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Comparative Studies of Two Membrane Fractions Isolated from Chemotrophically and Phototrophically Grown Cells of *Rhodopseudomonas capsulata*

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Light and heavy membrane fractions have been isolated by equilibrium sucrose density centrifugation from *Rhodopseudomonas capsulata* 938 GCM grown aerobically in the dark (chemotrophically) and anaerobically in the light (phototrophically). The densities of the light and heavy fractions from phototrophic cells were 1.1004 to 1.1006 and 1.1478, respectively, and the densities of the light and heavy fractions from chemotrophic cells were 1.0957 to 1.0958 and 1.1315, respectively. Both fractions were active in photochemical and respiratory functions and in electron transport-coupled phosphorylation. The light membrane fraction isolated from chemotrophic cells contained the reaction center and the light-harvesting pigment-protein complex B 870, but not the variable light-harvesting complex B 800-850. A small amount of the complex B 800-850 was present in the light fraction isolated from phototrophically grown cells, but it was not energetically coupled to the photosynthetic apparatus. From inhibitor studies, difference spectroscopy, and measurement of enzyme activities it was tentatively concluded that the light membrane fraction contains only the reduced nicotinamide adenine dinucleotide-oxidizing electron transport chain having a KCN-insensitive, low-potential cytochrome *c* oxidase, whereas the heavy fraction contains additionally the succinate dehydrogenase and a high-potential cytochrome *b* terminal oxidase sensitive to KCN. The light membrane fraction was more labile than the heavy fraction in terms of phosphorylating activity.

Cells of *Rhodopseudomonas capsulata* grown under strict aerobic conditions in the dark contain, besides the cytoplasmic membrane, a few tubular intracytoplasmic membranes in the polar region of the cell (22).

As soon as the formation of the photosynthetic apparatus is induced, vesicular intracytoplasmic membranes (chromatophores) are formed (8a, 21). These intracytoplasmic membrane vesicles are the main location of the photosynthetic apparatus (21), and the respiratory functions are concentrated in the cytoplasmic membrane (21). Rate zone centrifugation of the membrane fractions on sucrose gradients has shown that the chromatophores sediment faster than the cytoplasmic membrane (22). The buoyant densities of the membrane fractions are variable under different culture conditions. In particular, the light fraction can move to different positions in the sucrose gradient (A. F. García and G. Drews, unpublished data). The activities of phosphorylation and NADH-dependent respiration are present in both fractions, but have different specific activities (12). Under conditions which induce the formation of the photosynthetic apparatus and limit growth, radioactive amino acids were incorporated into both membrane fractions at specific sites with different kinetics, resulting in different protein patterns (8a). In this communication we will describe characteristic differences in the composition of the photosynthetic apparatus and the respiratory electron transport chain of both membrane fractions.

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MATERIALS AND METHODS

Bacteria and culture conditions. *R. capsulata* wild-type strain 37b4 (German collection of microorganisms, Göttingen, strain 938), the carotenoidless, phototroph-positive mutant strain Ala$^+$ derived from *R. capsulata* 37b4, and the culture medium have been described previously (11, 35).

The cells were cultivated in a 14-liter fermentor (Microferm; New Brunswick Scientific Co.) in the dark at 30°C under strong aeration with mechanical stirring (500 to 600 rpm). The formation of the photosynthetic apparatus was induced by lowering the oxygen partial pressure in the dark for 30 min (stirring at 200 rpm, low aeration). The culture was then gassed with purified nitrogen (99.999%) and irradiated (40,000 lx; in-

candescent bulbs behind the fermentor). The culture was harvested at an optical density of 0.3 to 0.5 (0.5-cm light path at 660 nm). Biosynthetic processes were stopped by the addition of chloramphenicol (100 μg/ml), NaN_3 (2 mM), and phenylmethylsulfonyl fluoride (PMSF; 1 mM) (final concentrations).

Membrane isolation. The cells were disrupted at 10,000 lb/in^2 in a French pressure cell (300 mg [wet weight] of packed cells per ml of 50 mM Tris-hydrochloride buffer [pH 7.8] plus 2.5 mM $MgCl_2$). DNase was added, and the extract was centrifuged at 14,000 rpm for 30 min in a Sorvall centrifuge RC-2B (Rotor SS 34) (11). The supernatant material was layered on top of a discontinuous sucrose gradient consisting of 3 ml of 60% sucrose, 4 ml each of 50, 40, 30, and 20% sucrose in Tris-hydrochloride buffer plus $MgCl_2$ and centrifuged to equilibrium of distribution (38,000 rpm, 17 h; 60 Ti rotor; Beckman ultracentrifuge L65) (12).

Measurement of enzymatic activities. Succinate dehydrogenase (EC 1.3.99.1) and NADH dehydrogenase (EC 1.6.99.3) were determined as described previously (13). NADH and succinate oxidation and ascorbate–2,6-dichlorophenol indophenol (DCPIP)–cytochrome oxidoreductase (terminal oxidase; EC 1.9.3.7) were determined by polarographic O_2 measurement by using a YSI 53 biological oxygen monitor (Yellow Springs Instruments Co., Yellow Springs, Ohio). The oxygen uptake was calculated on the basis of an oxygen concentration of 237 μM in the air-saturated buffer at 30°C (6, 8). NADH or succinate oxidation is the membrane-bound capacity to oxidize the respective substrate by oxygen. Photophosphorylation was measured in a scintillation counter by using the ATP-induced light emission produced by the luciferin-luciferase reagent (LKB products) as described previously (12). The membrane samples were taken directly from the sucrose gradient. Oxidative phosphorylation was measured coupled to NADH oxidation.

Photochemical activities. A Perkin-Elmer double-beam spectrophotometer model 356, in the split beam mode, was used for the estimation of the light-minus-dark difference spectra. The light for cross-illumination was passed through an interference filter with maximum transmission at 872 nm (38%) and a half bandwidth of 14 nm. The method has been described previously in detail (5, 10, 13, 30, 31).

The absorption spectra were measured in a Cary 14 double-beam spectrophotometer. The fluorescence emission spectra were registered with the equipment described previously (11a).

Analytical procedures. Bacteriochlorophyll (BChl) was estimated by extraction of cells or membranes with an acetone-methanol mixture (7:2, vol/vol). The extinction coefficient was $\epsilon = 76\ mM^{-1}$ at 772 nm (7). Protein measurements were performed by the method of Lowry et al. (24).

The concentration of reaction centers in membrane fractions was determined from light-induced absorption changes at 880 nm and calculated by using an extinction coefficient of $\epsilon = 113\ mM^{-1} \cdot cm^{-1}$ (7, 37). One reaction center contains 4 mol of BChl (37).

RESULTS

Spectroscopic observations. Cell-free extracts from the wild-type strain of *R. capsulata*, isolated from chemotrophically and phototrophically grown cells, were subjected to equilibrium density centrifugation. Under both growth conditions two major membrane fractions were obtained which, according to their position in the gradient, will be designated upper and lower fractions. The density of both fractions in the gradient increased during the induction period, indicating a decrease of the lipid/protein ratio (12). Neither fraction from aerobically grown cells contained the variable light-harvesting B 800-850 pigment-protein complex (Fig. 1, curves a and b). However, this component was present in different ratios in both fractions from phototrophically grown cells (two generations of phototrophic growth; Fig. 1 curves c and d).

Fluorescence emission spectra. The fluo-

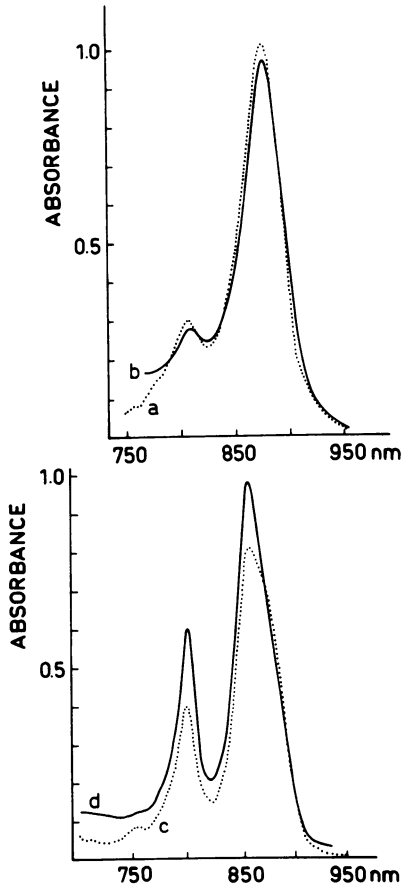

FIG. 1. *Near infrared absorption spectra of the purified fractions isolated from chemotrophically grown cells (a, b) and from cells grown phototrophically for 4 h (c, d). Curves: a, upper fraction, banding at 26% sucrose (equilibrium density centrifugation); b, lower fraction, banding at 38% sucrose; c, upper fraction, banding at 29% sucrose; d, lower fraction, banding at 40% sucrose.*

fluorescence emission spectra of the membrane fractions isolated from aerobically grown cells showed peaks in the near infrared at 902 to 903 nm (77°K) and 887 to 891 nm (300°K), respectively, indicating the fluorescence emission band of the B 875 BChl (Fig. 2a and b). The fluorescence emission spectra of the lower bands from phototrophically grown cells taken at 300°K showed a peak at 885 nm corresponding to the fluorescence emission of the B 875 BChl and a shoulder at 850 nm corresponding to the fluorescence emission of the B 850 BChl (Fig. 2d).

The upper band isolated from phototrophically grown cells showed at 300°K a major fluorescence band at 883 nm (Fig. 2c) and therefore mostly originated from the B 800-850 complex (11a). It is remarkable however, that the fluorescence emission spectrum at 77°K showed an emission band at 902 nm and a prominent shoulder coinciding with the emission maximum of the isolated B 800-850 complex, whereas under the same experimental conditions the lower band showed only one peak near 907 nm. This reflects a low-resonance energy transfer in the upper fraction (11a). Since comparable lower membrane fractions with the same B 870/B 800-850 ratio showed at 77°K only the peak at 902 nm, we conclude that in the upper fractions of phototrophically grown cells either the energy transfer at low temperatures is physically hindered by larger distances separating the complexes B 875 and B 800-850 in the newly formed membrane, or there are two membrane fractions enriched in the reaction center plus B 870 and the B 800-850 pigment complex, respectively, which also have very similar density properties but which are structurally independent.

Enzyme activities. The upper fractions of chemotrophically and phototrophically grown cells consist of closed membrane vesicles (J. R. Golecki, unpublished data). They show low ATP-forming activity by either photophosphor-

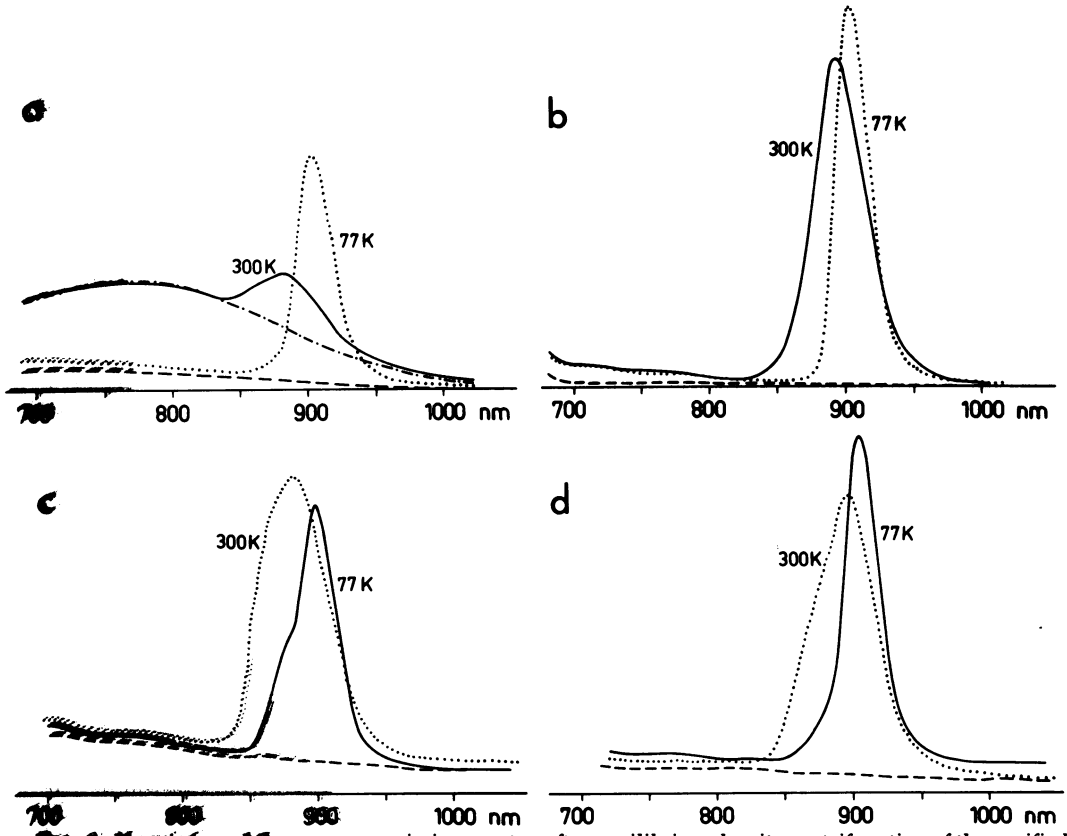

FIG. 2. Near infrared fluorescence emission spectra after equilibrium density centrifugation of the purified upper and lower membrane fractions isolated from chemotrophically (a, b) and phototrophically (c, d) grown cells. See Fig. 1 for conditions of growth; broken line, base line of spectrophotometer; the emission curves at 77°K are taken at higher magnification. Panels: a, upper fraction, peaks at 887 nm (300°K) and 903 nm (77°K); b, lower fraction, peaks at 892 nm (300°K) and 902 (77°K); c, upper fraction, peaks at 883 nm (300°K) and 902 nm (77°K); d, lower fraction, peaks at 895 nm (300°K) and 907 nm (77°K).

ylation or by NADH-dependent oxidative phosphorylation (Table 1). The activity of ATP formation was found to be between 5 and 10 times higher when the samples were taken from the gradient after 3 to 4 h instead of after 17 h (Table 1). The upper fractions from both chemotrophically and phototrophically grown cells did not show any succinate dehydrogenase activity, but they catalyzed NADH-supported oxygen uptake. Ascorbate-DCPIP oxidation was also not measurable in the upper fractions. In contrast, succinate and NADH oxidation were both present in the lower fractions of phototrophically and chemotrophically grown cells. The difference in oxidative capacity in the upper fraction might indicate that only NADH oxidation coupled to one terminal oxidase is active. This idea is supported by the observation that the upper fraction showed low sensitivity to KCN, whereas the lower fraction showed a biphasic behavior (Fig. 3). Similar results were obtained for the upper band (data not shown) isolated from phototrophically grown cells. The effect of antimycin A on the oxidation of NADH points also to differences in the respiratory electron transport chain of the upper and lower membrane fractions isolated from aerobically grown cells. At a 10 μM concentration of antimycin A, the oxygen uptake in the lower fraction was 46% of the control, whereas at this concentration the upper band was not inhibited. This effect of antimycin A at low concentrations points to a difference between the NADH-supported oxygen-consuming reactions with the two fractions. The effects of KCN and antimycin A shown here are in agreement with those of more than one electron transport pathway reported previously by other authors (3, 25, 32–34, 42).

Cytochrome spectra. Figure 4 shows the reduced-minus-oxidized spectra for the lower and upper bands in the cytochrome region. Although a definite identification of the species responsible for the different absorption bands must await further studies, the two fractions seemed to have a different cytochrome composition. At room temperature two bands were seen in the upper membrane fraction having their maxima at 556.5 and 550 nm. In the lower membrane fractions, the spectra clearly showed peaks at 552.5 to 553 and 560 nm, possibly corresponding to cytochromes *c* and *b*, respectively (18, 19). At cryogenic temperatures (77°K) a new band became detectable in the upper fraction with a maximum at 559.5 nm, although it was only seen as a shoulder on the long-wavelength side of the spectrum (not shown).

Size of the photosynthetic unit. The size of the photosynthetic unit (moles of BChl per reaction center) in the upper fraction was smaller than that in the lower fraction isolated from phototrophically grown cells. The upper fraction unit was, however, of the same magnitude as that measured in the lower membrane fraction isolated from aerobically grown cells (Table 2). The low variability of the size of the photosynthetic unit in the upper fraction is in accordance with the dominance of the B 875 BChl light-harvesting component in this fraction. The B 875 pigment protein complex is known as the invariable light-harvesting BChl (1, 23, 35, 36). The fourfold increase of the size of the photosynthetic unit in the lower membrane fraction of phototrophically grown cells as compared with the same fraction in chemotrophically grown cells is caused by the increase of the variable light-harvesting component B 800-850 (Fig. 1 and 2).

Light-minus-dark difference spectra. To learn more about the photochemical activity of the membrane fractions, we measured light-mi-

TABLE 1. *Enzyme activities of the different membrane fractions*[a]

Fraction	Phosphorylation[b]		Respiration[b]				
	Light induced	Oxidative	NADH dehydrogenase	NADH oxidase	Succinate dehydrogenase	Succinate oxidase	Ascorbate-DCPIP oxidase
Chemotrophic cells							
Upper fraction	0.0018	0.004	1.14	0.6	0.08	ND	ND
Lower fraction	2.36	3.78	5.94	9.4	12.3	3	12.6
Phototrophic cells							
Upper fraction	0.003	0.0025	1.62	0.42	0.06	ND	ND
Lower fraction	30.0	0.9	7.8	12.74	20.5	4.74	16.7

[a] The phototrophically grown cells were harvested 240 min after the beginning of incubation in the light (see text). During this period the BChl content of the purified lower fraction increased to 28 μg of BChl/mg of protein.

[b] Enzyme activities are expressed as micromoles of substrate oxidized or ATP formed per hour per milligram of membrane protein. ND, Not detectable.

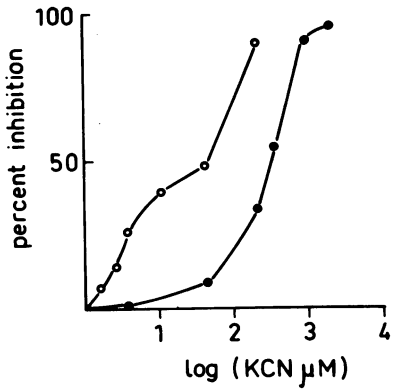

FIG. 3. *Inhibition of respiratory activity (oxygen uptake with NADH as substrate) by KCN in membrane fractions isolated from cells grown strictly aerobically in the dark. Initial activities (control): upper fraction (●) 0.64 μmol of $O_2 \cdot h^{-1} \cdot mg$ of protein^{-1}; lower fraction (○) 10.1 μmol of $O_2 \cdot h^{-1} \cdot mg$ of protein^{-1}.*

nus-dark difference absorption spectra. The difference spectra of upper and lower membrane fractions isolated from aerobically grown cells (Fig. 5a) showed light-induced reduction of cytochrome *b*, oxidation of cytochrome *c*, and bleaching of the reaction center BChl (590-nm band). Although the aerobically grown wild-type cells contained relatively large amounts of carotenoids in both membrane fractions, the difference spectra did not indicate a carotenoid band shift. The light-induced shift to longer wavelengths in the absorbance spectrum of carotenoids appears to be a result of a transmembrane electrical gradient (9). It is linked to the carotenoids of the B 800-850 pigment protein complex (39).

The carotenoids of the B 875 light-harvesting complex seem not to be involved (14, 26, 39). As a consequence of the absence of a light-induced carotenoid band shift in the upper and lower membrane fractions from chemotrophically grown cells, the difference spectra were very similar to the difference spectrum of a membrane fraction from phototrophically grown cells of the carotenoidless strain Ala$^+$ of *R. capsulata* (Fig. 5b).

The upper membrane fraction from phototrophically grown cells of the wild-type strain 37b contained carotenoids and the B 800-850 pigment protein complex. The difference spectrum, however, was similar to that shown in Fig. 5a (data not shown), indicating that the B 800-850 complex was present but not energetically linked to the reaction center.

This result further qualifies the low-temperature fluorescence spectra in which an inhibition of resonance energy transfer is apparent between

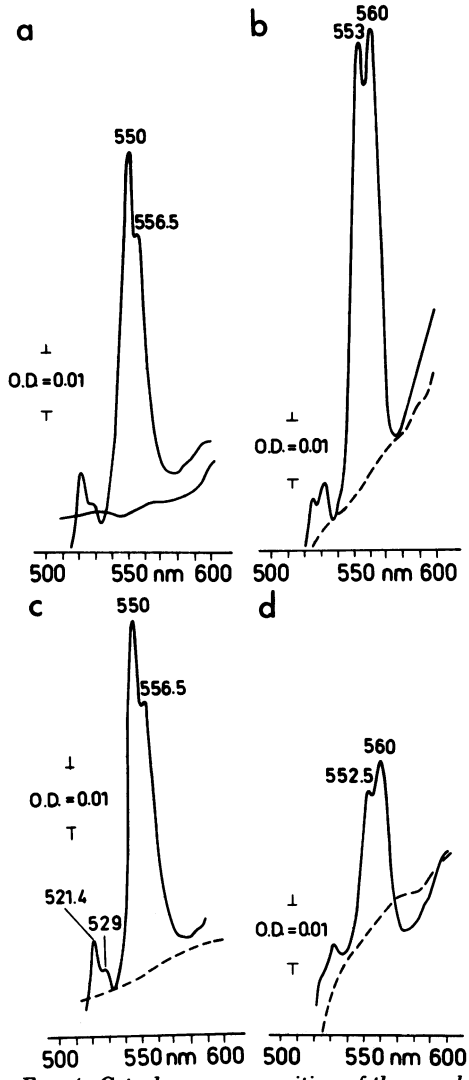

FIG. 4. *Cytochrome composition of the membrane fractions; reduced (dithionite)-minus-oxidized (ferricyanide) difference absorption spectra. Panels: a, upper fraction from chemotrophic cells; b, lower fraction from chemotrophic cells; c, upper fraction from phototrophically (180 min of induction) grown cells; d, lower fraction from phototrophically grown cells.*

the B 800-850- and the B 875-reaction center BChl complexes, detected in the upper band, from phototrophically grown cells. The light-minus-dark difference spectrum of the heavy membrane fraction from phototrophically grown cells showed a strong carotenoid band shift, as shown by other authors (17, 41).

DISCUSSION

The two membrane fractions were active in both photochemical and respiratory functions,

TABLE 2. *Size of the photosynthetic unit and specific BChl content of the different membrane fractions*

Fraction	μg of BChl per mg of protein	mol of BChl per reaction center
Chemotrophic cells		
Upper fraction	0.01–0.015	15[a]
Lower fraction	1.5–2.5	15
Phototrophic cells		
Upper fraction	2–3	10
Lower fraction	70	70

[a] This value was obtained after concentration of the upper band 15- to 20-fold by ultrafiltration. This process induced the appearance of some opalescence; therefore, the light scattering significantly increased the noise level during measurement and reduced the accuracy of the reported value.

but they differed in their lipid/protein ratio, in the composition of the pigment-protein complexes of the photosynthetic apparatus, and in the activities of the electron transport chain. The upper membrane fraction from chemotrophic cells lacked the variable light-harvesting pigment-protein complex B 800-850. The corresponding fraction from phototrophically grown cells contained variable amounts of this complex. The fluorescence emission (Fig. 2) and light-induced difference spectra (Fig. 5) of the upper membrane fraction support the idea that the B 800-850 complex, when present in the upper fraction, is not energetically coupled with the photosynthetic apparatus. This might be due to an incorporation of this complex into a membrane site physically separated from that of the photochemically active photosynthetic apparatus. This idea is supported by preliminary electron microscopic observations on the heterogeneity of the upper membrane fraction. It is interesting that in the upper fraction the ratio of B 875 BChl per reaction center is lower when compared with the steady-state values of a fully developed photosynthetic apparatus, for which values of 20 to 25 have been measured (1, 8, 35, 36).

A similar observation on the involvement of the B 800-850 pigment-protein complex in a low-density membrane fraction of *Rhodopseudomonas sphaeroides* was also made by Hunter and co-workers (15).

The electron transport system of *R. capsulata* is known to be branched, having two terminal oxidases (3, 25, 42). The low sensitivity of NADH oxidation to KCN, and the lack of ascorbate-DCPIP-oxygen oxidoreductase and succinate dehydrogenase activities in the upper membrane fraction suggest that only the NADH oxidation branch with a low-potential cytochrome *b* terminal oxidase is present. The unequal distribution of NADH and succinate-oxidizing electron transport chains and their components was previously reported from *R. sphaeroides* and *Rho-*

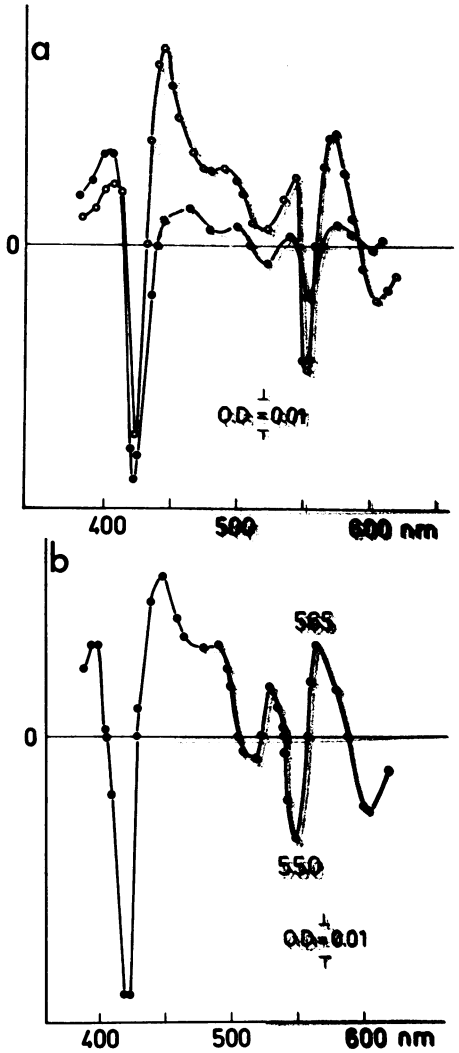

FIG. 5. *Light-minus-dark difference absorption spectra of membrane fractions isolated from chemotrophically grown cells of R. capsulata strain 37b4 and the strain Ala⁺ (see text). a, Upper fraction (●) from strain 37b4, repurified by a second sucrose density gradient run (protein content, 8.1 mg of protein·ml⁻¹); lower fraction (○) from strain 37b4, repurified as above (protein content, 4.4 mg of protein·ml⁻¹). b, Chromatophores from the blue-green mutant strain Ala⁺ (protein content, 4.6 mg of protein·ml⁻¹). The gradient for repurification was carried out in the SW 41 rotor of a Beckman ultracentrifuge L-65 in 50 mM Tris-hydrochloride buffer plus 2.5 mM $MgCl_2$. Sucrose concentrations: 0.6 ml of 60% sucrose, 1.4 ml of 50% sucrose, 2.4 ml each of 40, 30, and 20% sucrose. Finally, 2.0 ml of sample was layered on the top. The spectra were measured in the presence of 30 μM antimycin A.*

dospirillum rubrum (4, 16, 27-29, 38, 40). Among other *Rhodospirillaceae* there is ample evidence for branched pathways leading from succinate or NADH (or from both) to oxygen (2). Inhibitor studies suggested separate pathways for succinate and NADH oxidation (2, 3, 29, 38, 40) as well as the different extents of cytochrome reduction by substrates (20). One of the terminal oxidases of *R. capsulata* contains the high-potential cytochrome *b* and is sensitive to low concentrations of KCN. The other pathway leading to oxygen is connected to interaction of a low-potential cytochrome *b* with oxygen (2, 3, 42). According to Marrs and Gest, one of the pathways would be preferred by NADH oxidation (25). Our present results could indicate a deficiency of the terminal oxidase connected to the high-potential cytochrome *b* in the upper fraction. The upper fraction has a reduced content of a *b*-type cytochrome as shown by reduced-minus-oxidized spectra of the respective fractions.

The present and earlier results give evidence that the membrane system of *R. capsulata* and other facultative phototrophic bacteria is compartmentalized. We believe that the upper membrane fraction arises from the cytoplasmic membrane and partially from the tubular intracytoplasmic membrane of chemotrophically grown cells. It seems to be actively involved in the membrane assembly process and therefore in membrane differentiation. This low-density membrane fraction is only detectable in cells which grow or differentiate. The results are also in accordance with the observations that the reaction center and the B 800-850 complex are assembled at different sites of the membrane system and with different kinetics (Dierstein et al., in press).

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