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Intracellular Localization of Photosynthetic Membrane Growth Initiation Sites in *Rhodopseudomonas sphaeroides*

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Putative membrane invagination sites at which intracytoplasmic photosynthetic membrane growth is initiated in *Rhodopseudomonas sphaeroides* can be isolated in an upper pigmented fraction by rate-zone sedimentation. The intracellular localization of membranes present in the isolated fraction was investigated with the impermeant surface-labeling reagent pyridoxal 5'-phosphate, which has been shown to diffuse into the periplasmic space and to label proteins of both the peripheral cytoplasmic membrane and the mature intracytoplasmic membrane. A comparison of the extent of labeling at 25 and 0°C was consistent with the possibility that membranes present in the upper pigmented fraction arise from sites near the cell periphery. Pronase digestion of the surface-labeled membranes suggested further that the purified upper fraction consisted largely of open membrane fragments and that the majority of the intracytoplasmic membrane is labeled by this procedure. The pigmented membrane growth initiation sites were separated partially from undifferentiated respiratory cytoplasmic membrane also present in the upper fraction.

The intracytoplasmic photosynthetic membrane (ICM) of the photoheterotrophic bacterium *Rhodopseudomonas sphaeroides* possesses several advantages as a system for the study of the assembly of energy-transducing membranes (11). The ICM can account for a major portion of cell protein, and upon disruption this structure yields uniform vesicular structures (chromatophores) that are easily purified and contain most of the cellular bacteriochlorophyll *a* (Bchl) (11, 16). As a procaryotic structure, the formation of the ICM is controlled by a single genetic system unlike that of the more complex eucaryotic organelle membranes. Moreover, the major integral ICM proteins have been identified and consist of four light-harvesting (1) and three reaction center (17) polypeptide subunits. The levels of both these Bchl-associated proteins and the ICM can be altered dramatically by changes in oxygen tension and light intensity (11).

In addition to the rapidly sedimenting chromatophores, up to 15% of the Bchl is isolated in an upper pigmented band after direct rate-zone sedimentation of *R. sphaeroides* extracts on sucrose density gradients (14, 16). In vivo labeling with amino acids (14, 16) and the porphyrin precursor δ-aminolevulinate (7, 10) suggested that newly synthesized reaction centers and their associated core B875 light-harvesting complex are inserted preferentially into membranes present in the upper fraction. Their polypeptide components are then chased during membrane growth into the ICM with isolation characteristics of chromatophores (14, 16). Further characterization indicated that the size and composition of the photosynthetic units of the upper pigmented band (6), their stage of assembly (7), and their fluorescence yield properties (8) are those of partially developed (15, 20, 21) photosynthetic membranes. Overall, these results suggested that the upper band contains pigmented membrane derived from invagination sites on the respiratory cytoplasmic membrane (CM)-ICM continuum at which growth of the ICM is initiated. Slowly sedimenting membrane fractions in which pigment-protein complexes are inserted preferentially have also been demonstrated in *R. capsulata* (4) and *R. rubrum* (9).

The presence (16) of CM polypeptides (19) in the upper pigmented band has suggested that membranes within this fraction arise from the cell periphery. Sedimentation studies in cells undergoing induction of ICM formation and in osmotically lysed spheroplasts (14) have further suggested that the putative pigmented membrane invagination sites within the upper fraction are also peripherally localized.

In the present study, the intracellular localization of the membranes isolated with the upper pigmented band has been examined further by surface labeling of whole cells with pyridoxal 5'-phosphate (PLP). Although PLP enters into the periplasmic space, it is impermeant to the CM-ICM continuum (5) and forms aldimine linkages with free amino groups at the periplasmic aspect of the membrane that are labeled by reduction with [^{3}H]KBH_4. This procedure indicated that up to 76% of the ICM may be accessible to the external environment (5). A comparison of the diffusion of PLP and the extent of labeling at 0 and 25°C has suggested further that ICM growth initiation sites are localized at the cell periphery. Further evidence for the presence of both differentiating and undifferentiated peripheral membrane within the upper pigmented fraction is also presented.

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MATERIALS AND METHODS

Surface-labeling procedures. *R. sphaeroides* NCIB 8253 was grown phototrophically as in reference 19 at 1,800 lx to assure that the ICM penetrated deeply into the cytoplasm. Although the number of new invaginations relative to fully invaginated ICM is low under these circumstances, a reasonable yield of putative invagination sites is obtained in the

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upper pigmented band (16) free of contamination by ICM-derived chromatophores (6). For surface labeling (5), washed cells were reduced with BH_4, washed further in 1 mM Tris buffer (pH 7.5), and suspended to 0.14 g (wet weight)/ml in 50 mM potassium phosphate buffer (pH 8.5). PLP was added to 10 mM, and the cells were incubated in a shaking water bath at 40 oscillations per min. Aldimine linkages were reduced at 0°C with [^{3}H]KBH_4 (2 mCi per sample) diluted with sufficient unlabeled BH_4 to reduce all available sites. The radioactively labeled cells were washed once with the Tris buffer, disrupted in a French press, and fractionated as in references 14 and 16. Procedures for the determination of protein radioactivity on filter paper disks and the determination of membrane protein concentration were those given in references 16 and 19, respectively. The lipid extraction procedure used during the preparation of the fractions for liquid scintillation counting removed phosphatidylethanolamine, which is also labeled by this procedure.

Pronase (25 μg/ml) digestion of membrane fractions (1.0 mg of protein per ml) was performed in 50 mM Tris buffer (pH 7.5) in a total volume of 0.3 ml at 37°C for 60 min. The reaction was stopped with an equal volume of cold 10% (wt/vol) trichloroacetic acid. After 30 min at 0°C, trichloroacetic acid precipitates were resuspended, applied to filter paper disks, and treated for liquid scintillation counting as described previously (16).

Fractionation of upper pigmented band. The upper pigmented fraction isolated as in references 14 and 16 was diluted 1:3 in 10 mM Tris–20 mM EDTA buffer (pH 7.5) and sedimented at 280,000 × *g* for 60 min. The supernatant and resuspended pellet fractions which contained CM and pigmented membrane, respectively, were aggregated by sedimentation at 105,000 × *g* for 18 h and purified further by rate-zone sedimentation on a 5 to 55% (wt/wt) sucrose gradient prepared in the Tris-EDTA buffer. This partial separation was also achieved by Sepharose 2B gel filtration and centrifugation procedures as described previously (19). Both methods yielded preparations of comparable purity.

RESULTS

Localization of ICM growth initiation sites by surface labeling. Previous studies (14, 16) had suggested that peripherally localized membrane is present within the upper pigmented band observed by rate-zone sedimentation of *R. sphaeroides*. This was tested further by a comparison of membrane surface labeling of phototrophically grown cells at 25 and 0°C. It was expected that at the lowered temperature the diffusion of the labeling reagents deep into the ICM interior would be restricted in comparison to that of the peripheral portions of the periplasmic space. The distribution of radioactivity after direct rate-zone sedimentation of cell-free extracts is shown in Fig. 1A. Although chromatophores were not as highly labeled as the upper pigmented or cell wall-enriched fractions, the labeling of the chromatophore proteins at 0°C was reduced significantly in comparison to that at 27°C.

Since it appeared that labeled periplasmic proteins could account for much of the radioactivity in the upper pigmented band and this fraction also contains ribosomes (16), a further purification was carried out (Fig. 1B). The distribution of radioactivity after purification closely followed the profile of light-harvesting Bchl absorbance at 850 nm, indicating that pigmented membrane within the original upper fraction was highly labeled. In contrast, the ribosome fraction near the top of the gradient remained essentially unlabeled. The lack

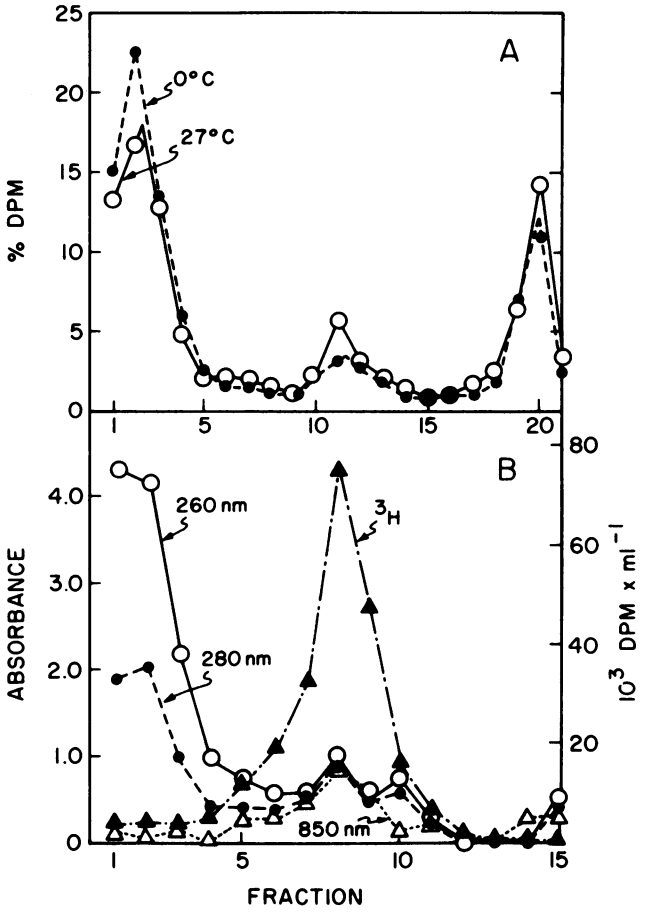

FIG. 1. Distribution of radioactivity in membrane protein after surface labeling of whole cells. (A) Comparison of aldimine linkage formation with PLP at 0 and 27°C. The PLP-treated cells were reduced with [^{3}H]KBH_4. Upper pigmented band, fractions 2 to 5; chromatophores, fractions 10 to 13; cell wall fraction, fractions 19 to 21. Fraction 1 represents the top of the gradient. Cellular phospholipid is distributed approximately equally in these membrane fractions (Radcliffe et al., in preparation). (B) Distribution of radioactivity in the surface-labeled proteins of upper pigmented fraction after sedimentation and further purification by sucrose density gradient centrifugation (9). Purified upper band, fractions 6 through 10; unfolded ribosomal particles, fractions 1 through 4. Fraction 1 represents the top of the gradient.

of ribosome labeling confirmed that the CM-ICM continuum is impermeant to PLP (5).

The surface-labeling kinetics of the upper pigmented and chromatophore fractions are shown in Fig. 2. The time required to achieve steady-state radioactivities in each fraction was increased three- to fourfold at 0°C (Table 1). Although the rates for half-maximal labeling did not differ greatly in the upper pigmented and chromatophore fractions, the extent of steady-state labeling at 0°C was reduced by about 25 to 40% in chromatophores. Overall, these results are consistent with restricted diffusion of the labeling system into the ICM interior at reduced temperature and a more peripheral in vivo localization for the more highly labeled pigmented membrane within the upper band than for ICM-derived chromatophores. Thus, about one-fourth to one-half of the ICM interior may be sequestered from labeling reagents at 0°C.

The availability of the labeled membrane preparations has

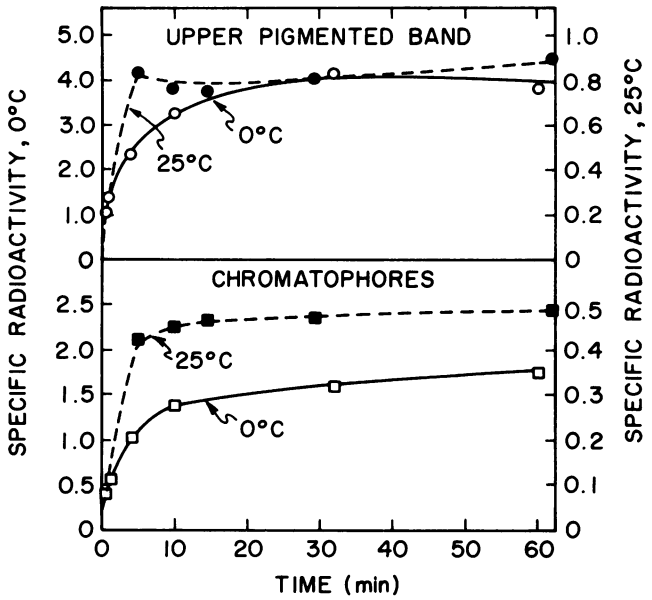

FIG. 2. Kinetics of radioactive surface labeling of membrane proteins in intact cells. The cells were treated with PLP at 0 and 25°C, and aldimine linkages were reduced with [^{3}H]KBH_4. Cells were labeled at 0°C in the presence of 2% (vol/vol) glycerol to further restrict diffusion of labeling reagents into the periplasmic interior of the ICM. Specific radioactivities, expressed as 10^4 dpm per mg of protein, are plotted such that the steady-state labeling achieved in the upper pigmented band at 0 and 25°C is equalized to facilitate comparison with chromatophores. Note that steady-state values in chromatophores are lower than those of the upper pigmented fraction.

also permitted a quantitative assessment of their periplasmic surface exposure. Previous studies (6, 14) had suggested that the upper pigmented fraction contains open membrane fragments with both surfaces exposed. Provided that all derivatized protein amino groups are removed by pronase, the digestion results in Table 2 would suggest that at least 77% of this fraction consists of membranes with an exposed periplasmic aspect; the remaining label was probably sequestered by membrane aggregation during purification. In contrast, other results presented in Table 2 indicated that only about 20% of the reduced pyridoxine 5'-phosphate conjugates at the periplasmic aspect of the ICM were exposed in the isolated chromatophores. This agrees with previous findings (3, 12) that about 80% of such chromatophores are isolated as sealed vesicles with their cytoplasmic aspect exposed and suggests further that the majority of the periplasmic aspect of the ICM was surface labeled by this procedure.

Partial separation of upper pigmented fraction into pigmented and unpigmented membrane. As described in Materials and Methods, the upper pigmented band has been separated partially into the pigmented membrane and CM whose presence was suggested previously (14, 16). Table 3 indicates that, typically, the Bchl content of isolated pigmented membrane was at least 2.5-fold that of CM and that the values in the latter approached those in reference 19 for peripheral CM. Although the Bchl level of the pigmented membrane fraction was only enriched <1.6-fold over that of the unfractionated upper band, this was due partly to some removal of the Bchl-rich B800-850 light-harvesting complex during purification. Previous analysis of the upper pigmented band (16) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis had indicated that polypeptide subunits of the Bchl-protein complexes were present and that these were superimposed upon the numerous polypeptide components of the CM (19). In the present investigation, the CM fraction derived from the upper pigmented band was enriched in the latter, whereas the pigmented membrane fraction was enriched in reaction center and light-harvesting polypeptides (not shown).

Studies of lipid phosphorus distribution in *R. sphaeroides* labeled uniformly with $^{32}P_i$ have suggested that about one-third of the phospholipid in cells grown at 1,800 lx is present in the upper pigmented band (C. W. Radcliffe, F. X. Steiner, and R. A. Niederman, manuscript in preparation). Table 3 shows that the pigmented membranes derived from the upper band have a high phospholipid content. The levels of total zwitterionic (phosphatidylethanolamine + phosphatidylcholine) to anionic (phosphatidylglycerol + cardiolipin) phospholipids in the pigmented membrane and chromatophore fractions were 1.90 and 1.41, respectively (Table 4). Table 3 also indicates that the ratio of the core B875 light-harvesting complex to that of the peripheral B800-850 antenna in the pigmented membrane fraction was twice that of chromatophores and that the pigmented membranes were

TABLE 1. Analysis of surface-labeling kinetics

Membrane fraction	Half-maximal labeling time (min)[a]		Relative specific radioactivity[b]			
			Trial 1		Trial 2[c]	
	25°C	0°C	25°C	0°C	27°C	0°C
Upper pigmented	<1.1	3.7	1.00	1.00	1.00	1.00
Chromatophore	<1.2	4.2	0.58	0.43	0.64	0.38
Cell wall	1.2	4.8[d]				

[a] Calculated from data presented in Fig. 2.
[b] Calculated from steady-state labeling presented in Fig. 2. The data at 25°C were obtained from an average of the points at 9.7, 14.5, 29.3, and 62.0 min, whereas those at 0°C are from points at 32 and 60 min. See Fig. 2 for specific radioactivity units.
[c] Reacted with PLP for 6 min and with [^{3}H]KBH_4 at 0°C in the absence of glycerol. Reaction was terminated with excess PLP.
[d] This decreased labeling rate at 0°C may reflect more marked differences in exposure of potential labeling sites at the two temperatures in outer membrane proteins than in the more hydrophobic proteins of the CM-ICM continuum.

TABLE 2. Pronase digestion of surface-labeled membranes

Membrane fraction[b]	Distribution of radioactivity (dpm per mg of protein)[a]		Radioactivity released (%)
	Precipitate	Supernatant	
Upper pigmented			
Pronase treated[c]	12,415	42,384	77.0
Control[d]	58,323	254	0.4
Chromatophores			
Pronase treated	31,593	8,967	22.1
Control	36,380	1,873	4.9

[a] Radioactivity of treated disks containing trichloroacetic acid precipitate and supernatant fractions was determined in the presence of NCS (16). Values are averages from two samples.
[b] Membranes were isolated as described in the legend to Fig. 1 from cells treated for 6 min with PLP at 27°C and reacted with [^{3}H]KBH_4 for 15 min at 0°C.
[c] Treated with pronase as described in the text.
[d] Incubated at 37°C for 1 h in 50 mM Tris buffer (pH 7.5) in the absence of pronase.

TABLE 3. Characterization of membrane fractions

Fraction[a]	Specific Bchl (μg/mg of protein)[b]	Phospholipid/protein (wt/wt)[b]	B800-850/B875 (mol/mol)[c]	Carotenoid absorption maximum (nm)[d]
Upper pigmented	13.4 (13.5)[e] (4.3)[f]	0.26	0.85	507.7
PM[g]	15.3 (18.7) (6.8)	0.31	0.71	507.3 (505.5)[f]
CM	6.3 (7.0) (0.5)	0.32	0.88	
Chromatophores	70.2 (68.2) (47.5)	0.27	1.46	508.5 (507.5)

[a] Except where indicated, the membrane fractions were isolated by the differential and rate-zone sedimentation procedures described in the text from cells grown as in reference 19 at 1,800 lx.

[b] Determined by procedures given in reference 19.

[c] B800-850 and B875 are the peripheral and core light-harvesting pigment-protein complexes, respectively, designated by near-infrared absorption maxima. Their levels were determined by computer-assisted deconvolution of near-infrared absorption spectra obtained on a Johnson Research Foundation DBS-3 spectrophotometer, using extinction coefficients given in reference 2.

[d] Maximum of longest-wavelength absorption band attributable to carotenoids determined by computer-assisted analysis of absorption spectra. Carotenoid absorbing at the shorter wavelength is that shown in reference 1 to be associated with B875.

[e] Upper pigmented fraction separated into pigmented membrane and CM fractions by gel filtration (19).

[f] Membrane fractions from cells of reduced Bchl content grown at 16,000 lx in which the B875/B800-850 ratio was increased by about twofold.

[g] Pigmented membrane fraction derived from upper pigmented band.

enriched in B875 carotenoids. Such elevated B875 levels are characteristic of partly developed ICM (15).

DISCUSSION

The intracellular origin of photosynthetic membrane growth initiation sites was examined in studies presented here on diffusion of the impermeant membrane surface-labeling reagent PLP through the periplasmic space and the interior of the ICM continuum. Free amino groups in whole cells that are exposed at the periplasmic aspect of the CM-ICM continuum were reacted with PLP for specific intervals at alkaline pH to form stable aldimine linkages. The reaction with PLP was conducted at 25 to 27 and 0°C to provide differences in the extent of diffusion of the reagent between the peripherally localized periplasmic space and its continuum into the ICM interior. The labeled cells were disrupted and the photosynthetic membrane growth initiation sites were isolated in an upper pigmented band, whereas the ICM was isolated as discrete chromatophore vesicles.

Differences in the extent of steady-state protein specific radioactivities at 25 and 0°C suggested a more peripheral localization for membranes of the upper pigmented band than for chromatophores. This is consistent with the expectation that restrictions to periplasmic penetration of the labeling reagents imposed by the reduced temperature would result in a greater extent of labeling of the surface of membranes located near the cell periphery than of the interior surface of an extensive tubular internal membrane structure. This corroborates previous evidence (14, 16) which suggested that pigmented membranes isolated with the upper fraction represent peripherally localized photosynthetic membrane growth initiation sites and that chromatophores arise from the ICM. Since only surface-labeled proteins were examined here, it is not possible to explain these results by the specific labeling of CM phosphatidylethanolamine whose diffusion into the ICM is more restricted at 0 than at 25°C. The close coincidence of the profile of light-harvesting Bchl with that of the radioactivity in the purified upper pigmented fraction is consistent with labeling of protein components associated with membrane invagination sites. The explanation of the results on the basis of diffusion of the labeling reagents within the CM-ICM interior is also supported by the demonstration with this procedure that most of the ICM was accessible to the external medium (5).

TABLE 4. Phospholipid composition of membrane fractions[a]

Fraction[b]	% Total phospholipid			
	Phosphatidylethanolamine	Phosphatidylglycerol	Phosphatidyl choline	Cardiolipin
Upper pigmented band derived				
Cytoplasmic membrane	39.1	32.5	13.7	3.4
Pigmented membrane	48.7	30.4	12.4	1.8
Chromatophore	40.4	36.3	13.7	2.2

[a] Cells were labeled uniformly with $^{32}P_i$ during growth as in reference 19 at 1,800 lx. Phospholipid composition was determined as in reference 18.

[b] Membrane fractions were isolated by the differential and rate-zone sedimentation procedures described in the text.

With regard to the sidedness and physical integrity of the membranes isolated in the upper pigmented band, photochemical studies indicated that, unlike the results obtained with vesicular chromatophores, the reaction center sites for interaction with soluble type *c* cytochrome are exposed in this fraction (6). Furthermore, this upper pigmented fraction lacked a light-induced transient absorption change resulting from a carotenoid spectral response to membrane potential (6). This suggested a lack of membrane vesicle integrity within this fraction. Electron micrographs of thin sections and pronase digestion studies (14) indicated that both the periplasmic and cytoplasmic aspects of these membranes were exposed. A quantitative assessment of this exposure was provided in the present study by following the release of radioactivity after pronase digestion of the upper pigmented fraction isolated from the [^{3}H]KBH_4-reduced, PLP-treated cells. Only portions of polypeptides originally exposed at the periplasmic aspect of the CM-ICM continuum were digested in this manner.

The results indicated that nearly 80% of the surface label was released from the upper pigmented fraction. If it is assumed that all derivatized protein amino groups on the exposed membrane surface are removed by this broad-specificity protease, this result would suggest that, as isolated, the periplasmic aspect is sequestered in less than one-

fourth of these membranes. Thus, the upper pigmented fraction is valuable for studies requiring exposure of both membrane surfaces. It is possible that, in the unaggregated state, even a greater exposure of both membrane surfaces exists.

These studies provide further support for the existence of three discrete regions within the CM-ICM continuum: mature, fully pigmented ICM isolated as chromatophores; undifferentiated respiratory CM that is relatively unpigmented; and partially pigmented ICM growth initiation sites. They suggest further that the latter two are localized at the cell periphery. Recently, differentiated CM regions that represent functionally distinct domains have also been separated in *Bacillus subtilis* (13).

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