Properties of a Cytochrome *c*-Enriched Light Particulate Fraction Isolated from the Photosynthetic Bacterium *Rhodopseudomonas spheroides*

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Differential centrifugation of suspensions of French-press-disrupted *Rhodopseudomonas spheroides* yielded a light particulate fraction that was different in many properties from the bulk membrane fraction. It was enriched in cytochrome *c* and had a low cytochrome *b* content. When prepared from photosynthetically grown cells this fraction had a very low specific bacteriochlorophyll content. The cytochrome *c* of the light particles differed in absorption maxima at 77 K from cytochrome *c*₂ attached to membranes; there was pronounced splitting of the α -band, as is found in cytochrome *c*₂ free in solution. Potentiometric titration at $A_{552}-A_{540}$ showed the presence of two components that fitted an $n=1$ titration; one component had a midpoint redox potential of +345 mV, like cytochrome *c*₂ in solution, and the second had E_0' at pH 7.0 of +110 mV, and they were present in a ratio of approx. 2:3. Difference spectroscopy at 77 K showed that the spectra of the two components were very similar. More of a CO-binding component was present in particles from photosynthetically grown cells. Light membranes purified by centrifugation on gradients of 5–60% (w/w) sucrose retained the two *c* cytochromes; they contained no detectable succinate–cytochrome *c* reductase or bacteriochlorophyll and very little ubiquinone, but they contained NADH–cytochrome *c* reductase and some phosphate. Electrophoresis on sodium dodecyl sulphate/polyacrylamide gels showed that the light membranes of aerobically and photosynthetically grown cells were very similar and differed greatly from other membrane fractions of *R. spheroides*.

The purple non-sulphur bacterium *Rhodopseudomonas spheroides* can grow photosynthetically under anaerobic conditions in the light, when it produces complex membrane structures that contain bacteriochlorophyll and carotenoids and the components of the photosynthetic electron-transport chain. These membranes pinch off to form small vesicles, called chromatophores, when the cells are disrupted. Under aerobic growth conditions the production of the photosynthetic pigments is inhibited and the cells contain almost no intracytoplasmic membrane. The adaptation of aerobically grown cells to assemble the photosynthetic apparatus under anaerobiosis can be studied under controlled conditions and so this organism is particularly useful in the study of the biosynthesis of energy-conserving membranes.

Crude membranes from the photosynthetically grown and aerobically grown cells have at least three *b*-type cytochromes in common, and also cytochrome *c*₂ [with a redox midpoint potential at pH 7.0 (E_0') of 290 mV] is present in membranes from both (Connelly *et al.*, 1973). This group of cytochromes includes all those that have been implicated in photosynthetic electron flow in this organism (see Dutton & Jackson, 1972). It has proved possible to reconstitute photo-

synthetic reactions in aerobic membranes *in vitro* by the addition of pure reaction-centre protein (Jones & Plewis, 1974) and light-harvesting protein (Hunter & Jones, 1976) to purified membranes from aerobically grown cells, confirming the existence of many photosynthetic electron-transport components in the aerobic cells.

In the present paper we describe a particulate fraction, enriched in cytochrome *c*, that has been obtained from both aerobically and photosynthetically grown *R. spheroides*, demonstrating further similarities in the organization of electron-transport carriers in cells grown under these widely different conditions.

Materials and Methods

Organisms and preparation of particles

The green mutant of *R. spheroides*, GVP, was isolated as described by Connelly *et al.* (1973) and the mutant 01, which is incapable of bacteriochlorophyll synthesis (Jones & Plewis, 1974), was obtained by similar methods. The carotenoid-less mutant, R-26, of *R. spheroides* was a gift from Dr. R. K. Clayton, Department of Development and Physiology, Cornell University, Ithaca, NY 14853, U.S.A. *R. spheroides*

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Scheme 1. Preparation of particulate and soluble fractions from *R. spheroides*

GVP and R-26 were grown photosynthetically in the medium described by Sistrom (1960) illuminated anaerobically by incandescent lamps. *R. spheroides* 01 was grown in the same medium in a fermenter, with vigorous aeration. Cells were disrupted by suspension in 10mM-Tris/HCl buffer, pH 7.5, and passing twice through a French pressure cell. Bulk particles were prepared by differential centrifugation (Connelly *et al.*, 1973). After sedimentation of the 'bulk' membrane fraction (chromatophores in the case of photosynthetically grown cells) the supernatants were centrifuged for a further 14 h at 120000g and the pellets collected and called the 'light membrane fraction' (Scheme 1).

Determination of redox midpoint potentials

The anaerobic procedures were those of Dutton *et al.* (1970) with a stirred cuvette gassed with a stream of Ar and fitted with platinum and calomel electrodes. On occasion samples at known redox potential were withdrawn by using gas-flushed syringes, and frozen in modified cuvettes in liquid N₂ for low-temperature spectroscopy, as described previously (Dutton, 1971; Saunders & Jones, 1975).

Spectrophotometry

The split-beam spectrophotometer for this work had a reciprocal dispersion of 2.6 nm; for spectra at 77 K the slits were set at 0.25 nm.

Assays

Bacteriochlorophyll was determined after extraction into acetone/methanol (Clayton, 1963). Protein was determined by the method of Lowry *et al.* (1951) with bovine plasma albumin as standard. Cytochrome *c* reductases were measured in a dual-wavelength spectrophotometer at $A_{550} - A_{540}$. Mammalian cytochrome *c* (4 µM) was used as substrate and 2.5 mM-KCN was present to prevent oxidation of reduced cytochrome *c* by bacterial particles; the cuvette contained 2.5 ml of Tris/HCl (10 mM, pH 7.5) and bacterial particles (about 0.2 mg of protein), and succinate (4 mM) or NADH (100 µM) was added to start the reaction.

Polypeptides of these particulate fractions were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Samples were first heat-denatured by brief boiling (about 30 s) in 1% sodium dodecyl sulphate and then samples containing approx. 70 µg of protein were applied to the top of a 6% acrylamide stacking gel above a 12% acrylamide slab gel containing 0.1% sodium dodecyl sulphate and run with a current of 60 mA for 6 h. Gels were stained by the method of Berg (1969) and destained by the method of Tanner & Boxer (1972). Standard polypeptide mixtures were run at the same time, containing horse heart cytochrome *c* (mol.wt. 12384), lactate dehydrogenase (subunit mol.wt. approx. 36000), glutamate dehydrogenase (subunit

mol.wt. approx. 50000) and bovine plasma albumin (subunit mol.wt. approx. 64000).

Ubiquinone was extracted from particles and assayed as described by Pumphrey & Redfearn (1960).

Results

The chromatophore pellets obtained from *R. spheroides* GVP or R-26 were observed to be heterogeneous. The upper part of the pellet was a buff

colour and the bottom of the pellet was deeply pigmented. They could be collected separately by triturating the top of the pellet gently with a glass rod and a little buffer and pouring off the resulting suspension. An even less-pigmented fraction was collected by recentrifuging at 120000g for 14h the supernatant decanted from above the chromatophore pellet. A further pellet was obtained that was called the light membrane fraction (see Scheme 1). A series of membrane fractions was obtained by treating the pellets from disrupted aerobically grown *R. spheroides* 01 in a similar way. The spectra of some of these membrane fractions are shown in Figs. 1 and 2.

Fig. 1. Oxidized-minus-reduced difference spectra of fractions obtained by differential centrifugation of disrupted-cell suspensions of aerobically grown *R. spheroides* 01. The reductant added to cuvettes was sodium dithionite (a few grains) and the oxidant was 200 μ M-potassium ferricyanide. For spectra at room temperature (293 K) the light-path was 1 cm; for spectra at 77 K the light-path was 0.2 cm. Spectra were recorded at different scale expansions and these are given below, where *A* refers to the absorbance in the distance between the bars illustrated. Explanation of spectra: (a) final supernatant, after 14 h centrifugation at 120000g, at a concentration of 0.6 mg of protein/ml (*A*=0.005); (b) pellet, after 14 h centrifugation, at a concentration of 5 mg of protein/ml (*A*=0.005); (c) fraction collected from the top of the bulk membrane pellet (obtained by 140000g centrifugation for 90 min) at a concentration of 4.0 mg of protein/ml (*A*=0.025); (d) bottom of the bulk membrane pellet (obtained by 140000g centrifugation for 90 min) at a concentration of 3.0 mg of protein/ml (*A*=0.025); (e), (f), (g) and (h) were recorded at 77 K; for (e), *A*=0.01; for (f), *A*=0.01; for (g), *A*=0.01; for (h), *A*=0.025.

Fig. 2. Oxidized-minus-reduced difference spectra of fractions obtained by differential centrifugation of disrupted-cell suspensions of photosynthetically grown *R. spheroides* GVP.

Conditions were as described in the legend to Fig. 1. Explanation of spectra: (a) final supernatant, after 14 h centrifugation at 120000g, at a concentration of 0.5 mg of protein/ml (*A*=0.005); (b) pellet, after 14 h centrifugation at 120000g, at a concentration of 3.6 mg of protein/ml (*A*=0.005); (c) fraction collected from top of the bulk membrane pellet (obtained by centrifugation at 140000g for 90 min) at a concentration of 2.0 mg of protein/ml (*A*=0.005); (d) bottom of the bulk membrane pellet (obtained by centrifugation at 140000g for 90 min) at a concentration of 2.0 mg of protein/ml (*A*=0.005). Spectra (e), (f), (g) and (h) were measured at 77 K; for (e), *A*=0.01; for (f), *A*=0.025; for (g) and (h), *A*=0.01.

The main membrane pellet from aerobically or photosynthetically grown cells contained both *b*- and *c*-type cytochromes, identified from their absorption bands at about 560 and 550 nm respectively, in a ratio of approx. 1:1. The upper part of this pellet contained cytochromes *b* and *c* in a ratio of approx. 1:2 and the small membrane fraction contained little cytochrome *b* (see Figs. 1*b* and 2*b*), but appeared to be rich in cytochrome *c*. The spectrum of this light-membrane cytochrome *c*, like that of the final super-

Fig. 3. Low-temperature reduced-minus-oxidized difference spectrum of cytochrome *c*₂ of *R. spheroides* GVP

The solution of cytochrome *c*₂, a gift from Dr. G. Hauska, University of Regensburg, Germany, had a concentration of 0.06 mg of protein/ml. One cuvette was reduced with a few crumbs of sodium dithionite, the other cuvette was oxidized with 20 μM-potassium ferricyanide. Spectra were recorded at 77 K.

Fig. 4. Redox properties of the cytochromes *c* of the light particulate fraction

(a) Potentiometric titration at $A_{552} - A_{540}$ of the cytochromes *c* of the light particulate fraction from photosynthetically grown *R. spheroides* GVP. The particles were suspended in 2-[(2-hydroxy-1,1-bis-(hydroxymethyl)ethyl)amino]ethanesulphonic acid (50 mM)/KCl (50 mM), pH 7.0, at a concentration of approximately 4.0 mg of protein/ml. Potassium ferricyanide (100 μM), diaminodurol (40 μM), phenazine methosulphate (20 μM) and phenazine ethosulphate (20 μM) were present as mediators and the assembly was gassed with Ar. Potentials were made more positive by the addition of potassium ferricyanide and more negative by successive additions of succinate, NADH and dithionite. Oxidative or reductive titrations produced similar results. Symbols: ○, experimental points; ●, points predicted by computer, assuming a two-component system with E_0' and ratio of components as derived in Fig. 4(b). (b) Re-plot of the data from Fig. 4(a), showing the resolution of the curve in two $n=1$ components. Lines drawn through the points are theoretical $n=1$ lines derived from the Nernst equation. E_0' from these lines was +343 mV (37% of total) and +110 mV (63%).

natant cytochrome *c*, showed splitting of the α -band in spectra measured at 77 K. The spectra of the cytochrome *c* of the chromatophore or main membrane pellets (Figs. 1*e*, 1*h*, 2*e* and 2*h*) did not exhibit such clear splitting: a shoulder rather than a second peak can be seen. Such splitting of the α -band at 77 K is, however, found in spectra of pure cytochrome *c*₂

from *R. spheroides* (see Fig. 3). Yet cytochrome c_2 is present as about 90% of the total cytochrome c of crude particles from *R. spheroides* when grown aerobically or photosynthetically (Connelly *et al.*, 1973; Saunders & Jones, 1974), although Saunders & Jones (1974) reported that a c -type cytochrome with a midpoint redox potential of +120 mV was found as a minor component from aerobically grown cells. It appears that when cytochrome c_2 is bound to the chromatophore or to the respiratory membrane the split α -band of its absorption spectrum at 77 K is lost.

Potentiometric titration at $A_{551}-A_{540}$ of the light membranes indicated the presence of two c -type cytochromes in this fraction (Fig. 4). One component had a redox potential at pH 7.0 of $+340 \pm 5$ mV, the other component has E_0' of $+110 \pm 8$ mV. The same two components were found in light membranes from either aerobically grown or photosynthetically grown cells and were present in ratios approaching unity.

Potentiometric titrations carried out on the upper layer of the main membrane pellets showed that this too was enriched in cytochrome c with E_0' approx. +120 mV.

Fig. 5. Low-temperature difference spectra of the high- and low-potential cytochromes c of the light particulate fraction from *R. spheroides* 01

A redox titration assembly containing the mixture of mediators and particles described in Fig. 4 was poised at the indicated potentials by the addition of NADH and ferricyanide. Samples were withdrawn in a gas-flow syringe, injected through a septum into an Ar-flushed low-temperature cuvette. In the other cuvette samples were either fully oxidized with ferricyanide or reduced with dithionite. The low-temperature cuvettes were immediately plunged into liquid N_2 and the difference spectra recorded. (a) +245 mV minus ferricyanide; (b) dithionite reduced minus 245 mV.

Attempts were made to determine the oxidized-minus-reduced difference spectra of each of the two components at 77 K. The light membranes were titrated to a redox potential of approx. +230 mV, half way between the E_0' of the two components, and samples were withdrawn in Ar-flushed syringes and rapidly transferred to the adapted gas-flushed, low-temperature cuvette. A reference cuvette was filled with membrane either fully reduced with dithionite or oxidized with ferricyanide and the difference spectra at 77 K were recorded (Fig. 5). The two difference spectra were similar, with splitting of the α -bands in both samples, although the splitting was less pronounced in the low-potential cytochrome.

The absorption spectra of light membranes from photosynthetically grown cells showed a small absorption band in the region approx. 560 nm. CO-difference spectra on these samples showed an intense band at 416 nm with a trough at 552 nm. Such spectra resemble those of cytochrome c' (Bartsch, 1978), which is believed to be involved in photosynthetic electron transport. The same CO-binding material was present, to a lesser extent, in light membranes from aerobically grown cells (see Fig. 6).

These light membranes were found to have slight oxidase activity, but no cytochrome oxidase was observed in reduced-minus-oxidized difference spectra or in CO-difference spectra, but the membranes had both succinate- and NADH-cytochrome c reductase activities. The light membranes from photosynthetically grown cells had about 1–2% of the specific bacteriochlorophyll of the usual chromatophore fraction.

When suspensions of light membranes from aerobically grown or photosynthetically grown *R. spheroides* were placed on 5–60% (w/w) linear gradients of sucrose containing 2 mM-EDTA and

Fig. 6. CO-difference spectra of the light particulate fraction of *R. spheroides* GVP

Particles (8 mg of protein/ml) suspended in 10 mM-Tris/HCl, pH 7.5, were divided between two cuvettes. Dithionite was added to both and a baseline recorded (---). CO was bubbled into the sample cuvette and the difference spectrum recorded (—).

Fig. 7. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of membrane fractions from photosynthetically and aerobically grown *R. spheroides*

The fractions were purified by centrifugation on sucrose gradients containing 2mM-EDTA before electrophoresis. Samples containing approx. 70 μ g of protein were applied to the top of a 6% acrylamide stacking gel above a 12% acrylamide slab gel containing 0.1% sodium dodecyl sulphate and run with a current of 60mA for 6h. The gels were stained in 0.25% Coomassie Blue dissolved in destainer and destained in 7.5% acetic acid/5% methanol. The composition of samples was: (a) standard proteins, bovine plasma albumin (mol.wt. 64 000), glutamate dehydrogenase (subunit mol.wt. 50 000) and lactate dehydrogenase (subunit mol.wt. 36 000) (indicated by arrows); (b) purified small-membrane fraction from the aerobically grown mutant *R. spheroides* 01; (c) purified cytoplasmic membrane fraction from the aerobically grown mutant *R. spheroides* 01; (d) purified small-membrane fraction from photosynthetically grown *R. spheroides* R-26; (e) purified chromatophores from photosynthetically grown *R. spheroides* R-26; (f) standard protein mixture as in (a).

centrifuged in a swinging-bucket rotor for 15h at 138000g, two major bands were resolved and collected from either suspension. The band with a density of 1.23 $\text{kg} \cdot \text{m}^{-3}$ corresponded to cell-envelope material (Niederman & Gibson, 1978; Barrett & Jones, 1978) and the cytochrome *c*-containing band with a density of 1.07 $\text{kg} \cdot \text{m}^{-3}$ corresponded to purified light membrane. In addition, a faint pink clear band was located at a slightly lower density than the main cytochrome *c*-containing fraction. The density of the light membrane band did not correspond to

that of chromatophore membrane nor to the bulk respiratory pigment-containing membrane of aerobically grown cells, which were located at a density of approx. 1.14 $\text{kg} \cdot \text{m}^{-3}$. Analysis of the purified light membranes with sodium dodecyl sulphate/polyacrylamide gels showed a striking similarity between membranes from photosynthetic and aerobic *R. spheroides*, although the major band, with a mol.wt. of approx. 48 000 was absent from aerobic membranes. Both lacked the characteristic triplet of reaction-centre bands with mol.wts. of approx. 26 000, 22 000

Fig. 8. Two components shown by potentiometric titration at $A_{551}-A_{540}$ of purified light particulate fractions of *R. spheroides* R-26

The particulate fraction was purified by density-gradient centrifugation and the band with an average density of $1.073 \text{ kg} \cdot \text{m}^{-3}$ was collected. Conditions were as described in Fig. 4. The lines drawn through the points are theoretical $n=1$ lines derived from the Nernst equation. E_0' from these lines are: +350 mV (54% of the total change); +115 mV (66% of the total change).

and 19000 (Clayton & Haselkorn, 1972) and the band of light-harvesting protein with a mol.wt. of approx. 9000 (Fraker & Kaplan, 1972; Clayton & Clayton, 1972), and the observed bands differed from those of chromatophores, respiratory-membrane or cell-envelope fraction (see Fig. 7). The light membrane fractions contained a greater number of higher-molecular-weight peptides than either the envelope bands or the bulk electron-transport membrane bands.

Other properties of the purified light membranes are shown in Table 1. From the low ratio of A_{260}/A_{280} it may be deduced that the light membranes are not enriched in nucleic acids and so are relatively free of ribosomal contaminants (Gibson, 1965). Further, they have a very low total phosphate and quinone content, indicating that they have very little phospholipid and markedly differ in electron-transfer components from the 'bulk' membranes. In agreement with this is the observation that succinate-cytochrome *c* reductase activity is apparently absent from the purified membrane, as might be expected in a system deficient in both quinones and cytochrome *b*. NADH-cytochrome *c* reductase activity has previously been found in soluble fractions from *R. spheroides* (Jones & Whale, 1970), where its function is uncertain. The activity is insensitive to antimycin A and to rotenone.

Potentiometric titration at $A_{550}-A_{540}$ of the purified light particulate fraction from *R. spheroides* R-26 showed that it contained the same two *c*-type components that were present in the crude light fraction (see Figs. 4 and 5). The midpoint potentials of the cytochromes were +350 mV and +115 mV and they were present as 47 and 53% of the total change respectively (see Fig. 8).

Discussion

Both aerobically grown and photosynthetically grown cells of *R. spheroides* have been shown in the present work to contain an insoluble protein complex with a polypeptide and quinone composition completely different from that of the bulk electron-transport membranes of this organism. It has a lower phosphate content than is commonly found in membranes; it may have originally been loosely attached to either the cell envelope or cytoplasmic membranes. Its function is obscure and could be concerned with membrane assembly or electron transport. It is interesting that much of the cytochrome *c*₂ of *R. spheroides* is localized in the periplasmic space (Prince *et al.*, 1975) and must be transported to this region through the cytoplasmic membrane when the electron-transport system is being assembled.

The midpoint potential of one of the two cytochromes *c* observed in this complex is +350 mV, very near the value of +345 mV that was found for a form of cytochrome *c*₂ present in chromatophore membranes (Dutton *et al.*, 1975). This latter form of cytochrome *c*₂ was thought to be dissociated from the reaction-centre bacteriochlorophyll complex, since it was oxidized only slowly by photo-oxidized reaction centres, on a time scale of seconds rather than microseconds (Dutton *et al.*, 1975). The present results suggest too that this cytochrome *c*₂ is located in a different environment from that of the main chromatophore cytochrome *c*₂, which has a midpoint potential of approx. +295 mV.

The cytochrome *c*₂ with a midpoint potential of about +115 mV is rather more difficult to identify with known carriers. There is a cytochrome *c* partly purified from *R. spheroides* that is reported to have a midpoint potential of +120 mV (Orlando, 1962), but this cytochrome has an absorption maximum in the α -band region at 553 nm, unlike the absorption maximum at 551 nm that we have found in our complex. It is possible that absorption of the cytochrome on a specific protein may effect such a shift in absorption spectrum without seriously altering the midpoint potential. We have already discussed in the Results section the change that takes place in the α -band of cytochrome *c*₂ when it is measured at 77 K, if it is dissociated from the chromatophore-binding site, although in this case the midpoint potential is changed by 60 mV. A *c*-type cytochrome with a midpoint redox

Table 1. *Properties of purified membrane fractions obtained after density-gradient centrifugation of the crude membranes prepared from aerobically or photosynthetically grown cells of R. spheroides*

Crude membrane preparations were layered on a 5–60% (w/w) linear gradient of sucrose containing 2 mM-EDTA and centrifuged for 15 h in a swinging-bucket rotor at 138 000g. N.D. indicates that activity was too small for detection.

	<i>R. spheroides</i> R-26		<i>R. spheroides</i> 01	
	Purified chromatophores	Light membranes	Respiratory membranes	Small membranes
Density of bulked fraction (kg·m ⁻³)	1.133	1.073	1.145	1.07
Protein (mg/ml)	14.9	13.4	11.5	10.0
A ₂₆₀ /A ₂₈₀ ratio	1.02	0.70	1.13	0.70
Phosphate (nmol/mg of protein)	1086	21.1	806	N.D.
Bacteriochlorophyll (nmol/mg of protein)	46.5	N.D.	N.D.	N.D.
Quinone (nmol/mg of protein)	28.3	0.88	8.16	N.D.
NADH-cytochrome <i>c</i> reductase activity (nmol of cytochrome <i>c</i> /min per mg of protein)	95.0	25.2	38.2	16.7
Succinate-cytochrome <i>c</i> reductase activity (nmol of cytochrome <i>c</i> /min per mg of protein)	64.1	N.D.	23.3	N.D.

potential of +120 mV was found to be present in membranes from aerobically grown cells of *R. spheroides*, giving about 10% of the total cytochrome change that was detected (Saunders & Jones, 1974). No function has been ascribed to this cytochrome, although Orlando (1962) speculated that it was involved in aerobic electron transport, since it was present in much greater amounts in aerobically grown cells. Our observation that the cytochromes *c*-120 and *c*-345 are present in approximately equal amounts in light membranes from both aerobically and photosynthetically grown cells is not in apparent agreement with his suggestion.

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