Vol. 207, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 6, 1995 Pages 265-272

EXPRESSION OF RAT LIVER ADOHCY HYDROLASE IN A *RHODOBACTER CAPSULATUS ahcY* MUTANT RESTORES PIGMENT FORMATION AND PHOTOSYNTHETIC GROWTH

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Received December 12, 1994

An amino acid alignment of fourteen S-adenosylhomocysteine hydrolases shows that sequences from six photosynthetic species and one species possibly derived from algae have an internal 36 to 41 amino acid sequence that is not present in hydrolase sequences from seven non-photosynthetic species. In the photosynthetic eubacterium *Rhodobacter capsulatus*, the StLB1 strain has a disrupted hydrolase gene, and hydrolase activity is not detectable. Photopigment synthesis and photosynthetic growth are significantly reduced in the StLB1 strain. Introduction of rat hydrolase cDNA into the StLB1 strain restored hydrolase activity, photopigment synthesis and photosynthetic growth. The results show that the 36 amino acid sequence of *Rhodobacter capsulatus* S-adenosylhomocysteine hydrolase does not have a photosynthesis specific function.

S-Adenosyl-L-homocysteine (AdoHcy) hydrolase (EC 3.3.1.1), an enzyme that catalyzes the reversible breakdown of AdoHcy into adenosine and homocysteine (1), has been purified from several species. Complete or nearly complete (mouse) sequence data are available for hydrolases from fourteen species as diverse as bacteria and humans and indicate that the enzyme is composed of identical subunits that vary between 45,000 and 55,000 daltons. An amino acid sequence alignment demonstrates that the enzyme is remarkably conserved throughout evolution even in species with quite different codon usage, such as the eubacterium *Rhodobacter capsulatus*, the slime mold *Dictyostelium discoideum*, and rat (2,3) (Fig. 1). The amino acid sequence of AdoHcy hydrolase from *R. capsulatus* shows 64% identity with the human enzyme and 62% identity with the parsley enzyme. Moreover, if conservative replacements are taken into account, a much higher degree of similarity (approximately 77%) is evident. The single

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0006-291X/95 $5.00

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 1) ...DDGGDATLLI HEGVKAEEEY KKSGAIPDPA STDNAEFQIV LSIIRDGLKS DPMKYHKMKD RLVGVSEETT...
 2) ...DDGGDATLLI HEGVKAEEEY KKNGALPDPS STDNAEFQIV LTIIRDGLKS DPTKYTRMKE RLVGVSEETT...
 3) ...DDGGDATLLI HEGVKAEEEF AKNGTIPDPN STDNAEFQLV LTIIKESLKT DPLKYTKMKE RLVGVSEETT...
 4) ...DDGGDVTLLI HEGVKAEEVF EKTGQLPDPS STDNAEMQIV LTIIRDGLKT DPKRYQKMKT RIVGVSEETT...
 5) ...DDGGDATLLI HEGVKAEEEF EKSGKVPDPE STDNPEFKIV LTIIRDGLKT DASKYRKMKE RLVGVSEETT...
 6) ...DDGGDATLYI LLGARVEAG- -ETDLIATPT SEDEV---CL FNQIKKRMVE SPGWFTQQRA AIKGVSEETT...
 7) ...DDGGDATLLV HKGVEYEKLY EEKNILPDPE KAKNEEERCF LTLLKNSILK NPKKWTNIAK KIIGVSEETT...
 8) ...DDGGDLTNLV ID-------- ---------- ---------- ---------- ---HHPELVP KIFGISEETT...
 9) ...DDGGDLTTLV HE-------- ---------- ---------- ---------- ---KYPQFLA GIKGISEETT...
10) ...DDGGDLTNLV HA-------- ---------- ---------- ---------- ---KYPQYLA GIRGLSEETT...
11) ...DDGGDLTNLV HS-------- ---------- ---------- ---------- ---KYPQLLK GIKGISEETT...
12) ...DDGGDLTNLI HT-------- ---------- ---------- ---------- ---KYPQLLS GIRGISEETT...
13) ...DDGGDLTNLI HT-------- ---------- ---------- ---------- ---KHPQLLS GIRGISEETT...
14) ...DDGGDLTNLI HT-------- ---------- ---------- ---------- ---KYPQLLP GIRGISEETT...

    |---Region 1---||-----Region 2, plant-specfic sequence------||-----Region 3------|
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	Percent Sequence Identity			
	Region 1	Region 2	Region 3	All Regions
1) *Petroselinum crispum*	100	100	100	100
2) *Catharanthus roseus*	93	88	94	93
3) *Nicotiana sylvestris*	93	71	96	93
4) *Medicago sativa*	93	73	92	90
5) *Triticum aestivum*	91	73	90	89
6) *Rhodobacter capsulatus*	69	22	64	62
7) *Plasmodium falciparum*	62	27	59	57
8) *Leishmania donovani*	64	--	63	63
9) *Dictyostelium discoideum*	63	--	61	62
10) *Caenorhabditis elegans*	63	--	64	64
11) *Xenopus laevis*	60	--	64	63
12) *Mus musculus*	64	--	64	64
13) *Rattus*	62	--	63	63
14) *Homo sapiens*	59	--	64	62

Figure 1. The plant-specific sequence of AdoHcy hydrolase and the conservation of amino acid identity among species. The top part of the figure shows the amino acid sequence of the plant-specific sequence and flanking regions. Each sequence is numbered and the species corresponding to the number is indicated in the lower part of the figure. The dots at the beginning of each sequence represent the sequence from the initial methionine of the complete sequence to the first amino acid shown in the figure; similarly, the dots at the end of each sequence represent the continuation of the sequence to the last amino acid that is present in the complete sequence. Dashes have been inserted to align the sequences. Region I begins with the initial methionine and extends through the amino acid preceding the plant-specific sequence. Region II is the amino acid sequence found in plants and related species. Region III begins with the first amino acid following the plant-specific sequence and extends through the last amino acid of the sequence. The bottom part of the figure shows within each region the percent identity of each sequence with *P. crispum* which was arbitrarily chosen as the reference sequence. Values for the percent identity were generated with the GAP program (27).

most obvious difference among these sequences is a conserved amino acid segment of 36 to 41 amino acids that is present in AdoHcy hydrolase from plants (4-8), the photosynthetic eubacterium *R. capsulatus* (3), and the malarial parasite *Plasmodium falciparum* (9) (Fig. 1). Interestingly, *P. falciparum* is proposed to have evolved from a photosynthetic (algal) ancestor, a conclusion based on 16s ribosomal RNA sequence data and the existence of a 35 kb plastid-like cytoplasmic element (10-12). Since the additional sequence is not observed in non-

photosynthetic (or non-photosynthetically derived) organisms such as human (13), mouse (14), rat (15), *D. discoideum* (2), *Xenopus laevis* (16), the nematode *Caenorhabditis elegans* (17) or the protozoan *Leishmania donovani* (18), we will refer to the additional sequence as the plant-specific sequence. The plant-specific sequences from *Catharanthus roseus* (5), *Medicago sativa* (8), *Triticum aestivum* (7), *Nicotiana sylvestris* (6), *P. falciparum* (9), and *R. capsulatus* (3) are 88%, 73%, 73%, 71%, 27% and 22% identical, respectively, to the sequence from *Petroselinum crispum* (4) (Fig. 1). The function of the plant-specific sequence, if any, is unknown.

Strains of *R. capsulatus* that have a disrupted AdoHcy hydrolase gene (*ahcY*) (3) provide an experimental system to test the expression and functionality of hydrolases that are structurally different from the endogenous *R. capsulatus* hydrolase. The StLB1 strain of *R. capsulatus* has a disrupted *ahcY* gene and AdoHcy hydrolase activity is not detectable (3). In addition, the synthesis of bacteriochlorophyll, which requires AdoMet methylation of Mg-protoporphyrin IX by the enzyme S-adenosyl-L-methionine:magnesium protoporphyrin methyltransferase, is significantly reduced in *ahcY* mutants of *R. capsulatus* (3). The observed reduction of bacteriochlorophyll biosynthesis is presumably caused by the large intracellular accumulation of AdoHcy that is a competitive inhibitor of S-adenosyl-L-methionine:magnesium protoporphyrin methyltransferase (19,20).

In this paper we show that rat AdoHcy hydrolase can be expressed in *R. capsulatus* and that expression of the rat AdoHcy hydrolase in an *R. capsulatus* strain that has a disrupted endogenous *ahcY* gene restores pigment formation and photosynthetic growth. This result indicates that the endogenous *R. capsulatus* AdoHcy hydrolase that contains the plant-specific sequence can be functionally replaced by an AdoHcy hydrolase that lacks the plant-specific sequence.

METHODS

Growth of *R. capsulatus*, analysis of photopigments, and the assay for AdoHcy hydrolase activity with the substrate 3-deazaadenosine have been described previously (3). Oligonucleotides were synthesized by Synthecell Corp., Columbia, MD.

To express the rat AdoHcy hydrolase in *R. capsulatus*, a vector similar to that used for expression of rat hydrolase in *Escherichia coli* (21) was constructed by subcloning the *Rhodobacter puf* promoter upstream from the rat AdoHcy hydrolase cDNA and optimizing the distance between the promoter and hydrolase cDNA. For the first step of the construction, a 607 bp *Xho*I/*Sal*I fragment from p1255ex (22) that contains the *Rhodobacter puf* promoter and the first part of the *pufQ* gene was subcloned into *Sal*I-cut and dephosphorylated pBluescript II KS plus vector (Stratagene). The clones were screened for orientation with *Xho*I/*Sal*I double digests. Because there is an *Xho*I site immediately upstream from the *Sal*I site and because only one *Sal*I site is regenerated upon insertion of the *Xho*I/*Sal*I fragment, the desired orientation, in which the *puf* promoter is immediately downstream from the vector *Xho*I site, was indicated by the presence of a 0.6 kb fragment in the double digests. The vector containing the *puf* promoter and part of the *pufQ* gene in the desired orientation was named pPufbs.

For the second step of the construction, the 2 kb *Eco*RI fragment from pKAH77 that contains the previously published AdoHcy hydrolase cDNA (15) was subcloned into *Eco*RI-cut and dephosphorylated pPufbs. Clones were screened for orientation by *Kpn*I digests and plasmids with the hydrolase initiation codon immediately downstream from the *pufQ* fragment were named pPufrat.

For the third step of the construction, DNA beginning with the "A" of the ATG initiation codon of *pufQ* and ending with the "C" immediately preceding the ATG codon of the rat hydrolase cDNA was deleted by loopout oligonucleotide-directed mutagenesis. Single-stranded DNA was purified from a culture of XL1-Blue/pPufrat that had been infected with VCS-M13 phage (Stratagene) and selected for resistance to kanamycin. The phosphorylated mutagenic oligonucleotide (5'-CAGTTTATCAGCCATTTCAGTTCCC) was annealed to the single-stranded pPufrat DNA and extended to complete second strand synthesis by incubation with the Klenow fragment of DNA polymerase I and T4 DNA ligase. XL1-Blue MR cells were then transformed with the heteroduplex DNA, and the transformants were grown on Colony/Plaque Screen (NEN Research Products, Boston, MA) nylon membranes. Replicate filters of the bacterial transformants were prepared according to instructions supplied by the manufacturer. One filter was screened for hybridization to a vector-specific oligonucleotide (5'-CAGGGTTATTGTCTC-ATGAGCGG), and the other filter was hybridized to an oligonucleotide (5'-AAGCCAGCGTCT-TCGCGCTCATG) complementary to the DNA to be deleted. Autoradiograms from each filter were overlaid, and colonies were picked that hybridized to the vector-specific oligonucleotide but did not hybridize to the oligonucleotide specific for the looped out DNA. Clones were further characterized by restriction digests (eg., the presence of only a single *Eco*RI site), and plasmid (pΔPufrat) from a candidate clone was sequenced with T3, T7 and RRA003 (5'-GTACATCTCCCGCATGC) primers using the dideoxynucleotide chain termination method (23). Sequence analysis from the T3 primer confirmed that the *Sal*I site GTCGAC was converted to GTCGAG and that the *puf* promoter was in the correct orientation. Sequence analysis from the T7 primer confirmed that the AdoHcy hydrolase cDNA was in the correct orientation, and sequence analysis utilizing primer RRA003 confirmed that the desired DNA had been deleted.

To facilitate plasmid selection in *R. capsulatus*, a spectinomycin resistance gene (24) was subcloned into the *Bam*HI site of pΔPufrat located in the multiple cloning region downstream from the hydrolase cDNA creating the plasmid pΔPufrat-SpR. The construct pΔPufrat-SpR was then mobilized by conjugation from *E. coli* into *R. capsulatus* strain StLB1 (3) according to previously described techniques (25). A spectinomycin resistant exconjugate was purified and characterized for hydrolase activity, proliferative properties, and photopigment synthesis.

RESULTS

To determine if the rat AdoHcy hydrolase would be active and allow the synthesis of photopigments in a photosynthetic organism, we introduced the rat AdoHcy hydrolase cDNA under control of the *R. capsulatus puf* operon promoter into an *ahcY* mutant strain of *R. capsulatus*. In confirmation of the report by Sganga et al. (3), AdoHcy hydrolase activity was not detectable in strain StLB1 that contains a defined disruption of *ahcY* (Table 1). However, AdoHcy hydrolase activity was detectable when strain StLB1 *trans*-expressed the rat AdoHcy hydrolase cDNA clone on plasmid pΔPufrat-SpR (StLB1/pΔPufrat-SpR). The activity in this strain was approximately one-sixth of that found in the parent strain St. Louis that contained a wild-type *ahcY* loci.

The Sganga et al. (3) study also demonstrated that bacteriochlophyll synthesis was severely impaired in strain StLB1. As shown in Fig. 2, spectral analysis indicates that StLB1

Table 1. AdoHcy hydrolase activity in wild-type strain St. Louis, *ahcY* mutant strain StLB1, and the reconstituted *ahcY* mutant strain (StLB1/pΔPufrat-SpR) grown photosynthetically

Strain	Specific Activity*	Average Specific Activity
St. Louis	1.32, 1.06, 0.82	1.07
StLB1	ND	
StLB1 + rat hydrolase	0.24, 0.19, 0.12	0.18

* Values are nmoles of 3-deazaadenosylhomocysteine produced per min. per mg of protein determined for three different samples. ND, not detectable.

grown in PY complex medium contains significantly lower steady state levels of photopigments compared to the parent strain St. Louis. However, photopigment levels are significantly increased, approaching normal levels, in strain StLB1/pΔPufrat-SpR that expresses rat AdoHcy hydrolase.

We next addressed whether restoration of pigment biosynthesis in StLB1/pΔPufrat-SpR was sufficient to restore photosynthetic growth. The photosynthetic growth properties at two different intensities of light for the wild-type parent strain St. Louis, the *ahcY* disrupted strain StLB1, and strain StLB1/pΔPufrat-SpR that expresses the rat hydrolase are shown in Fig. 3. When grown in PY complex medium under high light intensity (8,000 lux), the doubling time of strain StLB1/pΔPufrat-SpR that expresses the rat hydrolase was 3 hours, a doubling time that

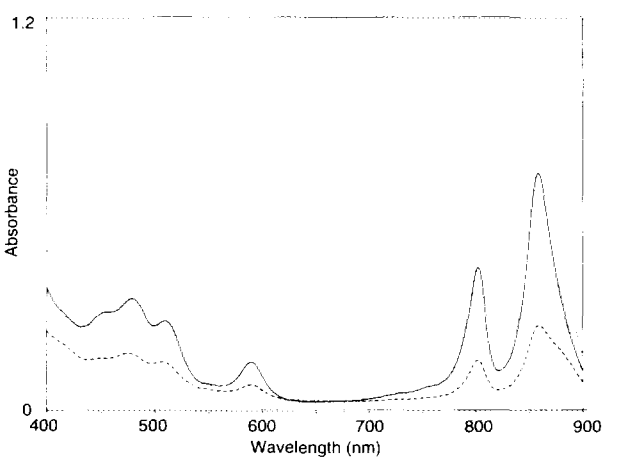

Figure 2. The absorbance spectrum of photopigments synthesized in wild-type strain St. Louis (*dotted line*), *ahcY* mutant strain StLB1 (*dashed line*), and StLB1 expressing rat AdoHcy hydrolase (*solid line*). *R. capsulatus* strains were grown photosynthetically in a medium of 0.3 % peptone and 0.3 % yeast extract. An equal number of cells were scanned for each *in vivo* absorbance spectrum (28).

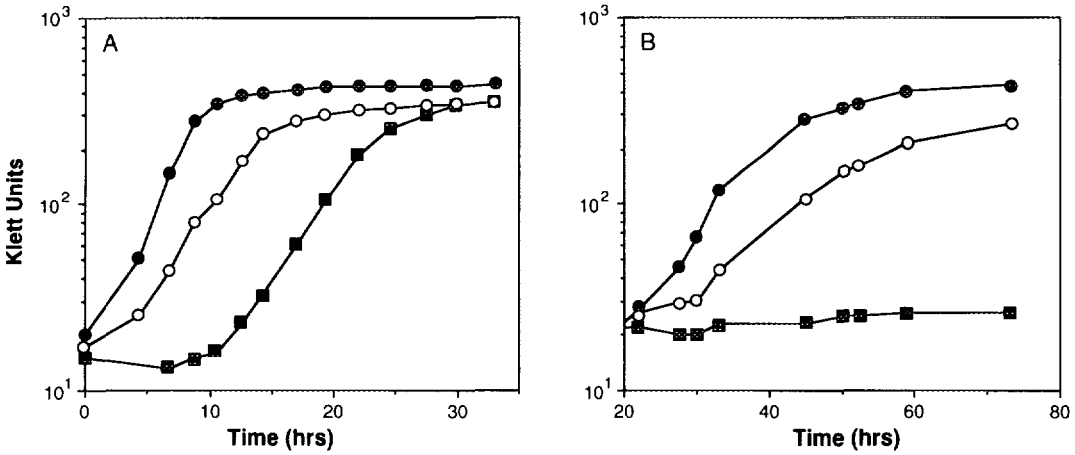

Figure 3. Photosynthetic growth curves for wild-type strain St. Louis (●), *ahcY* mutant strain StLB1 (■), and StLB1 expressing rat AdoHcy hydrolase (○). *R. capsulatus* strains were grown in a medium of 0.3 % peptone and 0.3 % yeast extract at either 8000 (part A) or 100 (part B) lux.

is intermediate between that of the wild-type strain St. Louis (1.8 hr) and that of *ahcY* disrupted strain StLB1 (4 hr). A more dramatic recovery of photosynthetic growth is observed when the cells are grown under low light intensity (100 lux). In this case StLB1 does not make enough photopigments to grow appreciably whereas both the wild-type strain St. Louis and StLB1/pΔPufrat-SpR exhibit reasonable doubling times of 4.5 and 9 hours, respectively.

DISCUSSION

We have shown that expression of rat AdoHcy hydrolase can functionally complement the *R. capsulatus ahcY* mutant strain StLB1. The observed level of rat hydrolase activity was one-sixth that found in wild-type cells and was sufficient to support the synthesis of significantly elevated levels of photopigments relative to that observed with the *ahcY* disrupted strain StLB1. StLB1/pΔPufrat-SpR grew photosynthetically in both high and low light intensities at a rate that was significantly faster than that observed for StLB1 but still less than that observed for the wild-type parent.

The presence of the plant-specific sequence in hydrolases from plants and *P. falciparum*, as well as its absence in non-photosynthetic species such as vertebrates, indicates an early evolutionary deletion or insertion in the hydrolase. The question arises as to why the insert has continued to persist in phototrophs as diverse as photosynthetic bacteria and higher plants. One possibility is that the plant-specific sequence might be specifically related to methylation of photosynthetic pigments and its regulation. However, the observation reported here that the synthesis of bacteriochlorophyll and concomitant photosynthetic growth are restored in an *R.*

capsulatus ahcY mutant that expresses a vertebrate hydrolase suggests that a photosynthesis specific function cannot be assigned to the plant-specific sequence present in *R. capsulatus* hydrolase. However, it should also be noted that AdoHcy hydrolase from tobacco is known to bind the plant hormone cytokinin that regulates a number of diverse cellular growth processes such as cell division (6,26). Although we cannot assign a function to the plant-specific sequence for the *R. capsulatus* enzyme, it remains to be determined if the plant-specific sequence might have a regulatory function in higher plants involving the binding of cytokinin or some other regulatory component.

ACKNOWLEDGMENTS

This project was supported in part by NIH grants GM 40941 and GM00618 to C.E.B. J.J.B. was also supported by an NIH predoctoral fellowship.

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