

Discovery of zinc-bacteriochlorophyll in *Rhodobacter sphaeroides*

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Introduction

At the heart of photosynthesis are the chlorophyll pigments responsible for absorption and transmission of light energy. How the cell synthesizes and distributes these pigments to the photosynthetic apparatus (Fig 1) of *Rhodobacter sphaeroides* (Fig 2) is the focus of this study.

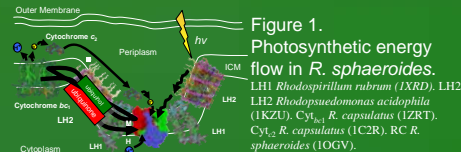


Figure 1. Photosynthetic energy flow in *R. sphaeroides*. LH1 *Rhodospirillum rubrum* (IXRD), LH2 *Rhodospseudomonas acidiphila* (1KZU), Cyt₂ *R. capsulatus* (IZRT), Cyt_{1,2} *R. capsulatus* (1C2R), RC *R. sphaeroides* (10GV).

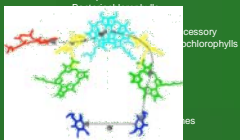
The protein complex at the core of the photosynthetic apparatus is called the reaction center (RC) and consists of 3 proteins named H, M and L which bind four bacteriochlorophylls (BChl), two bacteriopheophytins (BPhe), one carotenoid, two ubiquinones, and one non-heme iron atom (Fig 3) (7).



Figure 2. *Rhodobacter sphaeroides*. Left, wild type; right, *bchD* mutant.

Figure 3. RC branched electron transfer chain.

Electrons transfer down right hand branch from special pair BChl to accessory BChl then onto BPhe, Q_a and Q_b, which is reduced to quinol. Adapted from Watson AJ, et al. (2005). *Biochim Biophys Acta* 1710: 34-46.



The biosynthetic pathway to produce BChl and BPhe begins with chelation of Mg²⁺ into protoporphyrin IX by an enzyme complex of BchH, I, BchK, L and D subunits, thus setting the product, Mg-protoporphyrin IX, on the path to becoming BChl (Fig 4) (1).

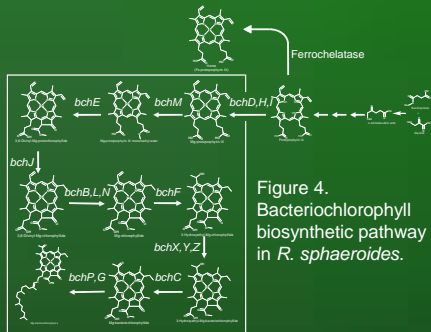


Figure 4. Bacteriochlorophyll biosynthetic pathway in *R. sphaeroides*.

In this study we characterize a *bchD* knockout (3), which abolishes all Mg-chelatase activity and renders the cell incapable of creating Mg-protoporphyrin IX or BChl. We have discovered that these mutants make an uncommon pigment called zinc-bacteriochlorophyll (Zn-BChl), in place of BChl, and assemble it with their RC.

Results

1. BChl is absent in *bchD* mutant but a pigment with similar spectral properties is present.

To determine whether the *bchD* mutant made any photosynthetic pigments, cell pigments, cell extractions were run on HPLC (Fig 5A). BChl was conspicuously absent from these cells, but a major peak not seen in wild type cells that had a retention time 2.5 minutes longer than BChl was isolated and analyzed spectrophotometrically (Fig 5B). This pigment was found to have a very similar absorbance pattern to BChl but with several major absorbance bands blue-shifted from BChl by 6-8 nm.

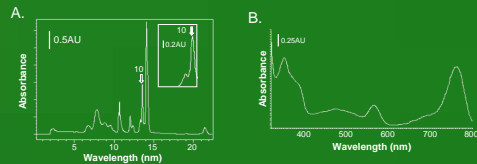


Figure 5. Isolation of pigment from *bchD* mutant. A. HPLC trace of whole cell acetone/methanol extractions. B. Absorption spectra of peak 10.

2. Identification of Zn-BChl.

To further characterize this pigment we used MALDI-TOF mass spectrometry on the isolated sample. The major molecular ion peak (M⁺) was (M⁺) was 950.6 m/z, which agrees well with the predicted mass of Zn-BChl (M⁺=950.4), but not with any other metal BChl derivative (Fig 6A). Upon treatment with acid, which is known to demetallate BChl, another peak at 886.5 m/z appeared (Fig 6A, inset) which matches deprotonated BPhe (M⁺=886.5). Based on these results, the pigment produced by *bchD* knockout cells was identified as Zn-BChl (Fig 6B).

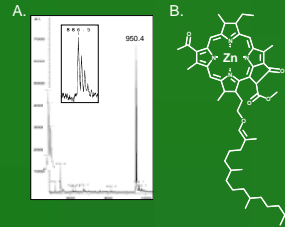


Figure 6. Identification of pigment as Zn-BChl. A. Mass spectrometric analysis of peak 10. B. Structure of Zn-BChl.

Pigment Characterization

3. Relative RC levels.

Although not able to grow photosynthetically we wanted to see if RC protein was still produced by this mutant, and if so, the levels relative to wild type. Western blots of membrane fractions were probed with antibodies against the H, M and L RC subunits (Fig 7). The results show all three RC proteins are present in *bchD* cells at approximately one third the wild type level.

Figure 7. Relative RC levels. Western blot of relative RC protein levels in wild type and *bchD* mutant.

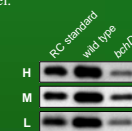
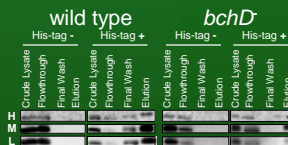


Figure 8. His-tag pull-down.

Western blot of four stages of pull-down. Crude lysate solubilized in LDAO detergent and bound to Ni²⁺-beads. Flowthrough is what didn't bind to beads. Beads are washed three times and retained proteins are eluted with 150mM imidazole. Samples run on 12% SDS-PAGE gel and blotted. His-tag - denotes lysate from cells containing plasmid with RC lacking His-tag. His-tag + denotes denotes cells containing plasmid with His-tagged RC H.



4. RC proteins interact and bind photosynthetic pigments.

We wanted to determine if the RC proteins were interacting and binding binding pigments. A His-tag pull-down assay revealed specific interactions between the RC proteins, indicative of complex formation (Fig 8).

To see if the RC proteins bound pigment, pull-down elutions were analyzed by absorption spectroscopy (Fig 9). The absorption bands indicate that the *bchD* mutant RC binds four Zn-BChl which have an absorption peak blue-shift: at 793nm instead of the normal accessory 804 nm peak and at 853nm instead of the normal special pair 865 nm peak. Interestingly, the BPhe peak at 758nm is missing in the *bchD* RC, indicating that the cells are unable to make BPhe and assemble it into their RC complex.

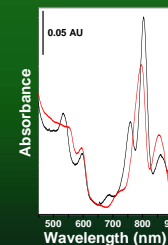


Figure 9. Absorption spectra of RC pull-downs. Black line, wild type; red line, *bchD* mutant.

Conclusions

This work shows that in a cell previously thought to be devoid of any of any photosynthetic pigments (3), there are small quantities of Zn-BChl that assemble into the RC complex. However never seen before in *R. sphaeroides*, such a Zn-BChl pigment was initially discovered in *Acidiphilium rubrum* where it is used as a photosynthetic pigment in the RC (6, 8). We have yet to assess functionality of *R. sphaeroides*' Zn-BChl RC but it is presumed that without BPhe there will be incomplete electron flow and no reduction of the quinones, rendering this RC non-functional.

We speculate that the biosynthesis of Zn-BChl is accomplished through circumventing the Mg-chelatase complex via Zn-chelation chelation activity of ferrochelatase. It has been shown that ferrochelatase can insert Zn²⁺ into the center of protoporphyrin IX with a K_m similar to Fe²⁺ (2, 5). Without competition from Mg-protoporphyrin IX, Zn-protoporphyrin IX could be accepted by BchM as a substrate to create the next intermediate in the pathway, Zn-protoporphyrin IX monomethyl ester, which which would be acted on by BchE and so on down the pathway (Fig (Fig 4), creating Zn-BChl (Fig 6B). This idea is supported by a report report that the last enzyme in chlorophyll synthesis can use Zn-chlorophyllide as a substrate with equal affinity to its normal substrate substrate Mg-chlorophyllide (4). This interchangeability of Zn²⁺ and Mg²⁺ seems to stem from the fact that they both possess similar similar molecular orbitals and bonding patterns which allow similar similar interactions with enzyme substrate recognition sites.

Future work will focus on elucidating the structure of the Zn-BChl BChl RC, testing its functionality and working out the mechanism of mechanism of Zn-BChl biosynthesis.

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