Characterizing the Effects of Ferric Ammonium Citrate on Heliobacterial Photosynthesis

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INTRODUCTION

Heliobacteria are a group of strictly anaerobic photosynthetic bacteria possessing a single Type I reaction center with a small antenna complement. Previously, we reported upon the steady-state absorbance changes associated with P800 photooxidation in whole cells of *Heliomicrobium modesticaldum* and suggested that our results were consistent with heliobacterial photosynthesis functioning in a light-limited manner. Briefly, when whole cells of heliobacteria are exposed to continuous light, the absorbance at 800 nm shows multi-phasic kinetics wherein a rapid initial bleaching reaches a maximum at around 10-30 ms, followed by a slower recovery until a steady-state is reached. The addition of ferric ammonium citrate (FAC) decreases the recovery phase, likely due to FAC oxidizing the membrane bound cytochrome and inhibiting forward donation to $P800^+$. In addition, FAC treatment results in an increase of the signal magnitude, suggesting that a significant portion of the P800 pool remains reduced under constant illumination. In this work, we quantify this so-called FAC effect and investigate its mechanism. Intriguingly, the FAC effect seemed dependent on sample preparation. Actively growing cells inconsistently showed an FAC effect, however, cells that had been harvested and frozen showed consistent results. The FAC effect showed significant concentration dependence, with a relative IC_{50} of 55-77 μM. When kinetics were measured at 436 nm and 550 nm to assay the *bc* complex and cytochrome *c* respectively, no significant light-induced absorbance changes were observed, suggesting that FAC had independently oxidized the cytochrome pool. Light saturation profiles indicated that the FAC effect results in nearly complete oxidation of the P800 pool. When combined with light saturation profiles of untreated cells, it is revealed that only 37% of the P800 pool is oxidized under saturating light conditions, consistent with our previous findings that heliobacterial photosynthesis is light-limited.

MATERIALS & METHODS

All reagents were ACS grade or better. All cell culture and biochemical manipulations were performed anaerobically used a Coy Type A anaerobic chamber (Coy Laboratory Products, MI, USA) containing an atmosphere of 10% H_2 / 90% N_2. All material were placed in the chamber 24 hours prior to placement in the chamber. Cultures of *Heliomicrobium modesticaldum* were grown in air-tight, stoppered, Hungate style, Bellco culture tubes in ATCC Medium: 2061 PYE at 49°C under a bank of either fluorescent lights. Cell growth was measured at 600 nm.

Cell were grown to late-exponential phase, harvested at 10,000 x *g* for 20 min. at 4°C, and the pellet resuspended with homogenization in 50 mM MOPS pH 7 with 10% glycerol. The cell suspension was stored at -80°C until use, where it would be thawed for 20 min. and re-homogenized. UV-vis spectra confirmed that bacteriochlorophyll *g* was not converted to chlorophyll *a* in this process. **This process was found necessary to producing consistent results from FAC treatment *(data not shown)*.**

Spectroscopic measurements were conducted using a JTS-10 spectrometer (BioLogic, Claix, France). Detection wavelengths were selected using interference filters (FWHM ≤ 10 nm) placed immediately after detection LEDs and before the focusing lens by virtue of a homebuilt filter holder. Actinic light was provided by orange LEDs centered at approx. 630 nm or red LEDs centered at approx. 720 nm. Samples were diluted to OD_{788} = 1.0 with 50 mM MOPS buffer, pH 7 and supplemented as indicated. Samples were prepared in a sealed cuvette (1 cm pathlength) under anaerobic conditions in the dark and were allowed to incubate at room temperature in darkness for 5 min before measurements were taken.

References

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Kramer, David M., et al. "Cyclic electron transfer in *Heliobacillus mobilis* involving a menaquinol-oxidizing cytochrome *bc* complex and an RCI-type reaction center." *Biochemistry*, vol. 36, no. 14, 1 Apr. 1997, pp. 4203–4211,https://doi.org/10.1021/bi962241i.

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During constant illumination, whole cells displayed a partial recovery after a large rapid bleaching at 800 nm. Upon adding FAC, the percentage of this recovery was reduced and signal size increased, implying that a large percentage of P800 was remaining in the reduced state during constant illumination.

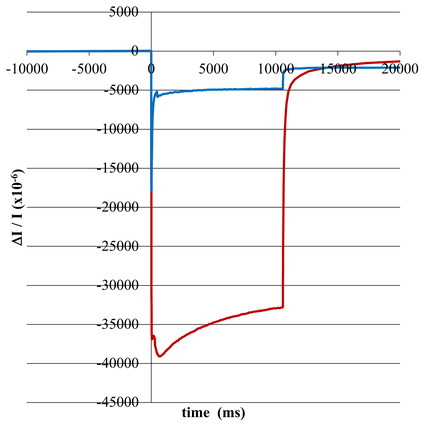

Figure 1. Light-induced absorbance changes, measured at 800 nm, depicting the P800 photooxidation of untreated *H. modesticaldum* whole cells (blue) and whole cells treated with FAC (red). Actinic illumination, initiating at 0 ms and ending at 10,000 ms, was provided by red LEDs centered at 720 nm at an intensity of 607 μE m^{-2} s^{-1}. 10 μs detection pulses were emitted every 50 μs – 100 ms by near IR LEDs filtered by an 800 nm interference filter.

FAC is a potent inhibitor of forward donation to $P800^+$, with an IC_{50} of 55 – 77 μM.

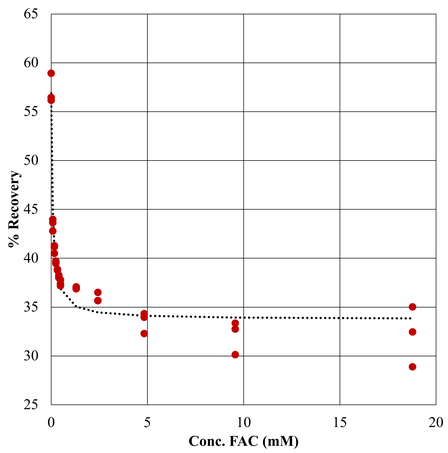

Figure 2. Inhibition of forward donation to $P800^+$ by FAC. Percent recovery is determined as the difference between the magnitudes of the initial and steady-state bleaching and expressed as the percentage of initial bleaching; a larger percent recovery indicates more efficient donation to $P800^+$. FAC inhibits donation to $P800^+$, resulting in a decreased percent recovery. Data was fit with the equation $Y = y_{min}(\frac{y_{max} - y_{min}}{1 + \frac{C}{IC50}})$; where Y is the percent recovery, y_{min} and y_{max} are the minimum and maximum observed percent recovery, C is the FAC concentration, and IC_{50} is the FAC concentration producing 50% inhibition. For this data, y_{min} = 33.7%, y_{max} = 56.9% , and IC_{50} = 76.6 μM.

RESULTS

The P800 pool size can be estimated utilizing light saturation profiles in cells treated with FAC. Even under saturating light conditions, only 37% of the P800 pool is oxidized in untreated whole cells.

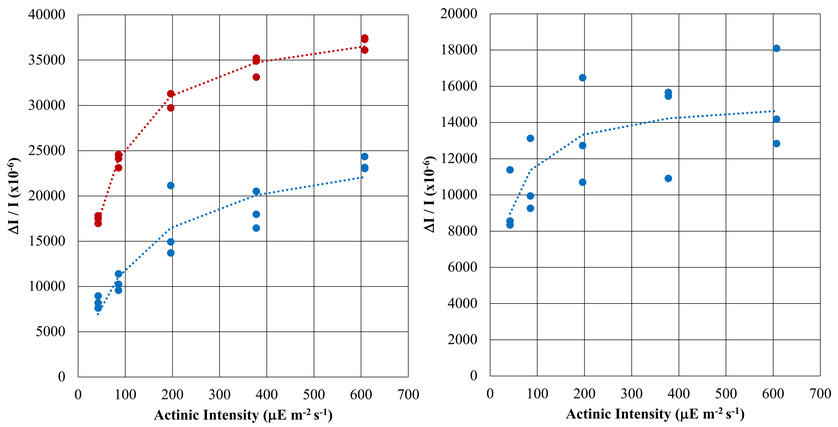

Figure 3. Light saturation profiles describe the effect of actinic intensity on the maximum *(left)* and steady-state *(right)* $P800^+$ signal magnitude; untreated cells in blue, FAC treated cells in red. Data is fit using the equation B = (I•B_{max}) / (I+K_{50}), where B is the observed signal magnitude, I is the actinic intensity, B_{max} is the maximum signal magnitude under saturating actinic intensities, and K_{50} is the actinic intensity producing 0.5 B_{max}. Relevant fitting parameters are indicated in ***Table 1***.

Untreated, maximum	FAC treated, maximum	Untreated, steady-state	% P800 oxidized
33,000	39,900	13,500	33.8
31,700	42,300	16,800	39.8
26,400	39,900	15,400	38.5
		Average	37.4
		Standard Deviation	3.2

Table 1. Fitting parameters from light saturation profiles. **The average percent of P800 oxidized under constant illumination when exposed to saturating light is approx. 37%.** The maximum P800 pool size was taken as the B_{max} determined from FAC treated cells. The amount of P800 oxidized under constant illumination was taken as the B_{max} from the "steady-state" light saturation profile.

Measurements at 436 nm, representing the *bc* complex, and 550 nm, representing cytochrome *c*, on FAC treated cells showed only minimal light-induced absorbance changes compared to untreated cells, suggesting that FAC functions by oxidizing the cytochrome pool.

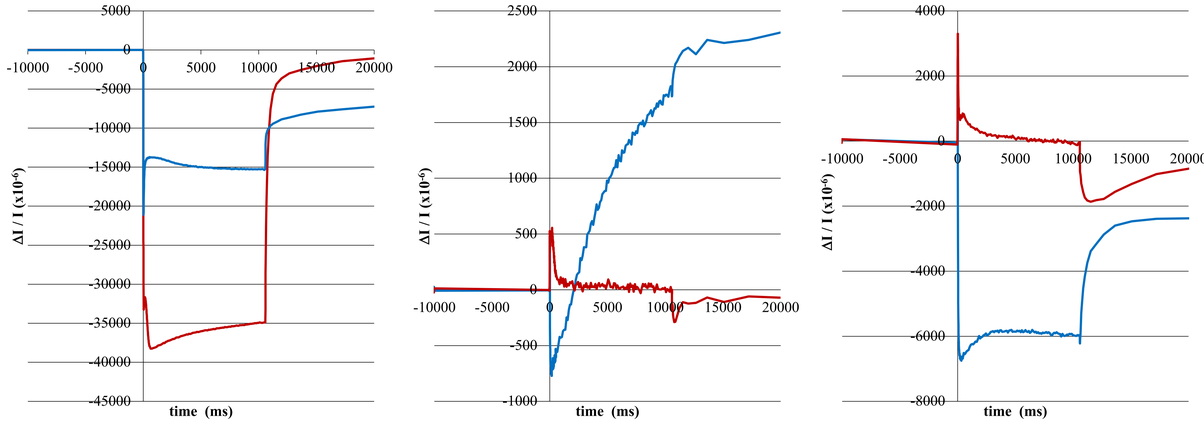

Figure 4. Light-induced absorbance changes of *H. modesticaldum* whole cells at 800 nm *(left)*, 550 nm *(middle)*, and 436 nm *(right)*; untreated cells in blue, FAC treated cells in red. Actinic illumination, initiating at 0 ms and ending at 10,000 ms, was provided by red LEDs centered at 720 nm at an intensity of 607 μE m^{-2} s^{-1}. 10 μs detection pulses were emitted every 50 μs – 100 ms by near IR (800 nm) or white light (436 and 550 nm) LEDs filtered by the appropriate interference filter. Traces at 436 nm and 550 nm were corrected for background P800 changes by subtracting traces obtained at 589 nm (after Kramer, et. al. 1997).

CONCLUSIONS

1. **When *H. modesticaldum* is treated with FAC, a potent charged oxidant, the forward donation to $P800^+$ is inhibited due to the oxidation of the cytochrome pool by FAC. This conclusion is supported by the changes in kinetics at 436 nm and 550 nm, which are consistent with the oxidation of the *bc* complex and cytochrome *c* by FAC.**
2. **Despite some variation in the degree of inhibition , as measured by % recovery (see Figs 1 and 4), FAC is a potent inhibitor of forward donation, with an IC_{50} of 55-77 μM. This IC_{50} is in-line with values previously reported by Kashey, et. al. for azoxystrobin (5 μM) and terbutryn (100 μM). We tentatively attribute the variation in degree of inhibition to the similar mid-point potentials of FAC and cytochrome *c*.**
3. **Lights saturation profiles of FAC treated cells allow for the determination of the entire P800 pool size, whereas light saturation profiles of untreated cells describe the behaviour under in vivo conditions. Combined these results indicate that a mere 37% of the P800 pool is oxidized under saturating light conditions and a much smaller percentage is oxidized at full sunlight. These results are consistent with the light limited model of heliobacterial photosynthesis.**