Rapid Report

Discovery of Natural Photosynthesis using Zn-Containing Bacteriochlorophyll in an Aerobic Bacterium Acidiphilium rubrum

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We discovered natural photosynthesis using Zn-containing bacteriochlorophyll a in an acidophilic bacterium Acidiphilium rubrum. Chemical analysis of the cell extracts gave a 13:2:1 molar ratio of Zn-bacteriochlorophyll a:Mg-bacteriochlorophyll a:bacteriopheophytin a.

Most of the pigments are associated with fully active reaction center and light-harvesting complexes analogous to those in purple photosynthetic bacteria. The finding indicates an unexpectedly wide variability of photosynthesis.

Key words: Bacteriochlorophyll — Chlorophyll — Electron transfer — Photosynthesis — Photosynthetic bacteria — Reaction center.

Photosynthesis has been converting solar energy to chemical energy since its evolution on the early Earth, by use of the functions of Mg-containing porphyrin derivatives, namely, chlorophylls. The appropriate oxidation-reduction potentials and long-lived excited states of chlorophylls are favorable for the electron transfer reactions and the light-harvesting. These may be the reason for the photosynthetic organisms to choose chlorophylls among others. Among the chlorophyll derivatives containing metals other than Mg, only Zn-containing chlorophylls have chemical features comparable to Mg-chlorophylls (Watanabe and Kobayashi 1991). Zn-porphyrin derivatives are usually more stable than Mg-derivatives, and have been widely used in the studies of artificial photosynthesis (Wasielewski and Niemczyk 1984, Osuka et al. 1993). Zn-containing bacteriochlorophyll a (Zn-BChl, which more strictly should be termed Zn-bacteriopheophytin) has ever been introduced artificially into the isolated antenna proteins as replacement of light-harvesting Mg-BChl or of accessory Mg-BChl in the reaction center complex (Scheer and Hartwich 1995, Naveke et al. 1995). However, photosynthesis without (Mg-)chlorophylls has never been known. We here firstly report natural photosynthesis that utilizes Zn-BChl (see Fig. 3, inset).

Some species of bacteria which belong to the recently established genus Acidiphilium contain a pigment analogous to bacteriochlorophyll a (BChl) (Wakao et al. 1993, 1994, Kishimoto et al. 1995a). They are chemoheterotrophic aerobes and grow at pH 2.5–6.0. Sequence analysis of the small subunit ribosomal RNA (Kishimoto et al. 1995b) indicated that this genus belongs to the α-subclass of Proteobacteria that includes many of typical photosynthetic bacteria, such as Rhodospirillum (Rsp.) rubrum (Imhoff and Trüper 1992). A representative species, Ac. rubrum (Wichlacz et al. 1986), was found to accumulate a significant amount of this pigment and exhibit light-induced enhancement of CO₂-fixation (Kishimoto et al. 1995a). The bacterium, therefore, was assigned to a member of so-called “aerobic-photosynthetic” or “pseudo-photosynthetic” bacteria that biosynthesize photosynthetic apparatus even under aerobic dark conditions as reviewed by Shimada (1995).

In this communication we report that the major pigment in Ac. rubrum is Zn-BChl and that the photosynthetic apparatus consisting of this pigment is fully active.

Materials and Methods—Acidiphilium rubrum (ATCC 35905) cells were grown aerobically in darkness at 30°C under air bubbling in BYG medium (pH 3.5) consisting of mineral salts (without special addition of zinc), yeast extract (0.02%), and glucose (0.5%) (Wakao et al. 1993). The cells were washed, resuspended in 50 mM phosphate buffer (pH 7.5), and then disrupted by ultrasonication followed...
by centrifugation. The resultant membrane pellets were washed twice and resuspended in 50 mM Tris-HCl buffer (pH 7.5) and subjected to spectral analyses. Membranes of phototrophically grown cells of *Rsp. rubrum* were isolated by similar procedures.

Pigments were extracted with acetone-methanol (7:2-5:5, v/v) from fresh wet cells by sonication in darkness at 4°C. The extract was filtered and subsequently dried under vacuum or N₂ gas. The dried materials were dissolved in diethyl ether and then analyzed at 4°C by use of a silica gel HPLC column with n-hexane-isopropanol-methanol (100:2:1, v/v) as the eluent as described in Kobayashi et al. (1991). Carotenoids were determined by HPLC and field desorption mass spectrometry as described in (Takachi and Shimada 1992, Takachi 1993). Silica gel thin-layer chromatography was done according to Oelze (1985). In analysis of Zn- or Mg-BChl by fast atom bombardment mass spectrometry, purified pigments were mixed with p-nitrobenzyl alcohol as the matrix and analyzed according to de Pauw (1990). Metal contents of pigments were determined by inductively coupled plasma spectrometry.

Light-induced absorption changes of isolated membranes were measured after the excitation with a 532-nm, 10-ns laser flash from the second harmonic of Nd-YAG laser, or with a 3-μs xenon flash, using a split beam spectrophotometer with a 3-μs time resolution according to Iwaki and Itoh (1991).

**Chemical identification of Zn-BChl**—The absorption spectrum of isolated membranes of *Ac. rubrum* (Fig. 1, solid line), which included almost all the pigments of this organism, resembled that of the membrane of *Rsp. rubrum*, a representative of the purple photosynthetic bacteria (Fig. 1, broken line). The peaks at 377, 590, 792 and 864 nm of *Ac. rubrum* were blue-shifted by 5 to 15 nm from the corresponding peaks of Mg-BChl in *Rsp. rubrum*. The peaks of *Ac. rubrum* at 486, 515 and 549 nm were determined to be due to spirilloxanthin by HPLC and mass spectrometry as described in Materials and Methods. The close similarity in the spectral patterns suggests that *Ac. rubrum* has a set of pigment-protein complexes similar to those in *Rsp. rubrum*, which contains Mg-BChl and spirilloxanthin in the core-type light-harvesting complex (LH1) and the photochemical reaction center complex as well (Zuber and Cogdell 1995). The difference in peak positions between the two organisms, on the other hand, suggests that the pigment in *Ac. rubrum* differs from Mg-BChl.

Thin layer chromatography of the crude pigment extract from *Ac. rubrum* cells showed that the main band of the major purple pigment was preceded by a minor blue band of Mg-BChl (data not shown). HPLC analysis confirmed that the major pigment was different from Mg-BChl.

**Fig. 1** Absorption spectra of the membrane fractions prepared from *Ac. rubrum* (solid line) and *Rsp. rubrum* (broken line).

**Fig. 2** HPLC elution profiles of an extract of *Ac. rubrum* monitored at 388 nm (A) and absorption spectra of the major components (B). The spectra for peaks II (solid line in B) and III (broken line in B) correspond to P-763 (Zn-BChl) and Mg-BChl, respectively. Peak I and the component with a retention time of 3 min correspond to BPhe and spirilloxanthin, respectively.
We also detected small amounts of Mg-BChl and bacteriopheophytin a (BPhe). The isolated purple pigment (Fig. 2A, peak II) showed absorption maxima at 353, 389, 559 and 763 nm in diethyl ether and was tentatively designated P-763. The respective absorption maxima of P-763 were blue-shifted by 6 to 8 nm from those of Mg-BChl (Fig. 2B).

Inductively coupled plasma spectrometry indicated that the HPLC-purified P-763 contained large amount of Zn. Other metals, including Mg, were not found, or detected only in trace amounts. The Zn/P-763 molar ratio was 0.98 based on the assumption that the pigment had the same extinction coefficient at the far red peak as Mg-BChl (96 mM⁻¹ cm⁻¹ in diethyl ether according to Oelze 1985). Upon treatment with HCl, P-763 released Zn and was converted to a pigment identical to BPhe in terms of its absorption spectrum, HPLC retention time and 1H-NMR spectrum. Elementary analysis showed the same C : H : N molar ratio of 55 : 73 : 3.9 both for P-763 and Mg-BChl esterified with phytol, which was purified from the purple photosynthetic bacterium \textit{Rhodobacter (Rba.) sphaeroides}. The ratio of Zn-BChl (P-763) : Mg-BChl : BPhe estimated from the HPLC peak areas in Fig. 2A was 13 : 2 : 1. Although the ratio varied to some extent depending on preparations and extraction conditions, Zn-BChl was always the dominant component.

The chemical structure of P-763 was further confirmed by using fast atom bombardment mass spectrometry. The mass spectrum of P-763 showed a molecular ion peak (M⁺) at m/z 950.5 (Fig. 3), which is 40 mass unit larger than that of Mg-BChl from \textit{Rba. sphaeroides} (M⁺ = 910.6) measured concurrently (data not shown). These values well fit to their chemical formula predicted (P-763: C_{55}H_{73}N_{2}O_{4}Zn = 950.5; Mg-BChl: C_{55}H_{73}N_{2}O_{4}Mg = 910.6). The mass spectrum of P-763 also showed an intense peak at m/z 672.2 that can be assumed to arise from the loss of a phytol group (C_{20}H_{39}).

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Activity of Zn-BChl-containing photosynthetic apparatus—Photochemical activities of isolated membranes of \textit{Ac. rubrum} were examined by absorption changes of the pigments. Excitation by a 10-ns laser flash at 532 nm induced a rapid absorption change at 780 nm that recovered with a 40-ms half time (Fig. 4A, inset). The difference spectrum based on ΔA at 10 µs after the flash (Fig. 4A, open circles), with glass filters that pass light between 580 and 630 nm to excite Zn-BChl, was always the dominant component.

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The extents at 10 µs after a 532-nm laser flash, as shown by insert kinetics, were plotted versus wavelength. The flash was covered with glass filters that pass light between 580 and 630 nm to excite the 590 nm peaks of Mg-BChl and Zn-BChl. Membranes used were the same as those in Fig. 1.

Fig. 3 Mass spectrum of P-763 (Zn-BChl) purified from \textit{Ac. rubrum} in a range of m/z 400–1100. Inset, structural formula of Zn-containing bacteriochlorophyll a (Zn-BChl) esterified with phytol found in \textit{Ac. rubrum}. Replacement of central Zn atom by Mg gives bacteriochlorophyll a (Mg-BChl) and that with two hydrogen atoms gives bacteriopheophytin a (BPhe).

Fig. 4 Light-induced absorption change and its dependency on light intensity in membranes of \textit{Ac. rubrum} and \textit{Rsp. rubrum}. (A) Difference absorption spectra induced by laser flash. Open circles, \textit{Ac. rubrum}; broken line, \textit{Rsp. rubrum}. The extents at 10 µs after a 532-nm laser flash, as shown by insert kinetics, were plotted versus wavelength. (B) Dependence of the extent of absorption changes (ΔA) at 780 nm on the intensity of excitation light. Closed circles, \textit{Ac. rubrum}; open circles, \textit{Rsp. rubrum}. The extent were measured at 10 µs after a 5-µs xenon flash. The flash was covered with glass filters that pass light between 580 and 630 nm to excite the 590 nm peaks of Mg-BChl and Zn-BChl. Membranes used were the same as those in Fig. 1.
circles) showed a positive peak at 780 nm and negative peaks at 800 and 855 nm. The spectral shape resembles that of the membranes of *Rsp. rubrum* measured under the same experimental setup (Fig. 4A, broken line) but shows different peak positions. The absorption changes around 875 nm in *Rsp. rubrum* is known to be caused by oxidation of the special pair Mg-BChl in the photochemical reaction center and the sharp changes around 800 nm, by the blue shift of two Mg-BChl monomers (accessory BChl molecules) in response to oxidation of the adjacent special pair (Wang and Clayton 1973). The apparent similarity between these spectra suggests that *Ac. rubrum* has an active reaction center complex analogous to that of *Rsp. rubrum*. The peak positions assigned to the accessory and special pair BChl were blue-shifted by about 7 and 25 nm, respectively, in *Ac. rubrum* suggesting the normal functioning of Zn-BChl, instead of Mg-BChl.

Prolonged storage or mild treatment with detergents produced a membrane preparation which contained almost no Mg-BChl (less than 0.6% compared to Zn-BChl) due to the conversion of Mg-BChl to BPhe without significant loss of the activity (data not shown). This confirms that Zn-BChl is functional in the photochemical reaction center. The activity of photosynthetic electron transfer was also confirmed in intact cells of *Ac. rubrum* by the reversible photo-oxidation of c-type cytochrome(s) with a peak at 552 nm (data not shown). The result interprets the light-stimulation of growth or CO₂ incorporation observed in intact cells (Kishimoto et al. 1995a).

To evaluate the efficiency of light-energy conversion, absorption changes at 780 nm were measured at different intensities of 590-nm flash light, which almost evenly excited Zn-BChl in *Ac. rubrum* and Mg-BChl in *Rsp. rubrum* (see Fig. 1). In both types of membranes, the extent of absorption change showed a similar dependency on flash energy (Fig. 4B). The result indicates that the energy transfer in the light-harvesting complex and the photochemistry in the reaction center complex in *Ac. rubrum* proceed as efficiently as those in *Rsp. rubrum*. Zn-BChl appears to fully replace the function of Mg-BChl.

A question may remain why plants and bacteria ever known exclusively use Mg-(bacterio)chlorophylls. We may give speculations; Mg is ten-times more abundant in the environment, more soluble at neutral or alkaline pH's than Zn, or evolutionarily chosen etc. The strongly acidic growth conditions or/with some modifications in the metal-chelatase in the chlorophyll biosynthesis might be responsible for the natural abundance of Zn-BChl in *Ac. rubrum* membranes. However, the level of Zn in our growth medium was rather low and the pigment-protein complexes of the bacterium do not seem to be significantly different from those in purple photosynthetic bacteria. Actual reasons, therefore, remain to be studied. Photosynthetic apparature using Zn-BChl in *Ac. rubrum* will be very useful for the study of primary reactions of photosynthesis since Zn-BChl has chemical and optical properties distinctly different from those of Mg-BChl.

The finding is certainly a surprise by itself, and indicates the potential variability of biological photosynthesis. It also suggests the existence of wide varieties of natural photosynthesis in the past and probably in the future. Systematic survey of more varieties may open a profound future for our understanding of the biosphere.

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References


Photosynthesis using Zn-bacteriochlorophyll


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