15) while CKA added to *L*. *tarentolae* caused a slight growth inhibition (Fig. 16). Therefore, it is hypothesized that CKA is toxic to only to organisms that produce CK. In the case of *Leishmania*, CKA may be able to be used

as a viable option for therapy. *Leishmania* is a parasite in the genus trypanosomatid protozoa that causes the disease leishmaniasis. Leishmaniasis currently affects 350 million people in 88 countries (12). CKA is a small molecule that should be easy to inject into the body or be incorporated into a topical cream in order to inhibit the spread of leishmaniasis. However, further research is needed in order to determine if this would be feasible.

Further investigation elucidated that CKA does not affect sulfide uptake rates from whole cells of *Cba*. *tepidum* (Fig. 3.11). This is of great interest since SQRs are the main enzymes that turn-over sulfide in the cell resulting in persulfide production and donation of electrons to the quinone pool (9). The statistically insignificant change in sulfide uptake rates reported when CKA is added to whole cells implies that sulfide *in vivo* turn-over is also not affected by CKA. This result was taken to be evidence that CK is not the preferred electron acceptor of SQR.

Fluorescent measurements taken of whole cells exposed to CKA found that CKA significantly quenches cellular fluorescence (Fig. 3.12). Therefore, it is hypothesized that CKA is most likely inhibiting energy transfer to the reaction center complex by quenching light-energy absorbed by Bchl *c* and Bchl *a*. If light-energy is unable to reach the reaction center complex then cells would be not be able to generate reducing equivalents for carbon fixation. The lack of light-energy reaching the reaction center could be why CKA elicits a bacteriostatic effect on *Cba*. *tepidum*. Further studies would include adding other oxidized quinones anaerobically to cultures of *Cba*. *tepidum* in order to determine if they also quench cellular fluorescence. It would also be of interest to determine if CKA reduced by borohydride would elicit any fluorescent quenching. This would help concretely establish if the

fully oxidized state of a quinone is imperative for cellular fluorescence quenching to occur (19).

It is worthy to emphasize here that CK must play a much different role in *L*. *tarentolae* than it does in *Cba*. *tepidum*. Under aerobic conditions in *L*. *tarentolae*, CK is most likely an electron carrier involved in the electron transport chain (5). When the obligate anaerobe *Cba*. *tepidum* encounters oxidizing conditions, CK is most likely involved in quenching cellular fluorescence (18, 19). The evidence stated here shows that CKA is interacting with the chlorosomes and affecting their ability to transfer light-energy to the reaction center (Section 3.5.4). It has also been shown to be unlikely that CKA would be interacting with SQR (Section 3.5.3). Therefore, by association, native CK would not be expected to interact directly with SQR.

Localization and recovery of CKA involved the fractionation of cultures into whole cells, supernatant, crude cellular lysate, cytosol, membranes and chlorosome fractions (Fig. 3.13). However, this data set was inconclusive in its results because it is not certain if CKA is being isolated in its fully oxidized form, reduced form, or a mixture of both. It may be possible that cells are reducing CKA inside or near the chlorosomes. Reduced CKA absorbs light 3.5 times less efficiently at 270 nm than oxidized CKA (Fig. 3.6). This may be one reason why so little CKA could be observed in the chlorosome, membrane, and cytosolic fractions. In order to solve this problem, all quinone extracts would need to be fully reduced with borohydride prior to HPLC analysis. Doing this should help in better determining CKA localization and recovery yield. Reduced quinone extracts would be analyzed by HPLC using an absorbance of 242 nm: the wavelength of maximum absorbance for CKA in the reduced form (Fig. 3.6).

Finally, an inter-organismal precomputed bi-directional BLASTP search was used to compare the genomes of *Cba*. *tepidum* and *L*. *major* strain Friedlin in order to find possible gene candidates involved in CK biosynthesis (Table 1). One such gene was found just downstream of the *menA* gene CT1511 (Fig. 3.18). The gene product of *menA* is involved in the biosynthesis of menaquinone. CT1509 may be in the same operon as *menA*. Therefore, by association with *menA*, it would seem probable that CT1509 is involved in chlorobiumquinone biosynthesis.

Future experiments would include making a gene deletion of CT1509 in order to empirically determine if this gene is involved in CK synthesis. The first genetic transfer of plasmids into *Cba. tepidum* was done though conjugation using *E. coli* as donor cells (49). In order to take advantage of classical genetic analysis, CP medium was designed and the platting strain WT2321 developed (49). The broad-host-range IncQ group plasmids pDSK519 and pGSS33 were found to naturally transform *Cba. tepidum*. However, these plasmids are unstable at the optimal growth temperature of 47 $^{\circ}$C since the antibiotic resistance markers originated from mesophiles (20, 49). Therefore, strains were selected for and grown at 40 $^{\circ}$C when using antibiotic selection. Similar methods were used to create the first chromosomal gene inactivation strains in *Cba. tepidum* (20, 26). It is hypothesized that silencing CT1509 expression would not be a lethal. Instead, inactivating CT1509 would most likely produce a phenotype similar to *Cfx. aurantiacus* in which *Cba. tepidum* would no longer exhibit a redox dependent quenching mechanism. Accomplishing these future studies will build off the foundation of results stated in this thesis and significantly contribute to the growing body of knowledge on microbial sulfur oxidation in the *Chlorobi* and the function and biosynthesis of CK.

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