Coupling Between Bacteriochlorophyll and Membrane Protein Synthesis in *Rhodopseudomonas spheroides*

(chromatophore/δ-aminolevulinic acid/membrane synthesis)

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ABSTRACT Bacteriochlorophyll-containing membranes from Rhodopseudomonas spheroides contain proteins with estimated molecular weights of 26,000, 22,000, 19.000, and 10,000-6,000 (proteins 9, 10, 11, and 15) when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins 9, 10, and 11 may be associated with the reaction center form of bacteriochlorophyll and protein 15, with the light-harvesting form. These proteins were not detected in membranes from nonpigmented wild-type cells grown with high aeration. Proteins 10, 11, and 15 were not found in mutants with blocks in bacteriochlorophyll synthesis, including strain 8-17, which is blocked at the phytolation step. Protein 9 was found in significantly reduced amounts. Apparently, synthesis of the completed bacteriochlorophyll molecule is required for the occurrence of these proteins in the membrane.

Gel autoradiography was used to follow the synthesis of membrane proteins in mutant H-5, which requires δ -aminolevulinic acid for bacteriochlorophyll synthesis. Incorporation of labeled amino acids into proteins 9 and 15 was curtailed preferentially in cells deprived of δ -aminolevulinic acid.

Analysis of the chromatophore membranes from photosynthetic bacteria of the Athiorhodaceae group has shown several proteins to be unique to cells containing bacteriochlorophyll (1, 2). These proteins are absent from preparations of cells grown with high aeration and consequently devoid of bacteriochlorophyll. Also their appearance in the chromatophore membrane parallels pigment synthesis as cells adapt from the nonpigmented to the pigmented state (3). One of these specific proteins has been isolated from the chromatophores of Rhodopseudomonas spheroides and purified to apparent homogeneity (4). It has an estimated molecular weight of 14,000 and accounts for about 40-50% of the total chromatophore protein. The bulk of the bacteriochlorophyll is associated with this protein. Recently, three other chromatophore proteins have been identified as components associated with the photosynthetic reaction center (5).

It has been suggested that bacteriochlorophyll formation may be obligatorily linked to membrane protein synthesis (6). This hypothesis is supported by the observation that bacteriochlorophyll synthesis is accompanied by the preferential incorporation of labeled amino acids into chromatophore protein (6). The close relationship between bacteriochlorophyll and protein synthesis may be due to the occurrence of specific membrane proteins associated with the pigment.

In this report, we describe experiments that demonstrate the obligatory coupling between the synthesis of specific chromatophore proteins and bacteriochlorophyll formation by exploiting certain mutant strains of R. spheroides that are blocked in the bacteriochlorophyll biosynthetic pathway.

MATERIALS AND METHODS

Organisms and Growth Conditions. The wild-type strain was R. spheroides NCIB 8253. Mutants 8-17 and 8-32 were blocked in bacteriochlorophyll synthesis, and accumulated magnesium-containing precursors (7). Strain TA-R was a regulatory mutant that did not exhibit the normal repression of bacteriochlorophyll synthesis by oxygen (8). Mutant H-5 lacked δ -aminolevulinic acid synthetase activity and required δ -aminolevulinic acid for growth (9). Maintenance of stock cultures and other details of these strains are given in the appropriate references. For the preparation of membranes, cells were grown in malate-glutamate (MG) medium (10) supplemented with 0.4 mM L-methionine for growth of mutant TA-R and with 0.1% yeast extract and 0.1 or 1 mM δ -aminolevulinic acid for mutant H-5. Cultures were incubated aerobically in the dark under conditions of low or high aeration (8). The wild-type cultures contained 10-15 nmol of bacteriochlorophyll per ml with low aeration, but less than 0.1 nmol with high aeration.

Suspension Experiments with Mutant H-5. Concentrated cell suspensions were used to follow bacteriochlorophyll synthesis and incorporation of radioactive amino acids into membrane proteins of mutant H-5. For these experiments, cells were grown under high aeration in veast extract-MG medium with 0.1 mM δ -aminolevulinic acid and harvested during the logarithmic phase of growth. The cells were washed once in MG medium and suspended to an absorbance of 4 at 680 nm (equivalent to 2 mg dry weight of cells per ml) in yeast extract-MG medium with 1 mM δ -aminolevulinic acid. 100-ml Samples were incubated in the dark at 30° in 125-ml Erlenmever flasks on a rotary shaker operating at 200 rpm. After 3 hr, the cells were harvested, washed once in MG medium, and suspended in fresh yeast extract-MG medium either without δ -aminolevulinic acid or with 1 mM δ -aminolevulinic acid. In the pulse-labeling experiments, 150 μ Ci of a mixture of $[U^{-14}C]$ aminoacids were added to a 100-ml cell culture 3 hr after the second resuspension. After 20 min, chloramphenicol (0.7 μ g/ml) was added, and the cells were quickly harvested, washed, and stored frozen.

Membrane Isolation and Purification. For the preparation of membranes, cells were harvested in the logarithmic growth phase at an absorbance between 1 and 1.5, measured at 680



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FIG. 1. SDS-polyacrylamide gels of purified membranes of the wild-type R. spheroides. The gels are stained with Coomasie Blue.

nm. Membranes were purified by a modification of the method described by Worden and Sistrom (11). All operations were at 5-8°. Washed cells were suspended in 10 mM Tris HCl (pH 7.5) to an absorbance of 40 at 680 nm. DNase I was added $(0.4 \ \mu g/ml)$, and the cells were ruptured by two passages through a French pressure cell (Aminco) at 18,000 lb/inch². The extract was centrifuged at $15,000 \times g$ for 20 min to sediment whole cells and debris, and the resultant supernatant fraction was centrifuged at 144,000 $\times g$ for 60 min in a Spinco Type 65 rotor. The pellet was washed once and suspended in 30% (w/v) rubidium chloride in 10 mM Tris·HCl (pH 7.5). The suspension was centrifuged at 153,000 \times g for 6 hr in a Spinco SW41 rotor. The ribosome-free membrane fraction at the top of the density gradient was collected, washed, and then layered on a discontinuous density gradient of Ficoll (Pharmacia). This gradient was preformed by layering Ficoll solutions made up in 10 mM Tris HCl (pH 7.5) in the following volumes and concentrations: 2 ml of a 30%(w/v) solution, 4 ml of a 20% solution, 3 ml of a 10% solution, and 3 ml of a 5% solution. The gradient was centrifuged at $105,000 \times g$ for 10 hr in an SW41 rotor. The purified membranes were collected from the interface between the 10 and 20% Ficoll layers and then washed and stored at 5°.

Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis and Autoradiography. The purified membranes were prepared for SDS-polyacrylamide gel electrophoresis by treatment of 150-250 μ g of membrane protein with a solution of 3% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, and 40 mM Tris·HCl (pH 8.0). The mixture was heated in boiling water for 1.5 min. 10% Polyacrylamide gels containing 0.1% SDS were prepared by the method of Laemmli (12) in 175 \times 6 mm glass tubes. Electrophoresis was performed at 2 V per cm for the first hr and at 7 V per cm for the remaining 14–16 hr. The gels were fixed in 50% trichloroacetic acid for at least 4 hr, and then stained and destained by the methods of Weber and Osborn (13).

Gels containing radioactivity were fractionated in a Savant Autogeldivider, and fractions were collected for liquid scintillation counting in glass vials. 10 ml of a Triton X-100-toluene (3:1 v/v) scintillation cocktail mixture was added, and the samples were counted in a Beckman LS200 liquid scintillation spectrometer.

Gels containing radioactively labeled membrane proteins of mutant H-5 were sliced and prepared for autoradiography by described techniques (14). Samples containing 150–250 μ g of protein and 10,000–15,000 cpm were applied to the gels, and the strips were exposed for 4–5 weeks on Kodak No Screen x-ray film before development.

Stained gels and autoradiograms were scanned on a Joyce-Loebel microdensitometer.

Measurement of Protein and Phospholipid Synthesis. Total and membrane protein synthesis in cell suspensions of mutant



FIG. 2. Molecular weight estimations on SDS-polyacrylamide gels of the proteins unique to pigmented membranes. The standard proteins used and their molecular weights are: bovineserum albumin (67,000), ovalbumin (45,000), carboxypeptidase A (34,000), chymotrypsinogen A (25,000), trypsin (23,000), myoglobin (17,800), lysozyme (14,400), ribonuclease (13,700), cytochrome c (12,400), calf-thymus histone II (8,400), and insulin (6,000).

H-5 were measured by incorporation of $[U^{-14}C]$ aminoacids $(15 \,\mu\text{Ci/ml})$ into the proteins of the crude extract (supernate after the 15,000 $\times g$, 20-min centrifugation) and of the crude membrane (pellet after the 144,000 $\times g$, 60-min centrifugation) fractions, respectively. The label was added at the beginning of the suspension incubation. The amount of radio-activity incorporated into protein was determined (15).

Phospholipid synthesis was measured by incorporation of inorganic [³²P]phosphate (5 μ Ci/ml) into the lipid fraction. The phospholipids were extracted with chloroform-methanol (2:1 v/v) and treated with an acidified NaCl solution (16). The radioactivity in samples of the chloroform extract was determined by liquid scintillation counting.

Bacteriochlorophyll and Protein Determinations. Bacteriochlorophyll was determined in acetone-methanol (7:2 v/v)extracts, with a mM extinction value of 76 at 770 nm (17). Protein was determined by the method of Lowry *et al.* (18), with bovine-serum albumin as a standard.

Chemicals. The mixture of L- $[U^{-14}C]$ aminoacids (0.1 mCi/ml) was purchased from New England Nuclear Corp. Carrier-free inorganic [³²P]phosphate was obtained from International Chemical and Nuclear Corp. Rubidium chloride was purchased from Matheson Coleman and Bell. SDS obtained from Sigma Chemical Co. was twice crystallized from ethanol. Ovalbumin, beef-pancreas chymotrypsinogen A, bovine pancreas trypsin, sperm-whale myoglobin, and horse-heart cytochrome c were purchased from Mann Research. All other biochemicals were obtained from Sigma Chemical Co.

RESULTS

Membrane Proteins of Wild-Type R. spheroides. Stained SDS-polyacrylamide gels of purified membranes from wildtype cells grown with high and low aeration are shown in Fig. 1. The gel of membranes from the latter has four major bands, numbered 9, 10, 11, and 15, which are absent from preparations from cells grown with high aeration. Eleven other major bands and several bands of minor staining intensity were common to both types of preparation. Bands 9, 10, 11, and 15 from the pigmented membranes correspond to protein molecular weights of 26,000 $(\pm 10\%)$, 22,000 $(\pm 10\%)$, 19,000 $(\pm 10\%)$, and 10,000–6,000, respectively, as determined by the calibrated standard curve in Fig. 2. The standard curve is typically biphasic, with a breakpoint equivalent to about 17,000 daltons; this property of the SDSpolyacrylamide gel system has also been observed by Neville (19) and by Sandermann and Strominger (20). The weight assigned to band 15 is a rough estimate, since we find that molecular weight determinations below 17,000 are subject to considerable error.

It is known that lipids and pigments migrate to the area of band 15 in the SDS-polyacrylamide gel electrophoresis system (2) and that lipid material stains with Coomasie Blue in acrylamide gels (21). The presence of protein in band 15 was shown in the following way: cells were grown with low aeration in the presence of a mixture of $[U^{-14}C]$ aminoacids $(0.1 \ \mu Ci/ml)$. The purified membranes from these cells were extracted with chloroform-methanol. (2:1 v/v) and SDSpolyacrylamide gels of this nonlipid-containing membrane preparation were fractionated and counted. About 11% of the applied radioactivity migrated to the area of band 15.



FIG. 3. SDS-polyacrylamide gels of purified membranes of the wild-type (W-T), mutant 8-17, and mutant 8-32 strains grown with low aeration and of mutant TA-R grown with high aeration. The gels are stained with Coomasie Blue.

With unextracted membrane samples, about 18% of the counts were found in this band.

Membrane Proteins of Mutants 8-17, 8-32, and TA-R, Mutants 8-17 and 8-32 do not form bacteriochlorophyll, but accumulate bacteriochlorophyllide and Mg-2,4-divinyl-pheoporphyrin a_5 , respectively, under conditions of low aeration. Purified membranes from such cells were subjected to SDSpolyacrylamide gel electrophoresis (Fig. 3). The three major proteins, 10, 11, and 15, found in equivalent preparations from the wild type were clearly absent from the mutants. Protein 9 was detected in these stained gels as a faint minor band. Otherwise, membranes of the mutants showed gel patterns similar to that of wild-type cells grown with high aeration, with the exception of mutant 8-32, which showed an additional minor band migrating between bands 3 and 4.

In contrast to wild type, mutant TA-R forms bacteriochlorophyll with high aeration. When grown in this way, the pigment concentration in the mutant cells is similar to that in wild-type cells grown under low aeration (8). Accordingly, the SDS-polyacrylamide gel pattern of membranes from the mutant TA-R is similar to that of pigmented wildtype cells, and showed the characteristic bands, 9, 10, 11, and 15 (Fig. 3).

Bacteriochlorophyll and Membrane Protein Synthesis in Mutant H-5. Mutant H-5 lacks δ -aminolevulinic acid synthetase activity and requires added δ -aminolevulinic acid



FIG. 4. Protein, phospholipid, and bacteriochlorophyll synthesis in cell suspensions of mutant H-5. Arrows indicate when the cells were harvested and suspended in medium with and without δ -aminolevulinic acid. (A), incorporation of $[U^{-14}C]$ -aminoacids into membrane protein with δ -aminolevulinic acid (\bullet —— \bullet); bacterio-chlorophyll synthesis with δ -aminolevulinic acid (\bullet —— \bullet); bacterio-chlorophyll synthesis with δ -aminolevulinic acid (\bullet —— \bullet) and without δ -aminolevulinic acid (\bullet —— \bullet). (B), incorporation of $[U^{-14}C]$ -aminoacids into total protein with δ -aminolevulinic acid (\bullet —— \bullet) and without δ -aminolevulinic acid (\bullet —— \bullet). (B), incorporation of $[U^{-14}C]$ -aminoacids into total protein with δ -aminolevulinic acid (\bullet —— \bullet) and without δ -aminolevulinic acid (\bullet —— \bullet), and without δ -aminolevulinic acid (\bullet —— \bullet), and without δ -aminolevulinic acid (\bullet —— \bullet).

for growth and pyrrole synthesis (9). The SDS-polyacrylamide gel pattern of purified membranes from H-5 grown with 1 mM δ -aminolevulinic acid under low aeration was similar to that of pigmented membranes of the wild type (compare Fig. 5A with Fig. 1).

With mutant H-5, bacteriochlorophyll synthesis can be manipulated by varying the supply of δ -aminolevulinic acid; it was therefore used to study incorporation of $[U^{-14}C]$ aminoacids into membrane proteins in relation to pigment synthesis. In these experiments, concentrated cell suspensions were incubated with low aeration. In the presence of 1 mM δ aminolevulinic acid, bacteriochlorophyll synthesis continued for at least 7 hr, but halted abruptly upon withdrawal of δ -aminolevulinic acid (Fig. 4). Incorporation of label into membrane protein was significantly decreased with removal of δ -aminolevulinic acid, whereas incorporation into total protein and inorganic [³²P]phosphate incorporation into phospholipid were only slightly decreased.

Synthesis of the individual membrane proteins were examined by pulse-labeling the cells with $[U^{-14}C]$ aminoacids in the presence and absence of δ -aminolevulinic acid. SDSpolyacrylamide gels of the purified membranes were subjected to autoradiography, and the band intensities were measured on the developed films (Fig. 5C and D). Densitometer tracings show that bands 2, 3, 9, 10, 11, 14, and 15 were labeled in the presence of δ -aminolevulinic acid. In the absence of δ aminolevulinic acid, band 15 was only feebly labeled by comparison, and there was a reduction in the labeling of band 9. By integrating the peak area of band 15 in the scan of the autoradiogram, we calculate that it contained 51% of the ${}^{14}C$ label in preparations from cells incubated with δ -aminolevulinic acid but only 20% in preparations from δ -aminolevulinic acid-deprived cells. For band 9, these values were 9.1 and 6.1%, respectively.

The difference in labeling of band 15 can be largely attributed to a difference in its protein content. This difference was determined in the following way: the pulse-labeled membrane samples of mutant H-5 (each with 10,000 cpm) were



FIG. 5. SDS-polyacrylamide gels of purified membranes of mutant H-5. (A) Coomasie Blue-stained gel of membranes of H-5 grown with low aeration in medium containing 1 mM δ aminolevulinic acid. (B) Densitometer scan of the stained gel. (C) and (D) Densitometer scans of SDS-polyacrylamide gel autoradiograms of membranes of mutant H-5 after pulse-labeling with [U-14C] aminoacids in the presence (C) and absence (D) of δ -aminolevulinic acid.

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subjected to SDS-polyacrylamide gel electrophoresis. The pigmented areas corresponding to band 15 were sliced out, pulverized, and suspended in water for 36 hr. The suspensions were extracted with chloroform-methanol (2:1 v/v), and the radioactivities were determined in the extracts and in the protein-containing residues. The radioactivities in the latter fractions from cells incubated with and without δ -aminolevulinic acid were 1990 and 790 cpm, respectively. The radioactivities in the corresponding lipid-pigment extracts were, respectively, 880 and 600 cpm.

DISCUSSION

Electron micrographs of thin sections show that mutants of R. spheroides unable to synthesize bacteriochlorophyll lack the characteristic intracellular membranes associated with pigmented cells (22). On the basis of these observations, it was postulated that the completed molecule of bacteriochlorophyll is required for the assembly of these membranes. The present findings support this idea. Thus, mutants blocked at certain steps of the bacteriochlorophyll biosynthetic pathway lack three major membrane proteins found in the isolated membranes of pigmented cells. A fourth membrane protein (protein 9) is found in these mutants in significantly reduced amounts. Moreover, labeling of a least two of these proteins (proteins 9 and 15) by radioactive amino acids is preferentially repressed when bacteriochlorophyll synthesis is halted by lack of δ aminolevulinic acid in mutant H-5. All four proteins are synthesized by mutant TA-R, which makes bacteriochlorophyll under growth conditions in which pigment synthesis is normally repressed. Our findings support those of Oelze et al. (24), who have observed a similar association between bacteriochlorophyll and thylakoid formation in Rhodospirillum rubrum.

The membrane proteins 9, 10, and 11 are analogous to the reaction center proteins identified by Clayton and Haselkorn (5) and Feher (23) in the carotenoidless mutant of R. *spheroides*. They correspond in molecular weights as determined by SDS-polyacrylamide gel electrophoresis and show the typical 1:1:1 molar ratios, based on protein staining intensities. Protein 15 may be the same as the bacteriochlorophyll-containing protein isolated and purified from R. *spheroides* by Fraker and Kaplan (4) and the low molecular weight protein (11,000 daltons) identified by Clayton and Haselkorn (5). This protein is probably associated with light-harvesting bacteriochlorophyll (4, 5).

Among the four membrane proteins, the labeling of protein 15 with radioactive amino acids is the most affected when bacteriochlorophyll synthesis ceases in response to removal of δ -aminolevulinic acid in cultures of mutant H-5. Two possible reasons to account for this observation are: (i) the synthesis of all four membrane proteins is repressed when bacteriochlorophyll formation stops, but protein 15 is degraded at a faster rate than the others, or (ii) the formation of protein 15 may be more tightly coupled to bacteriochlorophyll synthesis and, therefore, its synthesis is preferentially repressed.

Our conclusion is that the appearance of specific proteins in chromatophore membrane depends upon the formation of the complete phytolated bacteriochlorophyll molecule. Magnesium pyrrole precursors, including bacteriochlorophyllide, are not sufficient. The mechanism by which the pigment molecule exerts its effect is obscure. It could act at the transcriptional or translational stage of synthesis of the proteins. Alternatively, the bacteriochlorophyll may be required for the integrated assembly of the proteins into the membrane structure. Although proteins corresponding to the specific membrane components were not detected in soluble fractions of the mutants (unpublished data), it is possible that such proteins might be rapidly destroyed in the absence of bacteriochlorophyll.

The development of the photosynthetic lamellae of the chloroplast is also closely linked to chlorophyll synthesis (25). Observations with *Chlamydomonas reinhardi* are particularly pertinent to those with the photosynthetic bacteria. The synthesis of certain chloroplast proteins of cytoplasmic origin occurs only as chlorophyll is being formed, and the data suggest that the pigment exerts its control at the transcriptional level (26).

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