Understanding Free Radicals

Student Author

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Abstract

All life persists in an environment that is rich in molecular oxygen. The production of oxygen free radicals, or superoxide, is a necessary consequence of the biogenesis of energy in cells. Both mitochondrial and photosynthetic electron transport chains have been found to produce superoxide associated with cell differentiation, proliferation, and cell death, thereby contributing to the effects of aging. Aerobic respiration in mitochondria consumes oxygen, whereas photosynthesis in chloroplasts or cyanobacteria produces oxygen. The increased concentration of molecular oxygen may serve to allow greater availability for the production of superoxide by cytochrome $bc$ complexes in photosynthetic membranes compared to those of mitochondrial membranes. The isolation of well-coupled chloroplasts, containing the cytochrome $b_{6,f}$ complex of oxygenic photosynthesis, is a vital initial step in the process of comparing the rate of production of superoxide to those of the homologous cytochrome $bc_{1}$ complex of aerobic respiration. It is necessary to determine if the isolated chloroplasts have retained their oxygen-generating capability after isolation by an oxygen evolution assay with a Clark-type electrode. A necessary second step, which is the isolation of cytochrome $b_{6,f}$ from spinach, has yet to be successfully performed. Oxygen measurements taken from chloroplasts in the presence of the uncoupler, NH₄Cl, exhibited a rate of oxygen evolution over three times greater at 344 +/- 18 μmol O₂/mg Chlorophyll a/hr than the rate of oxygen evolution without uncoupler at 109 +/- 29 μmol O₂/mg Chlorophyll a/hr. These data demonstrate that the technique used to isolate spinach chloroplasts preserves their light-driven electron-transport activity, making them reliable for future superoxide assays.

Stofleth, J. (2012). Understanding free radicals: Isolating active thylakoid membranes and purifying the cytochrome $b_{6,f}$ complex for superoxide generation studies, Journal of Purdue Undergraduate Research, 2, 64–69. doi:10.5703/jpur.02.1.10

Keywords
cytochrome $b_{6,f}$, free radicals, photosynthesis, superoxide formation
INTRODUCTION

Free radicals have received significant attention recently from the media and medical, fitness, and nutrition professionals. Free radicals are atoms or molecules with unpaired electrons, which make them highly reactive with surrounding biological molecules. They are known for their effects on aging, overall health, cellular damage, and link to the onset of varying diseases (Chakrabarti et al., 2011; Afanas’ev, 2010; Raha & Robinson, 2000). Free radicals also are important for activating various signaling pathways throughout the cell, such as the MAPK, ERK, JNK pathways that alter gene expression, as well as initiating cell death in coordination with superoxide dismutase (Pitzschke & Hirt, 2009; Buetler, Krauskopf, & Ruegg, 2004; Irihimovitch & Shapira, 2000; Shen & Liu, 2006).

Previously, mitochondrial respiration has been linked to the generation of oxygen free radicals, specifically those known as reactive oxygen species (ROS) or the superoxide anion, as part of normal electron leaking inefficiencies during steps of electron transfer between mitochondrial membrane proteins (Wang et al., 2008). Superoxide is produced during various steps of photosynthesis in a similar fashion as in mitochondria (Michelet & Krieger-Liszkay, 2011). Within the electron transport chains of these two processes, there exists a homologous protein complex of the cytochrome bc1 superfamily, which shares a similar role in electron transport and contributes to the formation of a proton (H+) gradient across their respective membranes by translocating H+, as shown in Figure 1 (Darrouzet, Cooley, & Daldal, 2004). The cytochrome b6f complex of oxygenic photosynthesis mediates the transfer of electrons from photosystem II to photosystem I, the two light harvesting protein complexes of photosynthesis (Baniulis et al., 2009). The ribbon structures in Figure 2 show the cytochrome b6f complex in (A), with homologous peptide subunits colored in the cytochrome bc1 complex in (B). The mechanism used by the cytochrome b6f complex to oxidize lipid-soluble plastoquinol (dPQH2) and reduce plastocyanin (PC), in conjunction with the translocation of two H+ per electron, is referred to as the Q-cycle (Cramer, Hasan, & Yamashita, 2011; Mulkidjjanian, 2010). Similarly, the homologous cytochrome bc1 complex of the respiratory electron transfer chain relies on the Q-cycle to transfer electrons from ubiquinol to cytochrome c (Cramer et al., 2011; Mulkidjjanian, 2010; Kramer, Roberts, Muller, Cape, & Bowman, 2004). The Q-cycle uses an unstable semiquinone...

Figure 1. The photosynthetic electron transport chain (A), showing the site of oxygen free radical formation (superoxide anion) at the cytochrome b6f complex. Also depicted is the site of oxygen generation at photosystem II, a major difference between respiration and photosynthesis. In (B) the mitochondrial respiratory chain is depicted, and the site of superoxide formation from the cytochrome bc1 complex is also shown. Cytochrome c oxidase (CoO) is shown consuming oxygen, in contrast to photosynthesis. Figure used with permission from S. Saif Hasan (Biology Dept., Purdue University).
Figure 2. The 220 kDa cytochrome b6 complex of oxygenic photosynthesis in (A) PDB 2ZT9, and the 250 kDa homologous cytochrome bc1 complex of aerobic respiration in (B) PDB 3CX5. Subunits and prosthetic groups with similarity between the two complexes are shown in matching colors, using PyMOL.

radical to reduce the b-hemes, but has the potential to pass off an electron to molecular oxygen in a “bypass reaction,” or leaking, to create superoxide (Kramer et al., 2004). The step in the Q-cycle thought to produce superoxide is shown in Figure 3. Both cytochrome b6 and cytochrome bc1 are capable of producing superoxide, but a comparison between the rates of production for the two has not been conducted (Muller, Crofts, & Kramer, 2002; Suh, Kim, & Jung, 2000; Sun & Trumpower, 2003; Sang et al., 2011). It would be useful to understand if a different rate of observed superoxide formation is due to the differences in structure between isolated complexes, and if the higher concentration of oxygen in photosynthetic membranes contributes to the rate of formation. This would advance the current field of research information on how plants and animals regulate superoxides differently and how these specific bc complexes contribute to the function and dysfunction of cells. It is especially useful as photosynthetic plants do not have the same effects of aging as organisms that rely solely on respiration as a source of energy transduction. Furthermore, this information would give researchers insight into the mechanistic differences that have evolved in how these two protein complexes have evolved, as well as the two energy transduction systems as a whole.

To accomplish this comparison, Danas Baniulis purified active cytochrome bc1 from yeast and measured the basal and inhibited rate of superoxide formation (D. Baniulis, personal communication, 2007) These rates will be compared to those for cytochrome b6f, both as an isolated complex and within the oxygen-evolving membranes. Here, we demonstrate that photosynthetic thylakoid membranes from spinach can be isolated with the ability to evolve oxygen, demonstrating that there has been no loss of function during isolation, and that they will be a viable option for superoxide evolution studies of the cytochrome b6f complex that is contained within them. Secondly, we describe the process and show results for isolating detergent-solubilized cytochrome b6f from spinach, and for the isolation of the copper-containing protein, plastocyanin from cyanobacteria.

A three-dimensional ribbon structure of PC from Anabaena variabilis (PDB: 2GIM) is shown in Figure 4. Plastocyanin is necessary for activity assays and superoxide production assays of the cytochrome b6f complex, as it is the electron acceptor for the complex (Cramer et al., 2011). The successful completion of the above protocols is a necessary step in the process of experimental setup for upcoming superoxide generation assays. Here, we show that isolated thylakoid membranes are active by their ability to evolve oxygen at a rate of 109 μmol O2/mg Chl a/hr, and are susceptible to ammonium chloride uncoupler addition by a tripled rate increase to 344 μmol O2/mg Chl a/hr. We were able to isolate 9 mg of cytochrome b6f from spinach, but problems with the electron transfer assay have precluded our ability to demonstrate activity and continue with further study. Lastly, 2 mg of plastocyanin was purified from cyanobacteria, providing a necessary electron acceptor for future assays. It is necessary to demonstrate activity in the isolated cytochrome b6f complex before proceeding forward with assays to measure superoxide generation.

MATERIALS AND METHODS

Organism

Prewashed, organic baby spinach leaves were bought from the supermarket. These were stored overnight at 4°C with no light exposure.

Chloroplast Preparation

Approximately 150 g of spinach leaves were homogenized in a blender in a sucrose solution containing 0.3 M NaCl, 30 mM Tricine (pH 7.8), 3 mM MgCl2, and 0.5 mM EDTA. The homogenate was filtered through four layers of cheesecloth and centrifuged to 8,000 x g. The pellet was resuspended in a buffer containing 0.2 M sucrose, 5 mM HEPES (pH 7.5), 2 mM MgCl2, and 0.5 mM EDTA. After centrifuging at 4,000 x g for 3 minutes, the pellet
was resuspended in a minimal volume of the same buffer. The chlorophyll a (Chl a) concentration was calculated in mg/mL using the following formula:

\[
\text{Chl a (mg/mL)} = 0.5 \times (0.2 \times (A_{665} + 20.21 \times A_{645}) + 5.8 \times A_{652})
\]

**Oxygen Evolution Assay**

Oxygen evolution measurements were collected on a Clark-type electrode under illumination of 2000–2500 microeinsteins/m²/s. A 1.8 mL final volume of assay medium containing 30 mM Tricine (pH 7.8), 0.1 M sucrose, 10 mM NaCl, 2 mM MgCl₂, 5 mM FeCN with chloroplasts at a Chl a concentration of 20 μg/mL was stirred under illumination. Changes in oxygen concentration resulting from chloroplast activity were plotted using LabVIEW software connected to the Clark-type electrode. The concentration of the uncoupler, ammonium chloride, was used at a final concentration of 1 mM in relevant assays.

**Cytochrome b₆f Preparation**

Spinach leaves were homogenized in a blender with a buffered solution, filtered through four layers of cheesecloth, and centrifuged at 10,000 x g. The pellets were resuspended in a hypotonic solution to induce chloroplast bursting and were homogenized with a drill press. 1 mL acetone extractions (80% v/v) were made and Chl a content was measured using the following formula for spectral data:

\[
\text{Chl a (mg/mL)} = 11,000 \times (A_{646} - A_{750}) + 7.34 \times (A_{663} - A_{750}) / 10
\]

The membranes were then washed in 2 M NaBr to remove peripheral membrane proteins. The membranes were centrifuged to separate and discard the solubilized proteins, and the pellet was then washed with a 30 mM Tris (pH 7.8), 50 mM NaCl, and 1 mM EDTA solution (TNE) and centrifuged again. Membranes were resuspended in a small volume of the TNE solution and combined with a solution of n-octyl-β-D-glucopyranoside 0.8% (w/v) and sodium cholate 0.1% (w/v) to extract integral membrane proteins into detergent micelles. The extract was ultracentrifuged at 55,000 rpm to remove insoluble cell debris. The soluble micelles containing extracted proteins were collected from the supernatant, subjected to a 37% saturation ammonium sulfate precipitation, and were ultracentrifuged to remove precipitants.

The supernatant was then loaded onto an 8mL propyl-agarose chromatography column at a rate of 0.5 mL per minute. The bound fraction was washed on the column with approximately 24-40 mL of a TNE (pH 7.8), 37% ammonium sulfate, 0.05% (w/v) n-undecyl-β-maltoside (UDM) solution, at 1 mL per minute until the flow-through became clear. The protein was eluted with a TNE, 20% ammonium sulfate, 0.05% UDM solution, and collected in 3 mL fractions.

The fractions containing cytochrome b₆f, recognizable by their dark brown color or their strong absorbance at 554 nm, were combined and the buffer was exchanged for TNE, 0.05% UDM (no ammonium sulfate). This was concentrated to less than a 5 mL volume and was then loaded onto 10%–32% sucrose gradients in TNE, 0.05% UDM for ultracentrifugation in a SW-41 rotor at 36,000 x g for 16 hours. Dimeric cytochrome b₆f was isolated from the proteolyzed monomer by identification of a brown band that migrates further down the gradient. After exchanging the buffer for TNE, 0.05% UDM the protein solution was concentrated to approximately a 2–3 mL volume. The concentration of cytochrome b₆f complex is determined by the following formula from ascorbate minus potassium ferricyanide (FeCN) difference spectra:

\[
\text{Cyt b₆f (mg/mL)} = (11,000 \times (A_{646} \text{ ascorbate} - A_{646} \text{ FeCN}) / 25
\]

**Plastocyanin Preparation**

 Stored peripheral membrane removal wash solutions saved from a step of the process for purifying cyanobacterial cytochrome b₆f were thawed for use (Baniulis, Zhang, Zakharova, Hasan, & Cramer, 2011). The solutions were subjected to a 60% ammonium sulfate precipitaton and centrifugation at 10,000 x g, and the supernatant was subjected to a 90% ammonium sulfate precipitaton and centrifugation. The pelleted precipitants were redissolved in 1 mM Mes (pH 6.5) with 25 μM FeCN. These solutions were dialyzed against a 1mM Mes (pH 6.5), 25 μM FeCN solution, with approximately a 10° dilution factor at completion in order to remove ammonium sulfate. The dialyzed fractions were loaded onto a 50 mL SP-sepharose FF chromatography column (GE or Sigma). Washes were done with the 1 mM Mes buffer containing no NaCl, followed by a 2 mM NaCl buffer wash. Plastocyanin was eluted on a gradient from 2 mM to 30 mM NaCl, light blue-colored fractions were pooled, and the protein was concentrated in a Centriprep YM-10 at 3000 x g. Concentration is determined by the oxidized minus reduced spectra at 597 nm, with an extinction coefficient of 4.7 mM⁻¹cm⁻¹ (Yoshizaki, Sugimura, & Shimokoriyama, 1981).

**Cytochrome b₆f Activity Assay**

Activity of the cytochrome b₆f complex was assayed on a Cary 4000 spectrophotometer kinetic collection. The assay mixture contained 125 μM FeCN, 5 μM

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**Figure 4.** Three-dimensional ribbon structure of the 11kDa soluble electron transfer protein, plastocyanin from Anabaena variabilis, PDB 2GIM. The reactive copper shown in the center is capable of accepting electrons from the cytochrome b₆f complex, and it is coordinated by two histidine residues. Image created with PyMOL.
plastocyanin, 5 nM cytochrome $b_{6f}$ in 50 mM Mops, pH 6.9, 0.05% (w/v) UDM. Reduction of ferricyanide was initiated by addition of reduced 25 μM decylplastoquinol (DPQH$_2$), and was monitored as the rate of increased transmittance by ferricyanide at 420 nm (Estabrook, 1961). This indicates an electron transfer pathway from dPQH$_2$ through cytochrome $b_{6f}$ to PC, and then terminally accepted by FeCN for which the change absorbance is monitored at 420 nm. DPQH$_2$ reduction can be monitored by its absorbance characteristics, as seen in Figure 6(D). The DPQH$_2$ solution in ethanol is fully reduced when there is only an absorbance peak at 290 nm. Any presence of a peak at 260 nm indicates that there is still oxidized decylplastoquinone (dPQ), which cannot donate electrons. A partially reduced dPQH$_2$/dPQ solution will misleadingly lower the recorded activity measurements for cytochrome $b_{6f}$.

RESULTS AND DISCUSSION

Oxygen Evolution in Photosynthetic Membranes

The ability of isolated photosynthetic membranes to evolve oxygen is demonstrated by the graph in Figure 5. The basal rate (blue) of oxygen generation was measured at $109 \pm 29 \mu$mol O$_2$/mg Chl a/hr under illumination. The illumination is necessary to allow photosystem I (PS I) and photosystem II (PS II) to harvest light and convert photon energy to electrochemical energy in the form of electrons. These reactions use H$_2$O for an electron acceptor to create molecular oxygen (O$_2$). The Clark-type electrode is able to detect the increase of pressure in the chamber due to oxygen and allowed us to calculate the rate that O$_2$ is created. Since we know that oxygen is capable of being produced, we can test the how leaky the membranes are by using an uncoupler like ammonium chloride. Since the membranes form tiny spherical lipid bilayers, they contain a sealed aqueous interior. If these membranes are fully functional and have the barrier properties of a normal membrane, they trap protons (H$^+$) created by PS II, and create an electrochemical potential that slows the rate of oxygen evolution. An uncoupler makes the spherical membranes behave as if they were porous and allows the H$^+$ gradient to dissipate, thus increasing the rate of oxygen evolution. When ammonium chloride, a classic uncoupler, was added to the assay mixture, the rate of oxygen evolution increased from 109 to $344 \pm 18 \mu$mol O$_2$/mg Chl a/hr, as seen by the red plot in Figure 5. This is a rate increase of 3.2 times the basal rate of oxygen created per unit of time, and it allows us to conclude that the membranes are not leaking H$^+$. This is important for future superoxide experiments, as leaky membranes are indicative of loss of function, and superoxide measurements gathered from faulty membranes will not yield reliable data. Since the protocol used to isolate thylakoid membranes can retain their oxygen-forming capabilities, we can proceed to further steps in the superoxide experiments.

Cytochrome $b_{6f}$ Isolation

The isolation of active cytochrome $b_{6f}$ complex is a necessary step for us to measure superoxide generation. First, we must be able to extract it from membranes into an artificial detergent, followed by steps to increase the purity of the sample and separate the proteolyzed or monomerized complex. Figure 6(B) shows the spectra for the purified sample at 554 nm, the ascorbate (reduced) minus the FeCN (oxidized) absorption characteristics of

Figure 5. The effect of the uncoupler, ammonium chloride on oxygen-evolving thylakoid membranes is seen by the contrast between the basal rate of oxygen evolution determined to be $109 \pm 29 \mu$mol O$_2$/mg Chl a/hr (blue), with ammonium chloride uncoupled membranes a rate increase of 3.15x at $344 \pm 18 \mu$mol O$_2$/mg Chl a/hr (red). Ammonium chloride was added at 250 seconds.

Figure 6. (A) shows the distinct colors of Cyt $b_{6f}$ (brown) and plastocyanin (blue) from chromatography column to concentrated sample. In (B) the absorption spectra for cytochrome $b_{6f}$is shown at 554 nm, indicating a concentration of 3.52 mg/mL. (C) shows the inverted ascorbate-FeCN difference spectrum. (D) demonstrates the different absorbance spectra for oxidized (260 nm) and reduced (290 nm) decylplastoquinone. The sample must be completely reduced before activity assays, leaving no absorption peak at 260 nm.
cytochrome f-heme. The absorbance difference spectrum allows us to calculate the amount of cytochrome b$_6$f in the sample by the calculation:

\[ \text{Cyt } b_6f (\text{mg/mL}) = \frac{A_{554}}{25 \times \text{dilution factor}} \times 110 \text{ (MW)} \]

With an $A_{554} = 0.08$ and a dilution factor of 100, there is 3.52 mg/mL of cytochrome b$_6$f. Since there was 2.75 mL final volume of concentrated protein, that gave us 9.7 mg of protein. We now know that we are able to isolate and purify the complex from spinach, but for it to be useful for further study, it must be capable of actively transferring $\text{H}^+$ from dPQH$_2$ to PC. Due to ongoing problems with the assay protocol, we have not demonstrated spinach cytochrome b$_6$f to be able to actively catalyze electron transfer. Since it is a well-established procedure, a relatively minor troubleshooting effort will likely yield an answer that allows us to exceed the current barrier. A photo of purified but inactive cytochrome b$_6$f is pictured in Figure 6(A) as the brown isolate from the brown band in the left-side chromatography column.

**Plastocyanin Preparation**

The plastocyanin preparation was successful in that it produced purified plastocyanin, which, when oxidized, yielded a light royal blue color seen in Figure 6(A). Unlike cytochrome b$_6$f, plastocyanin binds ubiquitously throughout the chromatography column and does not elute into a concentrated volume. We were able to purify about 300 μL of 650 μM PC. This is a less than optimal yield compared to past purifications, so we will need to optimize the process. The FeCN oxidized minus the absorbance difference spectrum yields an inverse absorbance peak at 597 nm, as seen in Figure 6(C). From the magnitude of this peak and an extinction coefficient of 4.7 mM$^{-1}$cm$^{-1}$, we calculated the PC concentration to be 650 μM. The success of this procedure represents a step forward in the process of developing the capability to measure superoxide generation by cytochrome b$_6$f.

**CONCLUSION**

Though thylakoid membranes were isolated and showed satisfying uncoupling capability with respect to oxygen evolution, the inability to confirm electron transfer activity in cytochrome b$_6$f has prevented any collection of superoxide generation data to date. Because this is an established technique for measuring activity, the outlook for solving this issue seems promising. All other steps in the process, such as plastocyanin purification and thylakoid isolation, have been successful and the experiment has made progress.

Once activity is observed in isolated cytochrome b$_6$f, the protein will be used in a fluorescence assay in the presence of superoxide dismutase, with dPQH$_2$ and PC. Inhibitors that probe different binding sites on the complex will give parallel data to that yielded from the cytochrome bc$_1$ complex. This data will allow for the first real comparative analysis of superoxide generation between homologous cytochrome bc$_1$ complexes.

**ACKNOWLEDGMENTS**

Special thanks to S. Saif Hasan for advice and guidance throughout this project.

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