Magnesium Insertion by Magnesium Chelatase in the Biosynthesis of Zinc Bacteriochlorophyll a in an Aerobic Acidophilic Bacterium Acidiphilium rubrum*

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To elucidate the mechanism for formation of zinc-containing bacteriochlorophyll a in the photosynthetic bacterium Acidiphilium rubrum, we isolated homologs of magnesium chelatase subunits (bchI, -D, and -H). A. rubrum bchI and -H were encoded by single genes located on the clusters bchP-orf168-bchI-bchD-orf320-crtI and bchF-N-B-H-L as in Rhodobacter capsulatus, respectively. The deduced sequences of A. rubrum bchI, -D, and -H had overall identities of 58.8, 40.5, and 50.7% to those from Rba. capsulatus, respectively. When these genes were introduced into bchI, bchD, and bchH mutants of Rba. capsulatus for functional complementation, all mutants were complemented with concomitant synthesis of bacteriochlorophyll a. Analyses of bacteriochlorophyll intermediates showed that A. rubrum cells accumulate magnesium protoporphyrin IX monomethyl ester without detectable accumulation of zinc protoporphyrin IX or its monomethyl ester. These results indicate that a single set of magnesium chelatase homologs in A. rubrum catalyzes the insertion of only Mg²⁺ into protoporphyrin IX to yield magnesium protoporphyrin IX monomethyl ester. Consequently, it is most likely that zinc-containing bacteriochlorophyll a is formed by a substitution of Zn²⁺ for Mg²⁺ at a step in the bacteriochlorophyll biosynthesis after formation of magnesium protoporphyrin IX monomethyl ester.

Naturally occurring cyclic tetrapyrroles such as chlorophylls and hemes usually contain a metal atom chelated to the nitrogen atoms at the center of the macrocyclic ring. Chlorophylls and bacteriochlorophylls (Bchls) have a chelated magnesium atom and function in light harvesting and energy-generating charge separation. Very recently, however, an exception was reported that a purple bacterium, Acidiphilium rubrum, possesses zinc-containing Bchl a (zinc-Bchl a) as its major chlorophyll (1). This finding modifies our common understanding that naturally occurring chlorophylls and Bchls ubiquitously have a chelated magnesium atom at the center of macrocyclic ring.

In the biosynthetic pathway of chlorophylls and Bchls, magnesium chelation into protoporphyrin IX (Proto) is catalyzed by magnesium chelatase to successively form magnesium-Proto monomethyl ester, and the magnesium atom is retained in the subsequent synthesis of chlorophylls. Magnesium chelatase genes have been cloned from higher plants, cyanobacteria and photosynthetic bacteria, and they are composed of three cogenate genes, chlI (bchI), chlD (bchD), and chlH (bchH) (2–5). Homologous or heterologous combination of these three cogeneate gene products overexpressed in Escherichia coli reconstituted the magnesium chelatase activity (3–5).

Considering many investigations on metal chelation of tetrapyrroles in vivo and in vitro, the following three possible mechanisms can be proposed for the formation of zinc-Bchl a in A. rubrum: 1) The zinc atom is directly inserted into Proto by the catalysis of a novel “zinc chelatase.” 2) The zinc atom is directly inserted into Proto by a presently existing metal chelatase such as magnesium chelatase or ferrochelatase. 3) After magnesium is inserted into Proto by magnesium chelatase, a zinc atom is substituted for the magnesium atom enzymatically or non-enzymatically. In all cases, the zinc atom is thought to be retained throughout the rest of the biosynthesis, since it is known that enzymes involved in the steps after magnesium insertion in the chlorophyll biosynthetic pathway, such as NADPH-protochlorophyllide oxidoreductase (6) and chlorophyll synthetase (7), can use both magnesium and zinc chlorophyll derivatives as substrates. Besides these possibilities, formation of zinc-Proto has been reported in several biological materials. Ferrochelatase is known to catalyze in vitro chelation of divalent metals including zinc, and may form zinc-Proto in vivo in erythrocytes of patients affected with lead poisoning and iron deficiency anemia (8). In photosynthetic organisms, formation of zinc-Proto has been demonstrated with cell-free systems of such as Rhodobacter sphaeroides (9) and greening cucumber cotyledons (10). However, it may be formed nonenzymatically. Furthermore, it is well known that among chlorophyll derivatives containing metals other than magnesium, only zinc-containing chlorophylls have chemical features comparable to those of magnesium-chlorophylls (11), and have been widely used in studies of artificial photosynthesis by virtue of good stability of zinc-porphyrin derivatives (12). Thus, we cannot exclude the possibility that in A. rubrum zinc-Bchl is synthesized from such nonspecifically formed zinc-Proto, although

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The nucleotide sequence(s) reported in this paper have been submitted to the GenBank®/EBI Data Bank with accession number(s) AB017350 and AB017351.

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§ The abbreviations used are: Bchl, bacteriochlorophyll; zinc-Bchl, zinc-containing bacteriochlorophyll; Proto, protoporphyrin IX; HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction.
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No expression of the gene Acidiphilium are chemoheterotrophic aerobes and extremely acidophilic bacteria showing optimum pH for growth at around 3.0. They belong to the α-subclass of Proteobacteria that includes many typical Bchl-utilizing photosynthetic bacteria (13). It is, therefore, likely that the Acidiphilium species utilize zinc-Bchl instead of acid-labile Bchl in order to adapt themselves to the acidic environment. In fact, our previous study (14) showed that the ratio of zinc-Bchl/Bchl in A. rubrum cells increases as the pH of the growth medium decreases to pH 3.5, suggesting that zinc-Bchl accumulation may be correlated with the acidic environment of A. rubrum.

In this study, we demonstrate that in A. rubrum magnesium is initially inserted into proto by a magnesium chelatase homolog to form magnesium-Proto monomethyl ester and it is most likely that in a later step the magnesium atom is substituted by zinc atom.

EXPERIMENTAL PROCEDURES

Methods, Strains, and Growth Conditions—A. rubrum (ATCC35905) was grown aerobically in darkness by shaking at 60 strokes per min at 30 °C in 5 × BYG medium (pH 3.5) containing mineral salts, 0.05% yeast extract, and 0.5% fructose in L-shaped tubes (1). The photosynthetic bacterium Rba. sphaeroides strain 2.4.1 was cultured photosynthetically as described previously (15). The insertion mutants of bchI (strain DB50), bchD (strain DB561), and bchH (strain ZY6) of Rhodobacter capsulatus (16, 17) were grown chemoheterotrophically or phototrophically in PYS or RCV 2/3 PVC medium as described (18).

Cloning of the A. rubrum bchI, -D, and -H Genes—Two sets of degenerate primers were designed from conserved regions of the Bchl/Chl and the BchI/ChlH protein sequences. For bchI, an upstream primer (DG-U1) 5'-GGI/GC/GA/A/G/GAAGGC/GC/TTGGA/GC/GCC/GC/G-CCGA/(G/C)(T/C)TGCC-3' and a downstream primer (DG-D1) 5'-GGGCC/GC/GC/GAG/A/G/C/T/C/G/C/CT/C(G/T)GGCC-3' were designed from the invariant amino acid motifs of GEKA-FEPGLLA and GPNEEDD/G/EBLPRQ, respectively. Likewise, for BchI, an upstream primer (DG-UH) 5'-GC/G/C/TTCGGG/G/C/T/C/G/C/CC/GC/G-CC-3' and a downstream primer (DG-DH) 5'-GTCC/G/C/G/C/T/C/G/C/CC/GC/G/CC/GC/G-CC-3' were made from the invariant amino acid motifs of FYGEGDPMR and YAANNPSEAT, respectively. These primers were applied by PCR to amplify DNA from the A. rubrum genome. LA Toq polymerase (Takara Shuzo) was used according to the manufacturer's instruction. Temperature regime used was as follows: 95 °C for 1 min, followed by 30 cycles of 98 °C for 0.5 min and 68 °C for 2 min. Fragments of 221 and 257 base pairs were PCR amplified and cloned by the standard procedure (19).

For isolation of the A. rubrum bchI gene, approximately 10 μg of A. rubrum genomic DNA was digested with BamHI and StuI, and separated by agarose gel electrophoresis. Since the radiolabeled bchI PCR product hybridized to ~5 kilobases of BamHI- and ~4 kilobases of StuI-digested genomic DNA, DNA fragments around these sizes were recovered from gels and cloned into pET-202-1 vector (Invitrogen). The two obtained mini-genomic plasmid libraries were screened with the bchI probe. For isolation of the A. rubrum bchH gene, a genomic clone of A. rubrum, designated pAR2, which functionally complemented bchI- and bchD-disrupted mutant of Rba. capsulatus, appeared to contain A. rubrum bchH gene (see “Results”). The cosmID pAR2 was digested with BamHI and StuI, and the digested fragments were cloned into pET20-2.1 vector. Obtained plasmid clones were screened with the radiolabeled bchH PCR fragment. Library screening, DNA manipulations, and hybridizations were performed according to standard procedures (19). Double-stranded plasmids containing cloned gene fragments were sequenced with Li-COR Model 4000L sequencer and the Thermo Sequenase cycle sequencing kit (Amersham Pharmacia Biotech). Results were analyzed by the GENETYX program (Software Development).

Hybridizations—Genomic DNAs were isolated from A. rubrum cells according to standard procedures (19). After digestion by restriction enzymes, 10 μg of each digested DNA was separated on a 0.7% agarose gel and then transferred to a nylon membrane. This blot was hybridized with the respective 32P-labeled PCR product for 16 h at 65 °C. After hybridization, the blot was washed twice with 0.2 × SSC, 0.1% SDS for 15 min at 65 °C.

Oxidation of A. rubrum bchI, -D, and -H Genes in E. coli—To construct pET24a (Novagen) derivatives containing the bchI, -D, and -H genes, specific oligonucleotides including the stop and start codons from the respective gene sequences were designed with preceding NdeI site containing a start codon of each gene. These oligonucleotides were used as primers to produce NdeI-digested DNA by PCR and to introduce NdeI restriction sites for cloning the PCR fragments into pET2A2a vector. After sequence confirmation, these constructs were transformed into E. coli BL21(DE3) (Novagen) strain. Expression of recombinant proteins were induced by addition of isopropyl-β-D-thiogalactopyranoside to the cultures at a final concentration of 1 mM at 37 °C. After 3 h, the cells were harvested and extracted by sonic disruption. The disrupted cells were centrifuged at 15,000 × g for 15 min and the resultant supernatants were subjected for SDS-polyacrylamide gel electrophoresis (20) and used for magnesium chelatase activity assay as described by Kannangara et al. (21). For assay of zinc chelatase activity, zinc chloride was added to the reaction mixture instead of magnesium chloride. For in vitro transcription and translation of recombinant proteins, DNA templates of the pET2A2a derivatives were reacted with T3 (Stratagene) and PROTEIN-TK (Ambion) according to the manufacturer's instruction.

Complementation of Rba. capsulatus Mutants—Each gene fragment of the pET2A2a derivatives was introduced into NdeI and blunt ended PstI sites of pJZ102 vector (gifted from Z.-Y. Jiang and C. E. Bauer), yielding pZJ-bchI, pZJ-bchD, and pZJ-bchH. Plasmid pJZ102 is a derivative of pBBR1MCS5 (accession number U25001), which contains Rba. capsulatus puc promoter for high level of expression in Rba. capsulatus, and the ATG in Ndel site of this vector is positioned as the start codon of pucB gene. The plasmids of pET-bch constructs were also digested with XbaI and relevant restriction enzymes, and resulting fragments containing each gene with a ribosome-binding site originated from pET2A2a were introduced just downstream of bch genes in the relevant pZJ-bch plasmids. All 6 combinations of genes, which were doubly ligated at the same transcriptional direction under puc promoter of pZJ102, were constructed, and designated pZJ-bchI-D, -I-H, -D-H, and -H-I. Likewise, all 6 combinations of triple ligated pZJ-bch plasmids were constructed by preparing 6 combinations of the double ligated fragments containing a ribosome-binding site and each gene in plasmid II-5K, followed by introduction of these cassettes into relevant pZJ-bch plasmids, yielding pZJ-bchI-D-H, -I-H-D, -D-I-H, -I-D-H, and -H-D-I. The above 15 pZJ-bch constructs and pZJ102 vector were transformed into E. coli strain S17-1 (22), permitting conjugal transfer of each of the plasmids into the insertional mutants of Rba. capsulatus (16, 17). Complementation of mutants of Rba. capsulatus strains was performed by conjugation, followed by selection of prototrophic colonies in RCV medium, and analyzed by PCR.

Fragment Analysis and High Performance Liquid Chromatography—A 4-day grown 50-ml culture of cells were pelleted by centrifugation at 10,000 × g for 10 min. Cell pellets were washed in 0.1 M Tris-HCl (pH 7.0), and pigments were extracted as described by Belanger and Rebeiz (23). For analysis of Bchls, the resulting hexane fractions which contains pigments free of carotenoids and esterified Bchls, was obtained with an excitation wavelength at 415 nm with a fluorescence detection at 780 nm. HPLC analysis of Bchls was achieved using the method of Shioi et al. (24). Sample was loaded onto a Asahipak ODP-50 column (250 × 4.6 mm inner diameter, Showa Denko, Tokyo) and eluted using a linear mobile-phase gradient from methanol, 1 mM ammonium acetate (8:2, v/v) to acetonitrile/acetone (7:3, v/v) with a flow rate of 0.8 ml/min at 27 °C. The elution of pigments was monitored fluorimetrically using excitation wavelength at 418 nm and 100% solvent B for 20 min (25). The elution of pigments was maintained isocratic for 10 min. The elution of pigments was monitored fluorimetrically using wavelength of excitation at 370 nm and emission at 780 nm.

For analysis of polar porphyrins, the resulting diethyl ether fraction, which contains pigments free of carotenoids and esterified Bchls, was subjected to spectral analysis and HPLC. Fluorescence spectra were obtained with an excitation wavelength at 415 nm with a fluorescence spectrophotometer (Hitachi Model 850). Porphyrins were separated by HPLC on a Zorbax ODS (Du Pont) column (250 × 4.6 mm inner diameter) at a flow rate of 0.8 ml/min. Pigments were eluted with linear gradient of solvent B (90% methanol, 0.1 mM ammonium acetate) in solvent A (0.1 mM ammonium acetate) as follows: 20–100% over 10 min and 100% solvent B for 20 min (25). The elution of pigments was monitored fluorimetrically using excitation at 595 nm and emission at 630 nm.
and its monomethyl ester, respectively. Zinc substitution was carried out directly into porphyrins in the extract of *Rba. sphaeroides* by the metal acetate method (27).

### RESULTS

Cloning of *bchI*-D, and -H from *A. rubrum*—For PCR amplification, we used two sets of degenerate primer pairs, designed from conserved regions of the BchI/ChlI and the BchH/ChlH protein sequences and subsequently cloned two fragments of *A. rubrum* genomic DNA. Sequence analysis revealed that the two fragments were highly identical to the *Rba. capsulatus* bchI (90.5%) and bchH (87%) genes, respectively. These fragments were used to screen a genomic library of *A. rubrum*. For isolation of the *bchI* gene, we obtained two overlapping plasmid clones designated pGBB4z and pGSS3z. Restriction mapping and sequence analysis revealed that these two clones comprised 4,965 bp of the *A. rubrum* genome which includes the genes *bchF*-orf168-*bchI*-bchD-orf210-*crtI*, showing that genes for BchI biosynthesis in *A. rubrum* were clustered like *Rba. capsulatus* (Fig. 1). For isolation of the *bchH* gene, by PCR amplification and Southern hybridization analyses, we found that a genomic clone of *A. rubrum* in cosmid pJR215, designated pAR2, which functionally complemented *bchN*-disrupted mutant of *Rba. capsulatus*, contained the *A. rubrum* bchH gene. From pAR2 cosmid DNA, we cloned two overlapping plasmid subclones, designated pHCE9z and pHCSS7z. These two clones comprised 7,482 base pairs of the plasmid subclones, designated pHC9z and pHCSS7z. These fragments were used to screen a genomic library of *A. rubrum*. Sequenced subclones are shown as solid lines. Putative genes and their transcriptional directions are indicated by arrows. The corresponding regions of *Rba. capsulatus* genes (16) are also depicted below.

**FIG. 1.** Organization of the gene cluster for Bchl biosynthesis in the genome of *A. rubrum*. For sequence determination of the *bchH* and *bchI*-D gene clusters, two sets of overlapping plasmids designated pHCE9z and pHCSS7z, and pGBB4z and pGSS3z, respectively, were isolated. Sequenced subclones are shown as solid lines. Putative genes and their transcriptional directions are indicated by arrows. The corresponding regions of *Rba. capsulatus* genes (16) are also depicted below.

The *A. rubrum* BchD significantly resembled the entire BchI sequences (30.4 and 28.8% amino acid identity to BchI of *A. rubrum* and *Rba. capsulatus*, respectively). Like *Rba. capsulatus*, the NH$_2$ terminus phosphate-binding motif conserved in BchD was lost in *A. rubrum* BchD. Immediately after the BchI homologous region, a proline-rich sequence at the center, followed by highly charged stretch of amino acids was observed in the *A. rubrum* BchD like other BchD homologs. The *A. rubrum* *bchH* gene encoded a protein of 1,200 amino acids with a calculated mass of 129.3 kDa and 50.7% amino acid identity to BchH of *Rba. capsulatus*. Three completely conserved His residues in other BchH homologs, which are considered to be associated with binding of substrates and catalysis of magnesium chelatase (28), were also conserved in the *A. rubrum* BchH (positions at 589, 593, and 734). Comparison of hydrophyty profiles of *A. rubrum* BchI, -D, and -H with those of *Rba. capsulatus* showed no significant difference between the two species (data not shown). Genomic Southern hybridizations with the radiolabeled fragments described in the previous section showed a single band, indicating that each gene is encoded by a respective single gene in *A. rubrum* (Fig. 2).

Overexpression and Reconstitution of the *bchI*, -D, and -H Gene Products—DNA fragments containing the *A. rubrum* *bchI*, -D, and -H genes were separately cloned into pET24a, and the *E. coli* cultures containing these constructs were induced with isopropyl-β-D-thiogalactopyranoside. *In vivo* expression in *E. coli* induced recombinant BchI and BchH proteins in the predicted molecular weight sizes on SDS-PAGE gel, whereas the BchD protein was not detected as in the case of *Rba. sphaeroides* (4), probably because of very low abundance (Fig. 3A). However, an *in vitro* transcription and translation experiment showed that all three proteins were expressed at the predicted molecular weight sizes (Fig. 3B). We tried to reconstitute metal chelatase activity by combination of all these *in vivo* and *in vitro* expressed proteins. However, unlike the case of *Rba. sphaeroides* (4), magnesium chelatase activity was hardly detected in various buffer systems and at the pH value tested (data not shown). In addition, when zinc was added to the reaction mixture instead of magnesium, a substantial quantity of zinc-Proto was always formed even without recombinant protein(s), indicating that zinc-Proto can be easily formed non-enzymatically *in vitro*. Therefore, we concluded that in the *in vitro* reconstitution experiment it was difficult to distinguish whether the *A. rubrum* chelatase catalyzes only the insertion of magnesium or both magnesium and zinc.

**FIG. 2.** Western blot analysis for the expression of Bchl biosynthetic enzymes in *A. rubrum*. A. *in vivo* expression in *E. coli* induced recombinant BchI and BchH proteins in the predicted molecular weight sizes on SDS-PAGE gel, whereas the BchD protein was not detected as in the case of *Rba. sphaeroides* (4), probably because of very low abundance (Fig. 3A). However, an *in vitro* transcription and translation experiment showed that all three proteins were expressed at the predicted molecular weight sizes (Fig. 3B). We tried to reconstitute metal chelatase activity by combination of all these *in vivo* and *in vitro* expressed proteins. However, unlike the case of *Rba. sphaeroides* (4), magnesium chelatase activity was hardly detected in various buffer systems and at the pH value tested (data not shown). In addition, when zinc was added to the reaction mixture instead of magnesium, a substantial quantity of zinc-Proto was always formed even without recombinant protein(s), indicating that zinc-Proto can be easily formed non-enzymatically *in vitro*. Therefore, we concluded that in the *in vitro* reconstitution experiment it was difficult to distinguish whether the *A. rubrum* chelatase catalyzes only the insertion of magnesium or both magnesium and zinc.

Complementation Analysis of *bchI*, -D, and -H Mutants of *Rba. capsulatus*—In addition to the reconstitution experi-

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2 K. Inoue, unpublished result.
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Complementation of the Rba. capsulatus mutant strains was performed by conjugation with E. coli strain S17–1 harboring pZJ102 plasmid derivatives, followed by selection for photosynthetic growth in RCV medium. Results are a summary of five independent experiments.

| Plasmid          | Mutant (strain) |
|------------------|-----------------|
| bchI (DB350)     | bchD (DB561)    | bchH (2Y6)    |
| pZJ102           | bchI           | bchD          | bchH          |
| pZJ-bchI         | bchI           | bchD          | bchH          |
| pZJ-bchD         | bchI           | bchD          | bchH          |
| pZJ-bchH         | bchI           | bchD          | bchH          |
| pZJ-bchI-D       | ++             | ++            | +             |
| pZJ-bchI-H       | +              | +             | +             |
| pZJ-bchD-I       | +              | +             | +             |
| pZJ-bchD-H       | +              | +             | +             |
| pZJ-bchH-I       | +              | +             | +             |
| pZJ-bchH-D       | +              | +             | +             |
| pZJ-bchH-I-D     | +              | +             | +             |
| pZJ-bchH-D-I     | +              | +             | +             |
| pZJ-bchH-D       | ++             | ++            | ++            |
| pZJ-bchH-I-D     | ++             | ++            | ++            |
| pZJ-bchH-D       | ++             | ++            | ++            |
| pZJ-bchH-I-D     | ++             | ++            | ++            |
| pZJ-bchH-D       | ++             | ++            | ++            |

++ in Table I, and no complementation of bchH mutant was observed. However, when the incubation period was prolonged, tiny colonies frequently or always appeared from all bchI, -D, and -H mutants strains. Although the frequency and the time required for colony formation varied, the Rba. capsulatus bchI mutant was essentially complemented by pZJ derivatives that contained the A. rubrum bchI gene in constructs (evaluated + or ± in Table I). With the bchD mutant, occasional or frequent complementation by pZJ-bchD-I and pZJ-bchI-D-H was observed. The complementation of the bchH mutant was observed in conjugants with pZJ-bchH, pZJ-bchH-D, pZJ-bchI-H-D, pZJ-bchH-I-D, and pZJ-bchH-D-I. We should note that even in low frequency and weak complementations such as observed with pZJ-bchI-H for the bchI mutant, and with pZJ-bchH and pZJ-bchH-I-D for the bchH mutant, such complementations were clearly distinguishable from reversion of the mutants, which recovered the ability of photosynthetic growth, because complemented colonies were formed from most of the conjugation spots and these were able to grow in the presence of selection antibiotics for pZJ102, while the revertants occasionally formed a few colonies from conjugation spots, but these were sensitive to antibiotic.

Among 15 pZJ derivatives, pZJ-bchH-I-D was most effective for complementation of all bchI, -D, and -H mutants. Fig. 4 shows HPLC analysis of BchI accumulated in the Rba. capsulatus bchI, -D, and -H mutants complemented by pZJ-bchH-I-D. Each complemented mutant accumulated only Bchl a (Fig. 4, traces 2, 3, and 4), and when compared with pigment extract from A. rubrum cells (trace 5), no accumulation of zinc-Bchl a was observed. HPLC analysis of other complemented mutants also showed accumulation of only Bchl a in the cells (data not shown), indicating that the A. rubrum BchI, -D, and -H proteins form an active magnesium chelatase complex in the Rba. capsulatus.

Pigment Analysis of A. rubrum Cells—To confirm whether a set of the three subunits of A. rubrum catalyzes insertion of only magnesium into Proto, we examined the formation of porphyrin intermediates in Bchl biosynthesis in A. rubrum cells. It is known that a small quantity of magnesium-Proto...
monomethyl ester accumulates in wild type cells of *Rba. sphaeroides* (29), and magnesium-Proto and its monomethyl ester accumulate abnormally in dark-grown etiolated cucumber cotyledons treated with 2,2'-dipyridyl (26). By using these extracts and commercially available zinc-Proto as standards, we examined the fluorescent properties of the extract from *A. rubrum*. magnesium-Proto and its monomethyl ester in diethyl ether extracts of 2,2'-dipyridyl-treated cucumber and magnesium-Proto monomethyl ester in *Rba. sphaeroides* extract have characteristic fluorescent properties with excitation and emission maxima at 418 and 594 nm, respectively (Fig. 5, traces 1 and 3). The peaks of emission and excitation in the spectra of zinc-Proto standard were slightly blue-shifted (excitation and emission maxima at 417 and 589 nm, respectively) with respect to those of magnesium-Proto (Fig. 5, trace 2). The excitation and emission maxima of *A. rubrum* extract were slightly blue-shifted (excitation and emission maxima at 417 and 589 nm, respectively) with respect to those of magnesium-Proto (Fig. 5, trace 2). The excitation and emission maxima of *A. rubrum* extract were 417 and 593–594 nm, respectively (Fig. 5, traces 1 and 3). In contrast, authentic zinc-Proto (trace 2) was eluted between magnesium-Proto and its monomethyl ester at 20.85 min. To estimate the retention time of zinc-Proto monomethyl ester, magnesium-Proto monomethyl ester in the extract of *Rba. sphaeroides* was chemically replaced with zinc (27) and the resulting zinc-Proto monomethyl ester was eluted at 24.63 min (trace 4). The elution profile of tetapyrroles extracted from *A. rubrum* was almost identical to that of *Rba. sphaeroides*, indicating that *A. rubrum* mainly accumulated the porphyrin intermediate magnesium-Proto monomethyl ester. Neither zinc-Proto nor zinc-Proto monomethyl ester was detected. It is, therefore, concluded that magnesium is inserted into Proto at the metal chelation step in zinc-Bchl biosynthetic pathway of *A. rubrum*.

**DISCUSSION**

The complementation experiment and pigment analysis described in this study demonstrated that *A. rubrum* synthesizes a set of cognate subunits of magnesium chelatase that catalyze the insertion of magnesium but not of zinc into Proto. Furthermore, since no zinc-Proto nor its monomethyl ester was detected in *A. rubrum* cells, the possibilities of nonspecific ferrochelatase-mediated or non-enzymatic zinc chelation into Proto are unlikely. It is, therefore, most likely that zinc-Bchl a is formed in *A. rubrum* by substitution of zinc for magnesium after the biosynthesis of magnesium-Proto monomethyl ester, supporting the third possibility described in the Introduction.
mutant, the *A. rubrum* Bchl can form an active complex with the *Rba. capsulatus* BchD subunit. On the other hand, to complement the *bchD* mutant, the concomitant existence of *A. rubrum* *bchI* and -D genes in the constructs was always necessary except for pZJ-*bchD*-I-H and pZJ-*bchD*-H-I, suggesting that *A. rubrum* BchD can only associate with *A. rubrum* BchI and not with *Rba. capsulatus* BchI. It is interesting to note that the transcriptional order of the *bchD* gene in double- or triple-ligated constructs seems to be critical for formation of the active complex. Except for low frequency complementation by pZJ-*bchD*-I, some *bch* gene(s) preceded the *bchD* in all successful cases, indicating that such precedence may be crucial for expression of the *bchD* gene. It is likely that such heterologous or homologous pre-activated BchI-BchD complexes can form an active magnesium-chelatase complex with *Rba. capsulatus* BchH. Since the *bchH* mutant was rescued by pZJ-*bchH* and pZJ-*bchH*-D with relatively low frequency, we presume that *A. rubrum* BchH can form an active but weak complex with *Rba. capsulatus* BchI and -D. We do not know why the transcriptional order of the *bchH* gene severely affected the degree of complementation. In any case, it was clearly demonstrated by HPLC analysis of Bchls that the *A. rubrum* *bchI*, -D, and -H gene products catalyze insertion of only magnesium into Proto in *Rba. capsulatus* in vivo.

Interesting questions are where and how the zinc substitution takes place during the biosynthesis of zinc-Bchl σ in *A. rubrum*, and whether a biological catalyst is involved in this reaction. The chemistry of metal-porphyrins will provide insight into these questions. Metalation of porphyrins with divalent metal ions depends on deprotonation of the pyrrole nitrogen atoms, together with the removal of metal-coordinated ligands such as water molecules (30). Zinc is the second easiest metal after copper for insertion into porphyrins (31), and it is easily introduced into porphyrin without any catalyst (9, 10). The difficulty of zinc insertion is increased as the macrocyclic ring is reduced from chlorin to bacteriochlorin (32). Although in an acidic environment zinc can be introduced by direct metal exchange into magnesium complexes of porphyrins, chlorins, and bacteriochlorins, these reactions require substantial heat energy for completion. Consequently, if zinc substitution takes place non-enzymatically, one of the most probable ways is spontaneous zinc insertion into hypothetical pheophytinized intermediates after magnesium-Proto monomethyl ester formation. Concerning the possibility of the involvement of biological catalyst, there is nothing known about the presence of distinct zinc chelatase or “metal-exchange enzyme” so far. The substrate specificity of ferrochelatase seems to be limited to porphyrin derivatives, and ferrochelatase has no metal exchange activity with magnesium-porphyrins; rather than it is significantly inhibited by them (33). However, if we assume that zinc insertion takes place non-enzymatically, there should be zinc incorporation and concentration mechanism in *A. rubrum* cells, because there is only a trace amount of zinc as a component of the yeast extract (0.5 g/liter) in the growth medium of *A. rubrum*. It is estimated that zinc in the medium is quantitatively incorporated in zinc-Bchl σ of *A. rubrum*, indicating that *A. rubrum* may vigorously incorporate zinc into the cells for synthesis of zinc-Bchl σ, and without this mechanism, a reaction of non-enzymatic zinc insertion into Proto may not proceed in the medium containing such a low concentration of zinc.

From our previous results, it seems likely that the acidic environment is correlated with an increased ratio of zinc-Bchl/ Bchl in *A. rubrum* (14). Since it is known that zinc is more

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soluble at acidic pH than neutral, and the magnesium-porphyrins are easily pheophytinized by acid, these circumstances may be correlated with the increased ratio of zinc-Bchl/Bchl in the cell. However, the intracellular pH of acidophilic bacteria was indirectly estimated to be neutral by the optimum pH of a cytoplasmic enzyme and by the distribution of a radioactive 5,5′-dimethyl-2,4-oxazolidinedione across the cell membrane (34–36). The relationship between the acidic environment and zinc replacement after magnesium-Proto monomethyl ester replacement (34–36). The relationship between the acidic environment and zinc replacement after magnesium-Proto monomethyl ester remains to be elucidated.

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