Localization of Photosynthetic Membrane Components in *Rhodopseudomonas sphaeroides* by a Radioactive Labeling Procedure†

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ABSTRACT: Reduction with [^{3}H]KBH_4 of Schiff's bases generated by reaction with pyridoxal 5′-phosphate (which cannot penetrate the intact cytoplasmic membrane) yields tritium-labeled derivatives of both proteins and lipids accessible on the periplasmic side of the cytoplasmic membrane. Application of this technique to phototrophically grown *Rhodopseudomonas sphaeroides* labeled both the cell envelope and chromatophore fractions. The technique was also applied to *R. sphaeroides* harvested at various times during an adaptation from heterotrophic to phototrophic growth conditions. The specific activity of the chromatophore fraction after 20 h of adaptation was 76% of that found at the beginning, indicating that the intracytoplasmic membranes and cytoplasmic membrane form a continuous membrane system, with the majority of the intracytoplasmic membranes accessible to the external medium throughout the adaptation. The identity of the proteins labeled by this technique was investigated in two fractions labeled after cell disruption: normal "inside-out" chromatophores and "right-side-out" membrane vesicles isolated by lysozyme–osmotic shock treatment of cells grown in high light intensity (15 000 lx). The results after sodium dodecyl sulfate–polyacrylamide gel electrophoresis and fluorography indicated that the 28 000-dalton subunit (and to a lesser extent the 21 000-dalton subunit) of the reaction center complex and two polypeptides in the light-harvesting region of the gel were heavily labeled in the chromatophores and were thus accessible on the cytoplasmic side of the membrane. At least one of the latter two polypeptides was also labeled in the membrane vesicles and was thus also accessible on the periplasmic side of the membrane. None of the reaction center subunits was significantly labeled in a reaction center complex prepared from the membrane vesicle sample.

Invagination of the cytoplasmic membrane to form the intracytoplasmic membrane system containing the photosynthetic apparatus was first proposed on the basis of electron microscopic observations of thin sections of phototrophically grown members of the Rhodospirillaceae (Giesbrecht & Drews, 1962; Drews & Giesbrecht, 1963; Cohen-Bazire & Kunisawa, 1963). The invagination hypothesis has received support from recent morphological studies on *Rhodospirillum rubrum* (Golecki & Oelze, 1975), *Rhodopseudomonas capsulata* (Lampe et al., 1972), and *Rhodopseudomonas sphaeroides* (Peters & Cellarius, 1972). Oelze & Drews (1970, 1972) have proposed that the ICM[1] and CM of *R. rubrum* represent reversible modifications of a single dynamic membrane system. Hence, in those species in which the membrane system has a vesicular appearance in electron micrographs, the ICM may exist in vivo as "buds" in a membranous continuum. Gibson (1965) and Collins & Niederman (1976) have presented evidence that a portion at least of the ICM vesicles may be structurally independent from (or only loosely bound to) the CM. The field has recently been extensively reviewed by Drews (1978) and Remsen (1978).

Recent results by Prince et al. (1975) have supported the view of a membranous continuum. Cytochrome c_2 is located on the periplasmic side of both the CM and the ICM and is trapped inside chromatophores[2] during cell disruption by French press or sonication. However, up to 82% of this cytochrome is lost during spheroplast formation in *R. sphaeroides* and *R. capsulata*, indicating that the internal space of the ICM must be continuous with the periplasmic space and that the vesicles are not sealed in vivo. This result has been confirmed in the case of *R. capsulata* by Hochman et al. (1975). Holmqvist (1979), on the other hand, has presented evidence for a discontinuity between the CM and ICM of *R. sphaeroides*, based on an electron microscopic examination of cells suspended in ferrous gluconate prior to fixation.

Reed et al. (1975) were the first to use ferritin-coupled antibodies prepared against the purified RC complex of *R. sphaeroides* in order to visualize the location of the RC in electron micrographs of EDTA-washed chromatophores. Feher and co-workers (Valkirs et al., 1976; Feher & Okamura, 1976) modified and extended this work by adding antibodies prepared against purified RC subunits (both the H subunit and a complex of the L and M subunits) to both chromatophores and partially disrupted *R. sphaeroides* spheroplasts. More recent studies have employed either proteolytic enzymes (Hall et al., 1978; Oelze, 1978; Takemoto & Gillies, 1978; Fieck & Drews, 1979) or lactoperoxidase-catalyzed ^{125}I- or ^{131}I-iodination (Snozzi, 1977; Zürrer et al., 1977; Cuendet et al., 1978; Oelze, 1978) to degrade or label accessible polypeptides in chromatophore preparations from various members of the Rhodospirillaceae. The degraded or labeled polypeptides were then detected after separation of the constituent polypeptides by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Other reagents used to chemically label photosynthetic membranes have included the following: *p*-diazonium[^{35}S]benzenesulfonic acid in plant chloroplasts (Dilley et al., 1972); 2,4,6-trinitrobenzenesulfonate in whole cells and chromatophores of *Chromatium vinosum* (Shimada & Murata, 1976, 1977); pyridoxal phosphate plus [^{3}H]KBH_4 in whole cells of *Chlorobium limicola* (Boyce et al., 1976). We

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[1] Abbreviations used: ICM, intracytoplasmic membrane; CM, cytoplasmic membrane; RC, reaction center; H, M, and L subunits, heavy, medium, and light subunits of the reaction center; LH, light harvesting; Bchl, bacteriochlorophyll.

[2] The term "chromatophore" will refer only to the subcellular membrane fraction derived from the intracytoplasmic membranes of phototrophic bacteria after cellular disruption.

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have used the latter technique with three types of preparations of *R. sphaeroides*: whole cells, normal "inside-out" chromatophores, and "right-side-out" membrane vesicles isolated by lysozyme–osmotic shock disruption of cells grown in high light intensity (Michels & Konings, 1978). Our results with whole cells support the membrane continuum theory of ICM structure. We also report herein the results of the labeling pattern found for specific photosynthetic membrane proteins. A preliminary report of this work has already appeared (Francis & Richards, 1979).

Experimental Procedures

Culture and Growth Conditions. R. sphaeroides N.C.I.B. 8253 was grown at 30 °C by stirring magnetically in medium MG of Lascelles (1966). Phototrophic cultures were normally grown semiaerobically in low light intensity (4500 lx). However, cells for the right-side-out membrane vesicles were grown in high light intensity (15 000 lx; Dierstein & Drews, 1974) by placing two 150-W spot lamps on either side and 30 cm from the center of a 1-L flask placed inside an 18.5 cm diameter water bath. The adaptation was carried out by first growing the cells heterotrophically at 30 °C under 65% O_2/35% N_2 in the dark. The pinkish white cells were then centrifuged and resuspended in 7 L of fresh MG medium (to an absorbance of 0.8 at 680 nm) and incubated at 30 °C under 4% O_2/96% N_2 with 4500-lx illumination. Samples of 1 L were collected after 2, 4, 8, and 20 h, during which time the culture became deeply pigmented. Light intensity was determined with a General Electric Type 213 light meter.

Labeling Procedure with Whole Cells. Cultures (1.8 L) were harvested at mid-log phase and resuspended in two 20-mL portions of 50 mM potassium phosphate buffer, pH 9.0 (hereafter referred to as phosphate buffer), at a (wet weight) cellular concentration of between 50 and 65 mg/mL. The labeling procedure was that of Rifkin et al. (1972) as modified by C. O. L. Boyce (R. C. Fuller, personal communication). The cell suspensions were treated with 0.1 mmol of pyridoxal 5′-phosphate for 30 min at 37 °C. They were then cooled to 4 °C and reacted with 0.1 mmol of KBH_4 for 15 min at 4 °C in a fume hood. In one portion of the cells (method 1), the borohydride was tritium labeled (specific activity 30 mCi/mmol); in the second portion (method 2), the borohydride was unlabeled. After reduction, the suspensions were reacted with an additional 0.05 mmol of pyridoxal phosphate and the cells were centrifuged and washed once with phosphate buffer. After resuspension again in 20 mL of phosphate buffer, the cells were disrupted by two passages through a chilled French pressure cell (American Instrument Co.) at 16 000 psi. The pyridoxal phosphate–KBH_4 treatment described above was then repeated except that in the first portion of cells (method 1) the borohydride was unlabeled and in the second portion (method 2) it was tritium labeled (specific activity 30 mCi/mmol). Both samples were then centrifuged for 20 min at 12000*g* to remove unbroken cells and larger cellular debris. Portions of the resulting supernatants were dialyzed overnight at 4 °C against 5 mM Tris-HCl buffer, pH 7.5, plus 5 mM NaEDTA (hereafter referred to as Tris/EDTA buffer) and used for the centrifugations of Figure 1. Whole cells harvested during the adaptation were treated in a manner similar to method 1, except that the pyridoxal phosphate–KBH_4 treatment *after* cell disruption was eliminated and the French press lysates were treated with 0.1 mg each of ribonuclease A and deoxyribonuclease I.

Purification of Subcellular Fractions. Dialyzed cell-free supernatants were layered on top of 28-mL discontinuous gradients made from solutions of 45, 20, and 10% (w/w) sucrose (in Tris/EDTA buffer) in a ratio of 1:2:1. The gradients were centrifuged for 90 min at 100000*g* in an International Model B60 ultracentrifuge with an A237 rotor. Three fractions were then withdrawn with a peristaltic pump: (a) the soluble protein fraction in the buffer above the 10% sucrose zone; (b) a pigmented band, containing ribosomal subunits plus small cell envelope fragments in the upper part of the 20% sucrose zone; (c) the chromatophore band at the 20/45% sucrose interface. The latter fraction was dialyzed overnight at 4 °C against the Tris/EDTA buffer and recentrifuged in 5–45% (w/w) linear sucrose gradients for 2 h at 140000*g* in a Beckman Model L5-75 ultracentrifuge with an SW40 rotor. The purified chromatophore bands were collected, dialyzed for 2 days at 4 °C against the Tris/EDTA buffer, and used for specific activity determinations (cf. Table I and Figure 2). The soluble protein fractions from the discontinuous gradients were similarly dialyzed and centrifuged for 3 h at 200000*g*; the supernatants were used for specific activity determinations (cf. Table I).

Labeling Procedure with Purified Membrane Preparations. The normal (inside-out) chromatophores were isolated from 1 g (wet weight) of a culture grown in low light intensity and purified in discontinuous sucrose gradients as described above. The chromatophore band was dialyzed for 4 days at 4 °C against 50 mM potassium phosphate buffer, pH 7.0. The pH was then adjusted to 9.0 with KOH and the volume brought to 20 mL with phosphate buffer. The suspension (ca. 1.0 mg of protein/mL; A_{850} = 5.0) was then treated with pyridoxal phosphate and [^{3}H]KBH_4 (specific activity 83 mCi/mmol) as described above for whole cells. Following the labeling procedure, the chromatophores were sedimented by centrifugation for 2 h at 140000*g* and washed once with phosphate buffer. The right-side-out membrane vesicles were isolated from cultures grown in high light intensity. One gram (wet weight) of cells was resuspended in 20 mL of a buffer containing 120 mM potassium phosphate, pH 8.0, 10 mM NaEDTA, and 10 mM sodium ascorbate and disrupted by a combination of lysozyme treatment and osmotic shock by the method of Michels & Konings (1978). Following removal of unbroken cells by centrifugation for 40 min at 800*g*, the membrane vesicles were sedimented by centrifugation for 30 min at 50000*g*. The pellet was resuspended in 20 mL of phosphate buffer; a spectrum (Cary Model 14) showed equal absorption at 875 and 850 nm, indicating that a high proportion of the B875 LH form of Bchl was present. The suspension (ca. 0.5 mg of protein/mL; A_{850} = 0.2) was then treated with pyridoxal phosphate and [^{3}H]KBH_4 (specific activity 83 mCi/mmol) as described above for whole cells. Following the labeling procedure, the membrane vesicles were sedimented by centrifugation for 1 h at 50000*g* and washed once with phosphate buffer.

The labeled chromatophores and membrane vesicles described above were used directly for the gel electrophoresis of Figure 3. The chromatophore fraction was then washed once with phosphate buffer containing 10 mM EDTA, resedimented, and used for the gel electrophoresis of Figure 5. The reaction center complex was prepared from the membrane vesicles by the method of Jolchine & Reiss-Husson (1974) and purified by Sepharose 6B gel filtration in 0.1 M sodium phosphate, pH 7.5, containing 0.3% lauryldimethylamine *N*-oxide. A near-infrared spectrum (Cary Model 17) of the major fraction revealed the following absorption maxima (relative absorbance in parentheses): 755 (1.00), 802 (1.87), and 860 nm (0.74). The light-harvesting complexes were prepared from both membrane preparations by the method

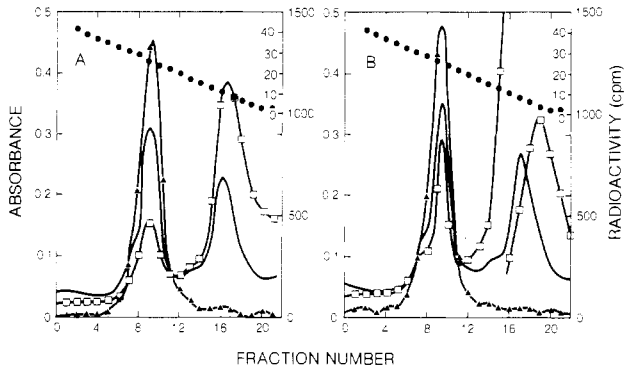

FIGURE 1: Sucrose density gradient centrifugation of broken-cell supernatants of *R. sphaeroides* labeled by (A) method 1 (reagents added before cell disruption) and (B) method 2 (reagents added after cell disruption). Samples were layered on top of 10-mL linear gradients formed between 5 and 45% (w/w) sucrose in Tris/EDTA buffer and centrifuged for 2 h at 140000*g* in a Beckman Model L5-75 ultracentrifuge with an SW40 rotor. The solid curve (A_{280}) was derived from the percent transmittance data recorded with an LKB Uvicord II flow monitor; (▲) A_{850} (recorded on a Cary Model 14 spectrophotometer after dilution approximately sevenfold); (□) cpm/0.1 mL of aliquot (determined in 5 mL of Aquasol with a Beckman Model LS-250 counter); (●) % (w/w) sucrose (inset scale; recorded with a Bausch & Lomb refractometer). In (B) the radioactivity data of fractions 16–22 have been reduced by a factor of 10.

of Clayton & Clayton (1972) as modified by Cogdell & Crofts (1978). The LH complexes were used without further purification.

Materials. Sucrose solutions were heated with activated charcoal and filtered before use. The [^{3}H]KBH_4, Aquasol, and PPO/DMSO were purchased from New England Nuclear, Lachine, Quebec. The [^{3}H]toluene was purchased from Amersham/Searle Corp., Oakville, Ontario. Pyridoxal 5′-phosphate, acrylamide, bis(acrylamide), sodium dodecyl sulfate, ribonuclease A, deoxyribonuclease I, and lysozyme were purchased from Sigma Chemical Co., St. Louis, MO. Sepharose 6B was purchased from Pharmacia (Canada) Ltd., Dorval, Quebec. The lauryldimethylamine *N*-oxide was a gift of the Onyx Chemical Co., Jersey City, NJ.

Results

Two different labeling methods were employed (cf. Experimental Procedures) in order to test the membrane permeability of the reagents. In each method, cells of *R. sphaeroides* were exposed to pyridoxal phosphate and KBH_4 both before and after disruption in the French press. In the first method, however, the KBH_4 added *before* cellular disruption was tritium labeled, while in the second method the KBH_4 added *after* disruption was labeled. Figure 1 shows the results of sucrose density gradient centrifugations of the broken-cell supernatants from both methods, after removal of unbroken cells and larger cell debris. Pigmented chromatophore bands were labeled by both methods; however, material in the upper portion of the gradient, containing soluble proteins, ribosomal subunits, and small fragments of the cell envelope (Niederman et al., 1972), was labeled to a much greater extent by method 2 (Figure 1B) than by method 1 (Figure 1A). The chromatophore fractions were purified as described under Experimental Procedures, and their specific activities (along with those of the soluble proteins) are given in Table I. The fractions containing ribosomal subunits and cell envelope fragments were also recentrifuged in sucrose gradients; only method 2 significantly labeled the ribosomal subunit band (G. R. Francis, unpublished observations).

The method 1 labeling technique was employed with whole cells of *R. sphaeroides* during an adaptation from heterotrophic to phototrophic growth conditions. The chromatophore fractions isolated during various stages of the adaptation were purified by sucrose density gradient centrifugation and their specific activities measured (Figure 2). While the specific activity does undergo a small initial drop between 0 and 2 h, it remains relatively constant for the rest of the adaptation, decreasing only to 76% of its initial value after 20 h of adaptation. Thus, the proportion of the ICM which becomes inaccessible to the external reagents can amount to no more than 24% of the total, assuming that the number of accessible reactive groups (e.g., ε-amino groups of lysine residues or phosphatidylethanolamine) per milligram of protein remains approximately constant throughout the adaptation.

In order to demonstrate which proteins were being labeled on the periplasmic and cytoplasmic sides of the ICM, we employed two purified membrane preparations with the labeling reagents: (a) a normal (cytoplasmic side out) chromatophore fraction obtained by French press disruption of cells grown in low light intensity (4500 lx); (b) a membrane vesicle fraction obtained by lysozyme–osmotic shock disruption of cells grown in high light intensity (15 000 lx). The latter vesicles

Table 1: Specific Radioactivities of Purified Membrane and Soluble Protein Fractions

sample[a]	sp act. uncor[b] (cpm/mg of protein)	sp act. cor[c] (dpm/mg of protein)
method 1 chromatophores	1.54×10^4	7.10×10^4
method 2 chromatophores	3.07×10^4	7.10×10^4
method 1 soluble proteins[d]	3.99×10^4	nd
method 2 soluble proteins[d]	2.23×10^5	nd
chromatophores[e]	3.45×10^5	1.16×10^6
membrane vesicles[e]	7.28×10^5	1.69×10^6

[a] Method 1, reagents were added before cell disruption; Method 2, reagents were added after cell disruption. [b] Determined in 5 mL of Aquasol with a Beckman Model LS-250 counter; protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. [c] Corrected for counting efficiency from a quenching curve obtained by counting aliquots of [^{3}H] toluene of known dpm in the presence of increasing amounts of unlabeled chromatophores; nd means "not determined". [d] Supernatants were counted before and after precipitation of the soluble proteins with 5% (w/v) trichloroacetic acid (in the presence of 0.5 mg/mL bovine serum albumin); the specific activities were determined from the difference in the two values. [e] Reagents were added to purified membrane preparations.

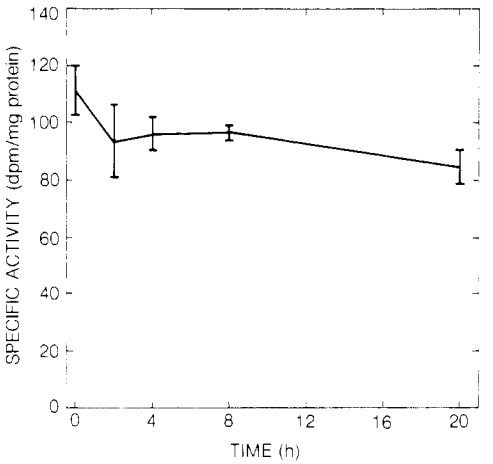

FIGURE 2: Specific activities (corrected for counting efficiency as described in Table I) of purified chromatophore fractions isolated at various times during an adaptation of *R. sphaeroides* from heterotrophic to phototrophic growth conditions. The solid line represents the average of two determinations.

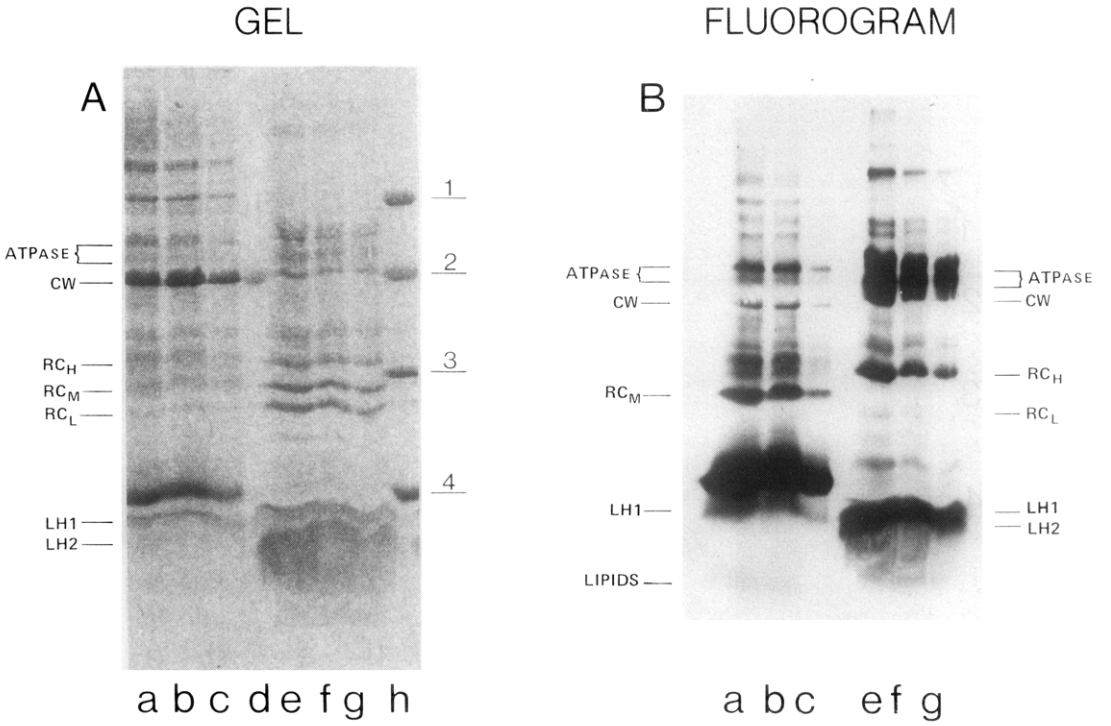

FIGURE 3: (A) Sodium dodecyl sulfate–polyacrylamide gel electrophoretic separation and (B) fluorographic detection of polypeptides labeled by the addition of the reagents to purified membrane vesicles and chromatophores. The method of Laemmli (1970) as modified by Schumacher & Drews (1978) was employed by using linear gradients of 10–15% (w/v) acrylamide polymerized in 1.5-mm slab gels. Membrane samples were diluted 1:2 with the sample buffer and heated for 40 min at 60 °C before application; electrophoresis was carried out at 20 mA for about 5 h. The gels were photographed before being prepared for fluorography by the method of Bonner & Lasky (1974). After being dried onto filter paper, the gels were placed in contact with Kodak XR-1 X-ray film for 2 weeks at −95 °C. The wells contained (a–c) membrane vesicles (300, 200, and 100 μg of protein, respectively), (d) ovalbumin (10 μg), (e–g) chromatophores (300, 200, and 100 μg of protein, respectively), and (h) protein standards (10 μg of each; all from Sigma Chemical Co., St. Louis, MO) [1, bovine serum albumin (68 000 daltons); 2, ovalbumin (43 000 daltons); 3, chymotrypsinogen A (25 000 daltons); 4, cytochrome *c* (12 400 daltons)]. Identified bands include the following: ATPase, α and β subunits of the coupling factor; CW, major cell wall polypeptide; RC_H, RC_M, and RC_L, heavy, medium, and light subunits of the reaction center complex, respectively; LH1 and LH2, two polypeptides associated with light-harvesting Bchl. The band marked lipids may represent labeled phosphatidylethanolamine.

have been shown to be oriented predominantly with their periplasmic side out and to include portions of the CM as well as the ICM (Matsuura & Nishimura, 1977; Michels & Konings, 1978; Lommen & Takemoto, 1978). The specific activities of both membrane preparations are given in Table I. The constituent polypeptides of both fractions were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 3A), and labeled polypeptides were detected by fluorography (Figures 3B and 4), a method developed by Randerath (1970) and Bonner & Laskey (1974) for the detection of tritium in polyacrylamide gels.

The results showed that in the chromatophore sample, polypeptides with mobilities similar to the α and β subunits of the coupling factor ATPase (55 000 and 51 000 daltons), the H subunit of the RC (28 000 daltons), and two polypeptides in the LH region of the gel (11 000 and 10 000 daltons) were heavily labeled and thus accesible on the cytoplasmic side of the membrane. Polypeptides that were less heavily labeled had mobilities similar to the L subunit of the RC (21 000 daltons) and to the major cell wall protein (42 000 daltons). In the membrane vesicle sample, however, the majority of the activity was located in an unidentified band with an apparent molecular weight of 12 500. Polypeptides with mobilities similar to the M subunit of the RC (24 000 daltons), one polypeptide in the LH region of the gel (11 000 daltons), the α and β subunits of the coupling factor ATPase, the cell wall protein, and the H subunit of the RC were less heavily labeled in the membrane vesicle sample.

Chromatophores which had been washed with EDTA-containing buffers in order to remove ATPase were also run in

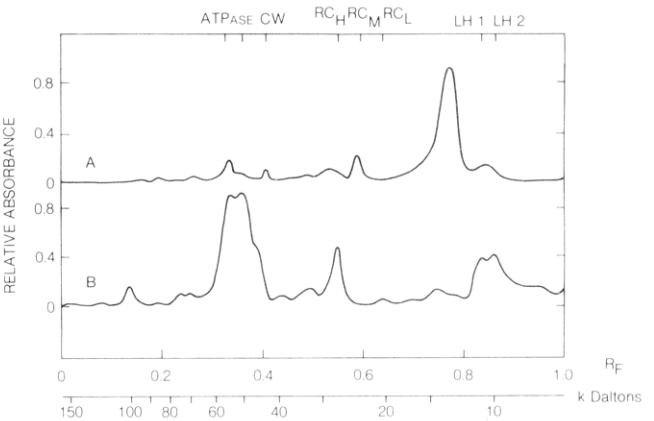

FIGURE 4: Densitometer (Transidyne Model 2950) scans of the fluorogram of Figure 3B. (A) Membrane vesicles (well c of Figure 3B) and (B) chromatophores (well f of Figure 3B) were scanned. The molecular weight scale (in kilodaltons) was constructed from a plot of the protein standard molecular weights vs. mobility (data of Figure 3A). For an explanation of the identified bands, see the legend to Figure 3.

polyacrylamide gels (Figure 5A). It can be seen that labeled bands corresponding to the two major ATPase subunits were almost completely removed by this procedure. In addition, complexes enriched in both RC and LH polypeptides were prepared from labeled membrane vesicles, and a complex enriched in LH polypeptides was prepared from labeled chromatophores (cf. Experimental Procedures). The results showed that only the H subunit (but not the M subunit) was labeled in the RC complex obtained from the membrane

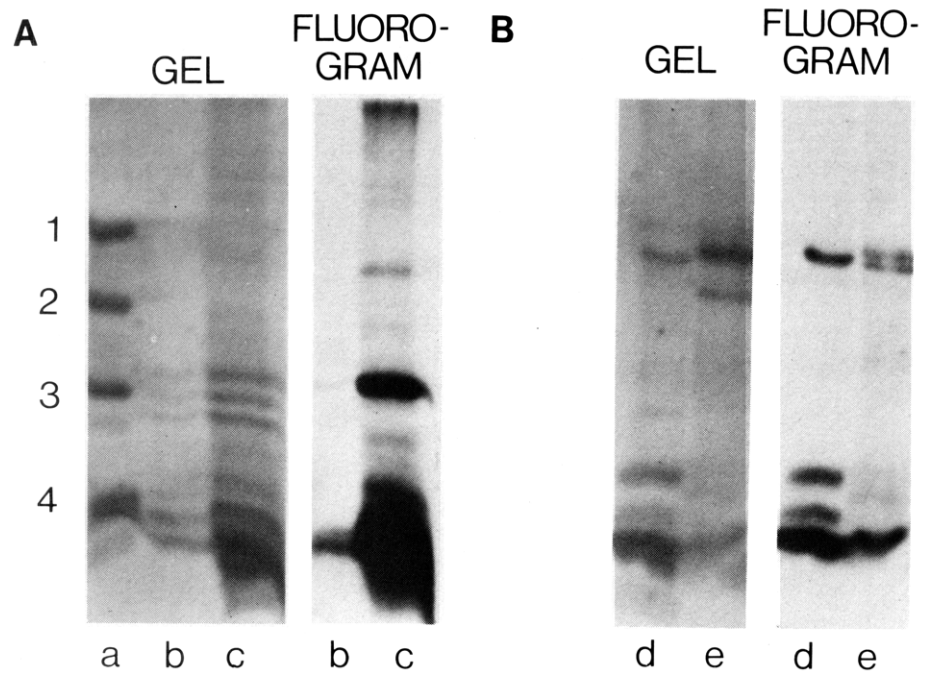

FIGURE 5: Sodium dodecyl sulfate–polyacrylamide gel electrophoretic separation and fluorographic detection of labeled polypeptides in EDTA-washed chromatophores, a RC complex prepared from the membrane vesicles, and LH complexes prepared from the chromatophores and membrane vesicles. The procedure was the same as that described in the legend to Figure 3, except that the gels were photographed after being prepared for fluorography (and dried) and the fluorograms were exposed for 5 weeks at −95 °C. The wells contained (a) protein standards (20 μg of each; cf. legend to Figure 3), (b) membrane vesicle RC complex (550 μg of protein), (c) chromatophores (300 μg of protein), (d) chromatophore LH complex (200 μg of protein), and (e) membrane vesicle LH complex (210 μg of protein).

vesicles, together with a smaller molecular weight (10000) LH polypeptide (Figure 5A). The latter was also the only labeled LH polypeptide visible in the LH complex isolated from the membrane vesicles (Figure 5B). From the appearance of the gels in Figure 5, it can be seen that the LH and RC complexes isolated from the membrane vesicles were not very pure. This may have been due to the fact that the proportion of photosynthetic proteins in the membrane vesicles was low, the majority of the proteins being cell envelope components. Nevertheless, these complexes have been enriched in both RC and LH polypeptides when compared with the unextracted membrane vesicle sample (Figure 3A).

Both of the LH polypeptides were labeled in the LH complex prepared from chromatophores (Figure 5B), although it appeared that either the smaller molecular weight (10000) polypeptide was being preferentially extracted or the extraction procedure was causing a change in the apparent molecular weight (from 11000 to 10000) of some of the higher molecular weight polypeptides. Another unidentified small molecular weight (14500) polypeptide was also labeled in the latter complex, while labeled polypeptides, perhaps corresponding to ATPase subunits, were present in the LH complexes prepared from both membrane samples (Figure 5B). Hence, the LH complex isolated from chromatophores can also only be considered to be enriched in LH polypeptides.

Discussion

The labeling reagents will react at the membrane surface with accessible amino groups of lipids and proteins. The modification of exposed hydrophilic portions of proteins by the addition of a negatively charged pyridoxal phosphate derivative might be expected to change their electrophoretic properties. However, labeled chromatophore samples (cf. Figure 3A) were found to have protein patterns indistinguishable from those of unlabeled samples. Therefore, this procedure should still be useful as a qualitative analytical tool for identification of labeled membrane proteins.

Confirmation of the reagents as impermeable membrane labeling agents (Rifkin et al., 1972; Boyce et al., 1976) was provided by the results of Figures 3B and 4 and the specific activity data of Table I. The activity in the method 1 soluble protein fraction may have been an indication of defects in the technique. Nevertheless, estimations (based on the data of Table I) indicated that at least 82% of the activity in the method 1 chromatophore fraction was due entirely to the exposure and labeling of components on the periplasmic side of the ICM. This figure is a minimum estimation since it is probable that a large portion of the measured activity in the method 1 soluble protein fraction was due to the presence of labeled periplasmic proteins or other nondialyzable labeled components dissociated from the cell envelope or ICM.

Although these results prove that some of the ICM in whole cells is accessible to the external environment, they do not indicate the extent of accessibility. This was estimated from the results of the adaptation experiment (Figure 2). It has been shown previously that prior to the appearance of the fully differentiated ICM during adaptation, the CM undergoes extensive invagination (Peters & Cellarius, 1972). The slightly pigmented membrane fractions derived from cells harvested at early stages (0–2 h) may correspond to vesiculated invagination sites in the CM and should therefore have been maximally accessible to the external reagents. The specific activity of the chromatophore fractions derived from cells harvested at later stages in the adaptation (4–20 h) would have been much less than the specific activity at earlier stages if the ICM had become discontinuous during this period. The small drop in specific activity of the chromatophore fractions observed during the entire course of the adaptation (Figure 2) indicated, however, that no more than about 24% of the membrane became inaccessible during this period. Our results thus confirm those of Prince et al. (1975) but not those of Holmqvist (1979), who concluded that the ICM vesicles are not normally continuous with the CM either during steady-state growth or during adaptation.

Our results on the localization of ICM proteins in *R. sphaeroides* confirm the results of others, working with either *R. sphaeroides* or *R. rubrum*, that the coupling factor ATPase (Reed & Raveed, 1972; Oelze, 1978) and the H subunit of the RC (Feher & Okamura, 1976; Snozzi, 1977; Zürrer et al., 1977; Hall et al., 1978; Oelze, 1978; Takemoto & Gillies, 1978) are accessible from the cytoplasmic side of the ICM. We also observed a less heavily labeled polypeptide in the L subunit region of the gel from the chromatophore samples (Figure 3B), and this may have been due to the partial exposure of this subunit on the cytoplasmic side of the ICM. Feher & Okamura (1976) reported that antibodies prepared against a purified complex of the L and M subunits of the RC reacted on both sides of the ICM in partially disrupted *R. sphaeroides* spheroplasts. Other workers could not, however, detect the accessibility of either the L or the M subunit to proteolytic enzymes (Hall et al., 1978; Oelze, 1978; Takemoto & Gillies, 1978) or lactoperoxidase-catalyzed iodination (Snozzi, 1977; Zürrer et al., 1977; Oelze, 1978) in either *R. sphaeroides* or *R. rubrum* chromatophores.

We were unable to confirm the accessibility of any of the RC subunits from the periplasmic side of the ICM. The heavily labeled polypeptide found in the M subunit region of the gel from the membrane vesicle sample (Figure 3B) must have been due to a non-RC protein of the CM with a similar molecular weight.[3] When a RC complex was prepared from the membrane vesicles, we found that only the H subunit (but not the M subunit) was labeled, and it so faintly that it was only detected after 5 weeks of fluorography (Figure 5A). It is likely that this faint labeling was due to some of the membrane vesicles having an opposite (inside-out) orientation. Lommen & Takemoto (1978) have estimated that about 20% of the cytoplasmic side of similarly prepared membrane vesicles was exposed to the external medium. Our results thus confirm those of Feher & Okamura (1976), who reported that the H subunit was not accessible to antibodies on the periplasmic side of the ICM, but not those of Takemoto & Gillies (1978), who concluded that the H subunit might span the membrane in *R. sphaeroides*. A conclusion similar to the latter was also reached by Snozzi (1977) and Zürrer et al. (1977) working with *R. rubrum*.

A number of workers have demonstrated the presence of two to three low molecular weight polypeptides (8000–12 000) thought to be associated with LH Bchl in chromatophore preparations of *R. sphaeroides* (Hall et al., 1973; Takemoto & Lascelles, 1974; Niederman et al., 1976; Broglie & Niederman, 1979), *Rhodopseudomonas palustris* (Firsow & Drews, 1977), and *R. capsulata* (Feick & Drews, 1978). Recently, Broglie et al. (1980) isolated and purified two LH complexes from *R. sphaeroides* by lithium dodecyl sulfate–polyacrylamide gel electrophoresis: one (B875) contained 12 000- and 8000-dalton polypeptides, and the other (B800 + B850) contained 10 000- and 8000-dalton polyeptides.[4] In the present study, we were able to resolve only two low molecular weight polypeptides (10 000 and 11 000) in our unextracted chromatophore sample (Figure 3A).[5] Both were labeled to about the same extent (Figures 3B and 4). However, the 10 000-dalton polypeptide was the major component in the LH complex isolated from the labeled chromatophore sample, although lesser amounts of labeled polypeptides (with apparent molecular weights of 11 000 and 14 500) were also present (Figure 5B, well d). Thus, the 10 000-dalton component may either be preferentially extracted into the LH complex or the extraction procedure may be causing a change in the apparent molecular weight (from 11 000 to 10 000) of a single LH polypeptide. That the latter alternative is more likely is indicated by the results with the LH complex isolated from the labeled membrane vesicle sample (Figure 5B, well e). The 10 000-dalton component was the only labeled LH polypeptide visible in the extract even though the 11 000-dalton component was the only labeled LH polypeptide visible in the unextracted membrane vesicle sample (Figures 3 and 4).[6] Both the 10 000- and 11 000-dalton components were visible in the RC complex isolated from the membrane vesicle sample (Figure 5A, well b); however, the 10 000-dalton component contained most of the label. It was also much more heavily labeled than the H subunit of the RC (presumably labeled in inside-out oriented membrane vesicles). Thus, although the total number of LH polypeptides and their apparent molecular weights are not clear, we conclude that at least one low molecular weight (10 000–11 000) LH polypeptide is accessible to the labeling reagents on both sides of the ICM.

In work with related bacteria, Oelze (1978) has reported that a single 9000-dalton LH polypeptide was attacked by chymotrypsin and Cuendet et al. (1978) have found that a similar low molecular weight polypeptide was labeled by iodination in *R. rubrum* chromatophores. Also, Feick & Drews (1979) have recently reported that three polypeptides of the LH II (B800 + B850) complex of *R. capsulata* mutant Y5 were degraded by trypsin in isolated chromatophore preparations.

The most heavily labeled polypeptide in our membrane vesicle sample had an apparent molecular weight of 12 500 (Figure 4). This band may have contained a cell wall component similar to a 7000-dalton lipoprotein covalently linked to the peptidoglycan (murein) layer of the cell wall of *Escherichia coli* (Braun, 1975). Lysozyme treatment of *E. coli* released a number of low molecular weight complexes of this protein with hydrolyzed portions of the peptidoglycan still attached (Schnaitman, 1971). Another major cell wall polypeptide (42 000 daltons; Parks & Niederman, 1978) was also present in large amounts in the membrane vesicle sample (Figure 3A). However, it was relatively lightly labeled (Figure 3B) and may have remained buried in the lipids of outer cell membrane fragments during the labeling procedure.

Studies on the determination of continuity or discontinuity between the ICM and CM of photosynthetic bacteria and the localization of membrane components are important for the

[3] Although the specific activity of method 1 chromatophores (isolated from cells in which the labeling reagents were added before cell disruption) was very much less than the specific activity of the membrane vesicles (cf. Table I), a similar fluorographic analysis of the separated polypeptides in the former sample revealed no label in any of the RC proteins, while at least three other unidentified bands were clearly labeled (G. A. Francis, unpublished observations).

[4] Only single polypeptides were detected in LH complexes (apparently containing B800 + B850) extracted and purified from chromatophores of *R. sphaeroides* by Clayton & Clayton (1972) and Sauer & Austin (1978). Moskalenko & Erokhin (1978) have demonstrated the presence of two polypeptides (9000 and 12 000 daltons) in a LH complex isolated from *R. sphaeroides* by the method of Clayton & Clayton (1972) using the gel system of Weber & Osborn (1969).

[5] We have observed three components when chromatophore samples were run in linear gradients formed between 10 and 15% (w/v) acrylamide in 1-mm slab gels (W. R. Richards, unpublished observations).

[6] The cells from which this sample was obtained contain relatively large proportions of the B875 LH form of Bchl with respect to the B800 + B850 LH form (Aagaard & Sistrom, 1972). Takemoto & Huang Kao (1977) also observed that the larger of two small molecular weight polypeptides was present in greater amounts in cells grown in high light intensity; however, they could not make a good correlation between the relative amounts of these two polypeptides and the two different LH forms of Bchl.

complete understanding of the mechanism of energy transduction in these organisms. If the ICM is continuous with the CM, the light-generated protonmotive force could be utilized either for ATP synthesis by coupling factors attached to *both* the CM and ICM or for transport and motility. Many of the components involved in energy transduction in photosynthetic bacteria have yet to be identified by detergent–gel electrophoresis and cannot, therefore, be localized within the membrane. The asymmetric orientation of the H subunit of the RC, however, indicates a possible role for it in energy transduction, e.g., as a coupling factor attachment site (Feher & Okamura, 1978). Our results also demonstrate that at least one of the LH polypeptides is symmetrically oriented so that individual molecules are accessible either from both sides of the ICM (if the polypeptide has two hydrophilic portions and completely spans the membrane) or from either side (if the polypeptide has a single hydrophilic portion which may be accessible at either membrane face).

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Site of Attachment of 11-*cis*-Retinal in Bovine Rhodopsin†

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ABSTRACT: A dipeptide containing the binding site for retinal in bovine rhodopsin has been isolated and its sequence determined. Rhodopsin containing [11-^{3}H]retinal was prepared in chromatographically pure form, and the [^{3}H]retinal was reductively linked to its binding site on opsin by using borane–dimethylamine. The [^{3}H]retinylopsin in octyl glucoside was exhaustively digested with Pronase, and its peptides were separated on silica gel in chloroform/methanol/ammonia [Bownds, D. (1967) *Nature (London) 216*, 1178–1181] followed by silica gel thin-layer chromatography in two solvent systems. The major retinyl peptide was shown to be alanyl-N^{ϵ}-retinyllysine by amino acid composition, ^{3}H content, and amino acid sequence analysis. The retinyl binding site is located in the carboxyl-terminal region of rhodopsin: when rod cell disk membranes containing [^{3}H]retinal rhodopsin were digested with thermolysin and then reacted with sodium borohydride or borane–dimethylamine, [^{3}H]retinal was reduced onto the F2 ($M_r \simeq 6000$) fragment, which derives from rhodopsin's carboxyl-terminal region.

Rhodopsin is the photoreceptor protein of rod cells in the vertebrate retina. Rhodopsin consists of an approximately 38 000-dalton protein, opsin, to which is bound a molecule of 11-*cis*-retinal. During the visual cycle, interaction of light with rhodopsin causes isomerization of the 11-*cis*-retinal to *all-trans*-retinal and its eventual dissociation from its binding site on the protein (Wald, 1968). For completion of the visual cycle and regeneration of the photosensitive visual pigment rhodopsin, a series of enzymes in the rod cell and pigment epithelium act to reconvert *all-trans*-retinal to 11-*cis*-retinal, which then recombines with the apoprotein opsin to regenerate rhodopsin (Bridges, 1976). The nature of the mode of association of retinal with the protein opsin has long been of interest due to the observation that retinal undergoes a red shift from its λ_{max} at 380 nm to as great as 560 nm in the formation of some visual pigments [see Hubbard et al. (1971)]. This shift is due both to the hydrophobic environment in the protein binding pocket and to the nature of the covalent linkage of retinal to the protein. A number of chemical studies have demonstrated that retinal is bound to the ϵ-amino group of a lysine residue in opsin via an aldimine or Schiff-base linkage [Bownds, 1967; Akhtar et al., 1968; reviewed by Knowles & Dartnall (1977)]. Spectroscopic studies reveal that the Schiff base is protonated [Lewis et al., 1973; reviewed by Callendar & Honig (1977)]. After the light-induced isomerization of protein-bound 11-*cis*-retinal to *all-trans*-retinal, the Schiff-base linkage becomes exposed to solvent and becomes susceptible to reaction with water or other reagents. It is then possible experimentally to covalently attach retinal to its protein-binding site by use of a variety of reducing agents which convert the Schiff-base linkage to a secondary amine (Bownds & Wald, 1965; Akhtar et al., 1965; Bownds, 1967; Hirtenstein & Akhtar, 1970; Zorn, 1971; Fager et al., 1972; Hall & Bok, 1976). In a pioneering study, Bownds (1967) enzymatically digested retinylopsin, isolated a set of overlapping peptides containing the retinyl binding site, and determined their compositions. There have been no subsequent reports elucidating the amino acid composition or primary structure of this region of the rhodopsin molecule. It has been the purpose of our study to isolate and characterize a small retinyl peptide from rhodopsin in order to assist in identifying the location of the retinyllysine in the primary structure of the protein.

Experimental Procedures

Materials

The following materials were obtained from Sigma Chemical Co.: *all-trans*-retinal, 9-*cis*-retinal, 13-*cis*-retinal, and N^{α}-acetyllysine. [11-^{3}H]Retinoic acid and 11-*cis*-retinal were gifts from Hoffmann-La Roche. Thermolysin, Pronase, and octyl β-D-glucopyranoside (octyl glucoside) were purchased from Calbiochem–Behring. Hydroxylapatite (DNA grade Bio-Gel HTP) and protein standard mixtures for molecular weight calibration of $NaDodSO_4$[1]–polyacrylamide gels were purchased from Bio-Rad. Silica gel HR was obtained from EM Laboratories, Inc. (Elmsford, NY), and silica gel G plates (250 μm) were obtained from Analtech, Inc. Diazomethane was synthesized from diazald (Fieser & Fieser, 1967). Diazald and borane–dimethylamine were purchased from Aldrich Chemical Co. Active manganese dioxide was prepared by the

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[1] Abbreviations used: DDT, dithiothreitol; [^{3}H]retinal ROS, ROS which have been bleached and then regenerated with [^{3}H]retinal; [^{3}H]-retinal rhodopsin, rhodopsin which has been prepared from [^{3}H]retinal ROS; $NaDodSO_4$, sodium dodecyl sulfate; Na_2EDTA, disodium ethylenediaminetetraacetate; TrTAB, tridecyltrimethylammonium bromide; Tris, tris(hydroxymethyl)aminomethane; ROS, rod cell outer segments; solvent I, $CHCl_3/CH_3OH/NH_4OH$ (70:27:3); solvent II, $CHCl_3/CH_3OH/NH_4OH$ (40:40:20); solvent III, 1-propanol/NH_4OH (70:30).

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