Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 6328–6332, July 1992
Biochemistry

Mutational and nucleotide sequence analysis of *S*-adenosyl-L-homocysteine hydrolase from *Rhodobacter capsulatus*

(*ahcY* sequence/gene evolution/bacterial photosynthesis)

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Contributed by Giulio L. Cantoni, April 6, 1992

ABSTRACT **The genetic locus *ahcY*, encoding the enzyme *S*-adenosyl-L-homocysteine hydrolase (EC 3.3.1.1) from the bacterium *Rhodobacter capsulatus*, has been mapped by mutational analysis to within a cluster of genes involved in regulating the induction and maintenance of the bacterial photosynthetic apparatus. Sequence analysis demonstrates that *ahcY* encodes a 51-kDa polypeptide that displays 64% sequence identity to its human homolog. Insertion mutants in *ahcY* lack detectable *S*-adenosyl-L-homocysteine hydrolase activity and, as a consequence, *S*-adenosyl-L-homocysteine accumulates in the cells, resulting in a 16-fold decrease in the intracellular ratio of *S*-adenosyl-L-methionine to *S*-adenosyl-L-homocysteine as compared to wild-type cells. The *ahcY* disrupted strain fails to grow in minimal medium; however, growth is restored in minimal medium supplemented with methionine or homocysteine or in a complex medium, thereby indicating that the hydrolysis of *S*-adenosyl-L-homocysteine plays a key role in the metabolism of sulfur-containing amino acids. The *ahcY* mutant, when grown in supplemented medium, synthesizes significantly reduced levels of bacteriochlorophyll, indicating that modulation of the intracellular ratio of *S*-adenosyl-L-methionine to *S*-adenosyl-L-homocysteine may be an important factor in regulating bacteriochlorophyll biosynthesis.**

S-Adenosyl-L-homocysteine hydrolase (AdoHcyase, EC 3.3.1.1) was first described in rat liver extracts as the activity responsible for the reversible hydrolysis of *S*-adenosyl-L-homocysteine (AdoHcy) to adenosine and homocysteine (Fig. 1) (1). AdoHcy is formed as a direct product of transmethylation reactions involving *S*-adenosyl-L-methionine (AdoMet) (2) and is known to be a potent inhibitor of most AdoMet-mediated methyl-transfer reactions.

AdoHcyase has been found in all cells that have been tested, with the exception of *Escherichia coli* and certain related bacteria (3–5). In the latter, the hydrolysis of AdoHcy to adenine and homocysteine requires two enzymatic steps catalyzed by a specific AdoHcy nucleosidase (6) and *S*-ribosyl-L-homocysteine hydrolase (7). AdoHcyase isolated from various sources always consists of a number of identical subunits, each containing 1 mol of tightly bound NAD^+ (8). The amino acid sequences of the rat liver, human placenta, and *Dictyostelium* enzymes have been deduced from cDNA sequences and show a high degree of sequence conservation (9–11). Site-directed mutational analyses coupled with *in vitro* biochemistry have demonstrated the presence of a nucleotide-binding domain (12, 13), but further conclusions regarding the protein structure have been hindered by lack of significant sequence diversity and a convenient system for genetic analysis.

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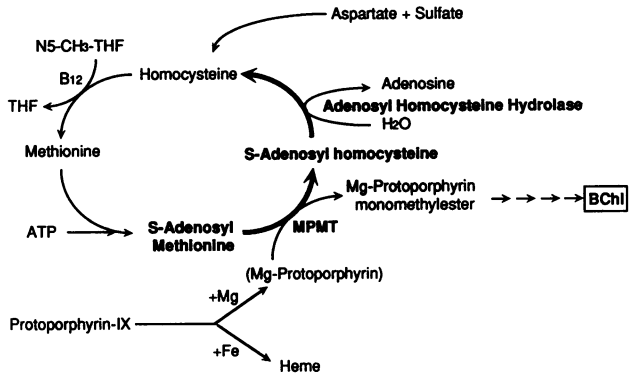

FIG. 1. Biosynthesis of homocysteine and biological methylation. The methylation of magnesium-protoporphyrin is shown as an example of one of many methylation reactions. THF, tetrahydrofolate; MPMT, *S*-adenosyl-L-methionine:magnesium protoporphyrin methyltransferase; BChl, bacteriochlorophyll.

In the purple non-sulfur photosynthetic bacterium *Rhodobacter capsulatus*, a regulatory cluster of genes involved in the maintenance and regulation of the photosynthetic apparatus has recently been identified (14). We report here that within this regulatory cluster the *ahcY* gene encodes the enzyme AdoHcyase. We also report some of the physiological and biochemical properties of interposon mutants of the *ahcY* gene. The amino acid sequence of bacterial AdoHcyase has one of the highest levels of conservation that has been observed between a human and a prokaryotic enzyme.¶

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. Wild-type *R. capsulatus* strain St. Louis and mutant derivatives were cultured at 34°C in the complex medium PYS, in malate minimal medium RCV, or in RCV supplemented with peptone and yeast extract (RCV 2/3 PY) (15, 16). *R. capsulatus* cells were grown either aerobically by shaking 10 ml of culture in a 250-ml side-arm flask at 300 rpm on a Gyrotory shaker, microaerobically by shaking 30 ml of medium in a 50-ml Erlenmeyer flask at 100 rpm, or anaerobically (photosynthetically) in stationary 16-ml screw-capped tubes completely filled with medium. Photosynthetic cultures were illuminated by banks of Lumiline 60-W bulbs (Sylvania) at

Abbreviations: AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; AdoHcyase, *S*-adenosyl-L-homocysteine hydrolase.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M80630).

saturating light intensity. *E. coli* strains were cultured in LB medium at 37°C by standard methods (17).

Interposon Mutagenesis. Site-directed mutagenesis of *ahcY* was accomplished by recombining a kanamycin-resistance cassette into the chromosomal copy of *ahcY*. Plasmid-encoded disruptions of *ahcY* were constructed by subcloning the 2958-base-pair *Bam*HI–*Xho* I fragment of the regulatory gene cluster from pMWS3.1 (14) into the *Bgl* II–*Sal* I site of pSP72 (Promega). This construct contains single *Eco*RI and *Bgl* II sites into which *Eco*RI and *Bam*HI fragments of pUC4-KIXX (18), which contain the kanamycin-resistance gene from Tn*5*, were ligated to produce interposon plasmids pBXLE2 and pBXLB1, respectively. The interposon constructs were transferred by conjugation into CB1127 (16), a generalized transducing strain of *R. capsulatus* from which the gene-transfer agent (GTA; ref. 19) was collected and used to recombine the interposons into the chromosome of a wild-type strain, *R. capsulatus* St. Louis, according to methods described by Scolnik and Haselkorn (19). The resulting mutants, StLE2 and StLB1, have the kanamycin-resistance cassette inserted into the *Eco*RI(2995) and *Bgl* II(3236) sites within the regulatory gene cluster region of their chromosome (Fig. 2 *Upper*).

Spectral Analyses. Crude photosynthetic membranes were isolated as described (20). Soluble membrane fractions were scanned for light absorbance between 400 and 900 nm with a Beckman DU-50 spectrophotometer. Total bacteriochlorophyll content was determined according to established procedures (21).

Sequence Analysis. Sequence analysis of *ahcY* was performed by subcloning the region between the *Eco*RV(2653) and *Apa* I(4310) sites into sites present in M13mp18 and -mp19 (22). Single-stranded DNA was isolated by established procedures and nucleotides of both strands were sequenced by the dideoxy chain-termination method (23) using both modified T7 DNA polymerase (Sequenase, United States Biochemical) and *Taq* polymerase (Taqtrack, Promega) in combination with deazanucleotide analogues. Universal and sequence-specific primers were prepared on an Applied Biosystems DNA synthesizer. Sequence data were analyzed on a MicroVax computer using version 7.0 of the University of Wisconsin Genetics Computer Group software.

Analysis of AdoHcyase, AdoMet, and AdoHcy. AdoHcyase activities were assayed in triplicate from clarified sonicated cell extracts obtained from cells grown to mid-logarithmic phase in RCV 2/3 PY medium. Assays were performed in the synthetic direction with 3-deazaadenosine as a substrate. 3-Deazaadenosine, a chemical analog of adenosine, was used because it is a good substrate for AdoHcyase and is not deaminated by adenosine deaminase (24). Each assay was performed in a 100-μl volume that contained 0.1 mM 3-deazaadenosine, 5 mM homocysteine, 20 mM Hepes (pH 7.2), and enzyme extract. The reaction mixtures were incubated at 37°C for 15 min and the reaction was terminated by the addition of 10 μl of 3 M perchloric acid. After incubation on ice for 15 min, the mixture was centrifuged for 5 min at 18,000 × *g* in a microcentrifuge at 4°C. The supernatant was removed, neutralized by addition of 1 M potassium carbonate, and centrifuged as described above. A 50-μl aliquot of this supernatant was then chromatographed on an Altex Ultrasphere ODS column (5-μm particles, 4.6 × 250 mm) by isocratic elution with 0.2 M ammonium dihydrogen phosphate (Aldrich) at a flow rate of 1 ml/min. Protein was determined by the bicinchoninic acid assay (Pierce).

AdoMet and AdoHcy levels were determined as follows. Cells were grown to mid-logarithmic phase (≈10^8 cells per ml) in RCV 2/3 PY medium, washed with RCV minimal medium, and then resuspended in RCV medium to a cell density of 10^9

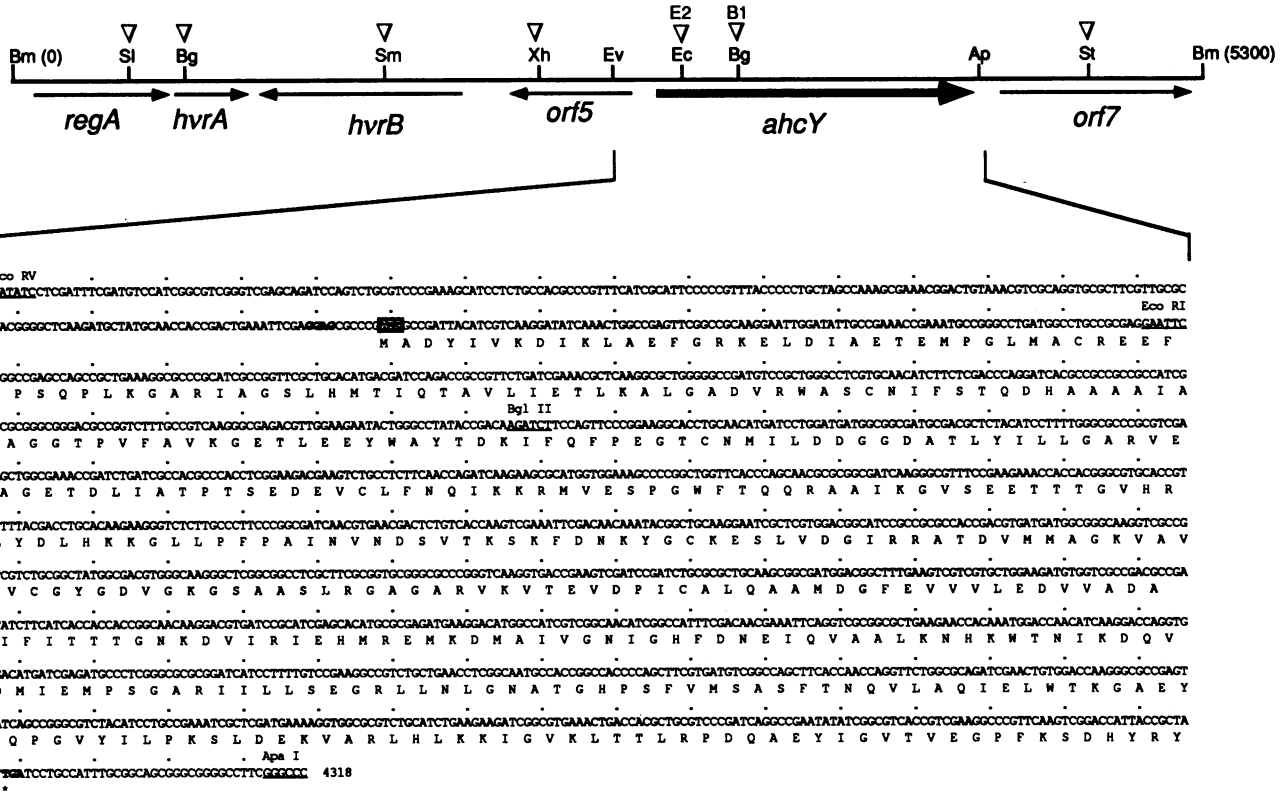

FIG. 2. Genetic and physical map of the photosynthesis regulatory gene cluster and sequence analysis of the *ahcY* locus. Arrows show the location of open reading frames in the 5300-base-pair regulatory gene cluster of *R. capsulatus*. Inverted open triangles denote the location of interposon insertion mutations constructed in this region. Open reading frames *hvrA*, *hvrB*, *orf5*, and *orf7*, as well as their chromosomal disruption, will be discussed elsewhere. Restriction sites: Bm, *Bam*HI; Sl, *Sal* I; Bg, *Bgl* II; Sm, *Sma* I; Xh, *Xho* I; Ev, *Eco*RV; Ec, *Eco*RI; Ap, *Apa* I; St, *Stu* I.

cells per ml. Fifty microcuries (1.85 MBq) of [^{35}S]methionine (SJ1015; Amersham) was added to a 200-μl aliquot of the cell suspension at 35°C, and the cells were incubated at 35°C for an additional hour. The cells were then chilled to 4°C and washed free of unincorporated [^{35}S]methionine with phosphate-buffered saline (10 mM potassium phosphate, pH 7.4/150 mM NaCl). The cell pellet was then immediately suspended in ice-cold 0.3 M perchloric acid, neutralized with an equivalent volume of 0.3 M potassium carbonate in 20 mM Tris·HCl (pH 6.8), and clarified by microcentrifugation.

Incorporation of [^{35}S]methionine into metabolites was determined after separation of the metabolites on a cation-exchange column. A Whatman Partisil 10 SCX column (4.5 × 250 mm) was equilibrated in 1 mM ammonium formate (pH 4.0, buffer A), the sample was applied, and the unbound compounds were removed by washing the column for 10 min with buffer A at a flow rate of 2 ml/min. Bound material was then eluted with a series of gradients made from buffer A and buffer B (0.2 M ammonium formate/0.8 M ammonium sulfate, pH 4.0) as follows: 0% B to 3% B in 20 min, 3% B to 20% B in 20 min, and 20% B to 80% B in 10 min. The column was then washed with 80% B for 10 min and reequilibrated with buffer A (25).

RESULTS

Sequence Analysis of *ahcY*. Sganga and Bauer (14) recently identified a region of the *R. capsulatus* genome that contains several genes responsible for regulating photosynthesis gene expression in response to oxygen and light intensity (Fig. 2). Insertion (interposon) mutagenesis of this cluster of genes was performed in an effort to characterize the involvement of this region in controlling expression of the photosynthetic apparatus. Several of the disruptions had subtle effects on the regulation of photosynthesis gene expression, which will be discussed in more detail elsewhere. Here we will discuss the dramatic decrease in photopigment production resulting from insertions at two closely linked sites (E2 and B1 in Fig. 2).

Sequence analysis showed that this region contained a single open reading frame that codes for a polypeptide with a molecular weight of 50,580 (Fig. 2). Analysis of homologs in the GenBank data base revealed that this protein displays a remarkable degree of amino acid sequence homology, approximately 65% identity and 77% similarity, to AdoHcyases that have been sequenced from rat (9), *Dictyostelium discoideum* (11), human (10), and *Caenorhabditis elegans* (GenBank, accession no. M64306) cDNA clones (Fig. 3). Comparison of the deduced amino acid sequence of the *R. capsulatus ahcY* gene product with those of its eukaryotic homologs reveals some interesting similarities as well as some significant differences. For example, a particularly high degree of sequence conservation exists across a region of the protein (*R. capsulatus* residues 247–278) that contains the putative NAD$^+$-binding domain, an observation that supports biochemical evidence that both the bacterial and eukaryotic AdoHcyase have bound NAD$^+$ (5, 8, 12, 13). Chemical modification studies by Gomi *et al.* (26) on rat AdoHcyase have demonstrated that 5′-*p*-fluorosulfonylbenzoyladenosine (FSBA) inactivates AdoHcyase by promoting disulfide bond formation between Cys-78 and Cys-112. They proposed that FSBA inactivated the enzyme by reacting with a cysteine at the AdoHcy site to form a sulfonylbenzoyladenosylated cysteine, which underwent rapid attack by a second cysteine sulfhydryl present in close proximity. Of interest therefore is the observation that cysteine at position 78 is conserved, whereas at position 112 it is replaced in *R. capsulatus* by tyrosine. Moreover, site-directed mutants where Cys-78 or Cys-112 is replaced by either serine or alanine are active (27). Together, the results of the mutagenesis study and the lack of conservation at Cys-78 and Cys-112 appear to rule out a direct involvement of either cysteine in catalytic activity.

Another notable feature of the bacterial enzyme is the presence of an additional 36 residues approximately one-third of the distance from the amino terminus that are not found in rat, *D. discoideum*, human, or *C. elegans* (Fig. 3). The presence of an additional amino acid segment has also been found in parsley (28). The additional segment in *R. capsulatus* does not have significant homology to the analogous segment in parsley.

In view of the similarity of the amino acid sequence encoded by the *R. capsulatus ahcY* gene to eukaryotic AdoHcyases, it was important to establish that the *ahcY* locus does, in fact, code for bacterial AdoHcyase. As shown

FIG. 3. Amino acid sequence homology between *R. capsulatus* AdoHcyase (Rca.AHCY) and AdoHcyase from the nematode *Caenorhabditis elegans* (Nem.AHCY), *Dictyostelium discoideum* (Dic.AHCY), rat (Rat.AHCY), and human (Hum.AHCY). Residues of the *R. capsulatus* protein that have corresponding identities in at least two of the homologs are starred. The putative NAD$^+$ binding site, amino acids 247–278 of *R. capsulatus*, is indicated. Numbers above the boxed cysteines refer to the numbering of the rat AdoHcyase.

in Table 1, the parent strain, *R. capsulatus* St. Louis, exhibits AdoHcyase activity that is comparable to that previously reported for the related bacterium *Rhodobacter sphaeroides* (4). In contrast, the StLB1 mutant has a complete absence of AdoHcyase activity even though the sensitivity of the assay is such that as little as 1% of the wild-type activity could have been detected. As a consequence, the intracellular AdoMet/AdoHcy ratio in StLB1 cells grown in complex medium is strikingly lower than that of the parent strain, *R. capsulatus* St. Louis (Table 1).

Mutational and Biochemical Analysis of AdoHcyase. The insertion mutation of *ahcY* in *R. capsulatus* is, to our knowledge, the first example of an AdoHcyase null mutation in either a prokaryote or a eukaryote and could therefore provide interesting insights into the relative importance of AdoHcyase activity in the metabolic physiology of bacteria. While two pathways, *de novo* synthesis from aspartate and sulfate and reutilization by AdoHcy hydrolysis (Fig. 1), can provide the homocysteine required for methionine biosynthesis in bacteria, their relative contributions may vary in different species and are not known for *R. capsulatus*. StLB1 cells, where homocysteine cannot be supplied by the hydrolysis of AdoHcy, should be entirely dependent on *de novo* synthesis to satisfy the requirement for a methionine precursor.

Table 2 shows that although the *ahcY* mutant StLB1 fails to grow in minimal medium, a normal growth rate is observed when this strain is grown in minimal medium supplemented with peptone and yeast extract. Growth is also restored when minimal medium is supplemented with methionine or homocysteine.

When grown under anaerobic or semiaerobic (<1.0% oxygen tension) conditions, photosynthetic bacteria synthesize large amounts of carotenoids and bacteriochlorophyll, which absorb light at 400–500 and 800–875 nm, respectively (Fig. 4, solid spectrum) (29). When grown under these conditions, the StLB1 mutant exhibits a most interesting characteristic in that it fails to synthesize appreciable amounts of photopigments (dashed spectrum), thereby indicating that bacteriochlorophyll synthesis is more sensitive to alterations in the AdoMet/AdoHcy ratio (Table 1) within the cell than is cell growth.

DISCUSSION

AdoHcyase Is a Highly Conserved Protein. In this paper, we report a prokaryotic gene encoding the enzyme AdoHcyase. Sequence analysis of AdoHcyase from *R. capsulatus* revealed that with the exception of a 36-amino acid segment, it exhibits ≈65% amino acid sequence identity to its eukaryotic homologs. To our knowledge, this is one of the highest levels of sequence conservation ever reported between proteins having a similar function in prokaryotic and human cells. The only other enzyme that approaches this level of sequence conservation is glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which exhibits ≈55% identity between the *E. coli* and the human enzymes (30). However, *E. coli* GAPDH is only 40% identical to other bacterial enzymes, leading to the suggestion that *E. coli* acquired GAPDH via horizontal transfer from a eukaryote (31). Along these lines it is interesting that the evolutionary ancestor of mitochondria is thought to be a member of the α-purple bacteria subdivision of which *R. capsulatus* is a member (32–34). By analogy, the high degree of sequence conservation of AdoHcyase might be a consequence of movement of *ahcY* from the mitochondria to the nucleus. Additional sequence information will be required in order to ascertain whether horizontal or organelle transfer has indeed occurred. A second, perhaps more obvious possibility is that lack of sequence divergence simply reflects an especially stringent requirement for this enzyme to retain its primary structure in order to function properly (11).

Table 1. Effect of mutationally disrupting *ahcY* on AdoHcyase activity, AdoHcy, and AdoMet

Strain	AdoHcyase activity*	^{35}S incorporation†		AdoMet/ AdoHcy
		AdoHcy	AdoMet	
St. Louis	7.7	27,500	743,000	27.0
StLB1	<0.1	286,000	487,000	1.7

Cells were grown aerobically in RCV 2/3 PY medium.
*Activity represents nmol of AdoHcy produced per min per mg of protein.
†Values represent dpm of [^{35}S]methionine incorporated into AdoHcy and AdoMet per 4 × 10^8 cells.

Table 2. Effect of metabolites on growth rate

Strain	Doubling time, hr			
	RCV	RCV + Met	RCV + Hcy	RCV 2/3 PY
St. Louis	7	8	7	3
StLB1	NG	12	16	3

Cells were grown aerobically at 35°C in the indicated medium. RCV, minimal medium; RCV + Met, RCV medium with 0.01 mM methionine; RCV + Hcy, RCV medium with 0.01 mM homocysteine; RCV 2/3 PY, RCV medium with peptone (2 mg/ml) and yeast extract (2 mg/ml). Cell density was measured with a Klett–Summerson photometer. NG, no growth.

***ahcY* Is Not an Essential Gene for *R. capsulatus* Grown in Supplemented Medium.** The ability of StLB1 cells to grow in a complex medium demonstrates that absence of AdoHcyase activity is not necessarily lethal and that the cell can tolerate a substantial alteration in the intracellular ratio of AdoMet to AdoHcy. The same conclusion was reached earlier by Fisher *et al.* (35), who reported that in *Alcaligenes faecalis* inhibition of AdoHcyase by neoplanocin A did not affect the growth of these organisms in broth cultures but inhibited growth in agar plates. The observation that addition of homocysteine or methionine allows cells disrupted for *ahcY* to grow in minimal medium is analogous to the findings of Kim *et al.* (36), who showed that addition of homocysteine rescued a mouse macrophage cell line from the cytostatic effects of 3-deazaaristeromycin, a potent inhibitor of AdoHcyase.

Effect of AdoHcyase on Bacteriochlorophyll Synthesis. Another result from this study is the dramatic inhibitory effect that *ahcY* disruptions have on bacteriochlorophyll biosynthesis. Methyl transfer has long been known to play an especially important role in bacteriochlorophyll biosynthesis

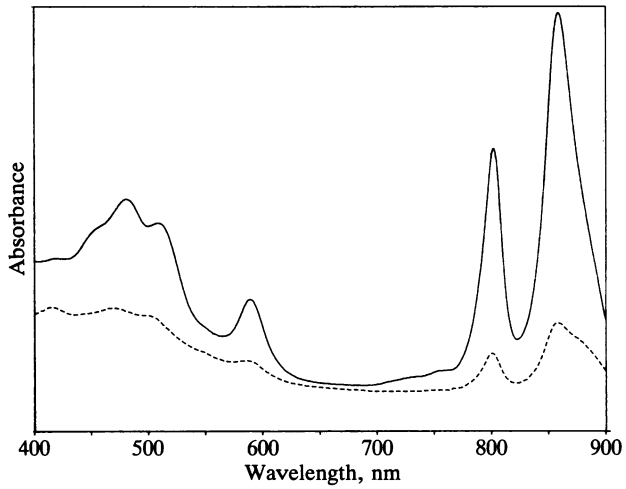

FIG. 4. Visible light absorption spectrum of photosynthetic membranes isolated from semiaerobically grown wild-type *R. capsulatus* strain St. Louis (—) and *ahcY* mutant strain StLB1 (- - -) when grown in RCV 2/3 PY medium. Major bacteriochlorophyll peaks are at 800 and 850 nm.

(Fig. 1) (37, 38). The biosynthetic pathways of bacteriochlorophyll and heme have common tetrapyrrole intermediates up to the stage of protoporphyrin IX, at which point the pathways diverge either by the addition of iron, resulting in the formation of heme, or by the addition of magnesium, resulting in the formation of magnesium-protoporphyrin IX. In photosynthetic bacteria, insertion of magnesium is thought to be coupled to a methyl-group transfer from AdoMet to the sixth propyl group of protoporphyrin IX (or magnesium-protoporphyrin IX) (39). The latter reaction results in the formation of magnesium-protoporphyrin monomethylester, which is the first stable intermediate unique to the bacteriochlorophyll biosynthetic pathway. This reaction is catalyzed by the enzyme AdoMet:magnesium-protoporphyrin methyltransferase, which has been characterized genetically (40, 41) as well as biochemically (42). Kinetic studies have shown that this methyltransferase is noncompetitively inhibited by AdoHcy, which is a direct product of the reaction (43–46), with the K_i value for AdoHcy (500 μM) being similar to the K_m value for AdoMet (106 μM) (42). Therefore, AdoMet:magnesium-protoporphyrin methyltransferase should be susceptible to alterations in the AdoMet/AdoHcy ratio. Furthermore, repression of *ahcY* transcription by high light intensity mimics repression of bacteriochlorophyll biosynthesis by high light intensity (J. J. Buggy, M.W.S., and C.E.B., unpublished data). This indicates that photosynthetic bacteria may control pigment biosynthesis in response to changes in light intensity, at least in part, by altering the intracellular AdoMet/AdoHcy ratio.

We wish to thank the members of the Photosynthetic Bacteria Group, Indiana University, and especially Dr. Howard Gest for helpful discussions and comments on the manuscript. This study was supported by a grant from the National Institutes of Health, GM40941-01.

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