Biochimica et Biophysica Acta, 357 (1974) 204–214

BBA 46773

RECONSTITUTION OF LIGHT-DEPENDENT ELECTRON TRANSPORT IN MEMBRANES FROM A BACTERIOCHLOROPHYLL-LESS MUTANT OF *RHODOPSEUDOMONAS SPHEROIDES*

O. T. G. JONES and K. MARY PLEWIS

Department of Biochemistry, University of Bristol, Bristol, BS8 1TD (U. K.)

(Received January 17th, 1974)

SUMMARY

A mutant, O_1, of *Rhodopseudomonas spheroides* has been prepared that is not capable of bacteriochlorophyll synthesis, but excretes pigments spectroscopically similar to green plant chlorophylls. The cytochrome content and respiratory activity of membranes from O_1 resemble those of aerobically grown wild type *R. spheroides*, but the mutant could not adapt to grow photosynthetically. Photosynthetic reaction centres were purified from the blue green mutant, of *R. spheroides*, added to membranes from O_1, and the detergent used in reaction centre preparation removed by carefully controlled reduction. A reaction centre membrane complex was formed in which the ratio of reaction centre to cytochrome *b* was near 1 : 2. Illumination caused oxidation of the membrane cytochrome *c* and reduction of cytochrome *b*. These changes were enhanced in the presence of antimycin A, suggesting that a cyclic electron flow system had been reconstituted. The implication of these results on the formation of the photosynthetic electron flow system is discussed.

INTRODUCTION

The purple non-sulphur bacterium *Rhodopseudomonas spheroides*, like many members of the Athiorhodaceae, grows anaerobically in the light or aerobically in the dark. When cells are grown aerobically the synthesis of bacteriochlorophyll and carotenoids is inhibited and the existing pigment is diluted out [1]. The changes in pigment synthesis are accompanied by profound changes in cellular structure; the aerobic cells do not contain the pigmented vesicles, termed chromatophores, that are found in photosynthetic cells [2–4]. Following many electron microscope studies it has become accepted that the chromatophores of *Rps. spheroides*, and of the closely related organism *Rhodospirillum rubrum*, arise as invaginations of the peripheral or cytoplasmic membrane (see ref. 5 for a recent review). Peters and Cellarius [6] have shown that both bacteriochlorophyll and the onset of photochemical activity were detectable in *Rps. spheroides* before distinct morphological invaginations of the cytoplasmic membrane could be observed. They suggest that an early event in adapta-

tion to anaerobic, light conditions is the incorporation of bacteriochlorophyll into the cytoplasmic membrane in such a way that this pigment is photochemically active. Pulse-labelling experiments where ^{14}C-labelled amino acids, followed by a chase of unlabelled compound, were added to *Rps. spheroides* growing exponentially anaerobically in the light also suggest that chromatophores are formed from a membrane precursor, believed to be the cytoplasmic membrane [7]. It has been pointed out [8] that in mutants of *Rps. spheroides* lacking bacteriochlorophyll no vesicles were formed,even in semi-anaerobic conditions, lending support to the idea that chromatophores synthesis is necessarily coupled to chlorophyll synthesis.

We have previously found by potentiometric titration [9] that aerobically grown *Rps. spheroides* contains the same three *b* cytochromes that are present in photosynthetically grown cells [10], and the same major cytochrome *c* component. Studies on the rate of oxidation of reduced cytochrome in anaerobic membranes prepared from aerobically or photosynthetically grown cells showed that at least two of the *b* cytochromes appeared to have the same half times of oxidation, following rapid mixing with O_2 in both types of cell, although the aerobic cell has developed what appears to be a mitochondrial type $a+a_3$ terminal oxidase [11] that is lacking in photosynthetic cells. It thus appeared possible that the respiratory electron transport of *Rps. spheroides* might have very much the same composition and orientation as the photosynthetic electron transport system and that it might carry out photosynthetic reactions if supplemented with a suitable form of bacteriochlorophyll.

In this communication we describe how purified reaction centres, prepared from photosynthetically grown *Rps. spheroides*, were added to membrane preparations from a mutant of *Rps. spheroides* that was incapable of bacteriochlorophyll synthesis and was grown aerobically in the dark. The reconstituted membranes appear to catalyse cyclic photosynthetic electron transport.

METHODS

Preparation of the bacteriochlorophyll-less mutant of Rps. spheroides

The mutant O_1, was prepared by treatment of cells of strain 2.4.1 with *N*-methyl-*N*-nitroso-*N*-nitroguanidine [12]. Photosynthetically incompetent cells were selected using a penicillin-screening technique [13]. Cells blocked in bacteriochlorophyll synthesis were further selected by their accumulation of pigments with spectra resembling that of chlorophyll *a*, together with the absence of detectable bacteriochlorophyll. The frequency of reversion suggested that a single point mutation was responsible for the failure to synthesise bacteriochlorophyll.

Growth of cells

The mutant was normally grown at 30 °C in liquid media [14] in a vigorously aerated fermenter. The rate of aerobic growth (about 3 h doubling time) was the same for mutant O_1 as for wild type cultures.

The carotenoid-less mutant of *Rps. spheroides*, R-26 [15] was grown anaerobically, in the light, in the same medium.

Particles were prepared from freshly harvested cells disrupted in a French pressure cell, as described previously [16].

Preparation of reaction centres

Purified reaction centres were prepared from membranes derived from cells of R-26, the blue green mutant of *Rps. spheroides*, by extraction with the detergent lauryldimethylamine oxide and $(NH_4)_2SO_4$ levitation [17]. The concentration of reaction centres was calculated following measurement of light-induced bleaching at 598 nm minus 575 nm, using $\Delta\Sigma$ mM = 21. (Prince, R. C., private communication). This reaction was carried out in a dual wavelength spectrophotometer with the photomultiplier protected by a filter of saturated $CuSO_4$ solution and the actinic light masked by a Kodak-Wratten far-red filter (Number 88A). No cytochromes of the *b*- or *c*-type were detected in this preparation by difference spectroscopy and it gave no reaction with antibody to *Rps. spheroides* cytochrome c_2.

Spectrophotometry

The split-beam spectrophotometer used in this work had a reciprocal dispersion of 25 Å/mm. For spectra at 77 °K the slit widths were set at 0.15 nm. The filter combination described above was used in any light-minus-dark difference spectroscopy on either the split beam or dual wavelength spectrophotometers.

Measurement of respiratory activity

Succinate and NADH oxidase activity were measured polarographically using a Clark-type oxygen electrode in a 5-ml reaction vessel maintained at 30 °C. Formation of [^{32}P]ATP by oxidative phosphorylation was estimated by a method adapted from Avron [18] and Nielsen and Lehninger [19] as described by Saunders and Jones [20]; the buffer used in these phosphorylation studies was that used by Baccarini-Melandri [21].

Protein assays

Proteins were determined by the method of Lowry et al. [22].

RESULTS

The mutant O_t is unable to grow anaerobically in the light; when grown with limited aeration in the dark it excretes into the medium ether-soluble pigments spectroscopically related to green plant chlorophylls with absorption maxima below 660 nm. No bacteriochlorophyll was detected in exponentially growing cells under conditions of high aeration or in acetone–methanol extracts of them, although during late stages of growth some bacteriochlorophyll was formed. Under similar aerobic growth conditions wild type cells contain considerable amounts of bacteriochlorophyll, even in the exponential phase. The mutant cells contain carotenoids, with absorption bands in vivo in the region 460–560 nm (see Fig. 1), that were chromatographically and spectroscopically similar to the carotenoids of aerobically grown wild type cells; the cytochrome absorption bands found in reduced-minus-oxidised difference spectra of mutant whole cells were also identical with those of the wild type (Fig. 2).

In a previous paper [9] we have shown that membranes from aerobically grown *Rps. spheroides* contain three cytochromes of the *b*-type that can be detected by potentiometric titration. We have recently found that multiple *b*-type cytochromes

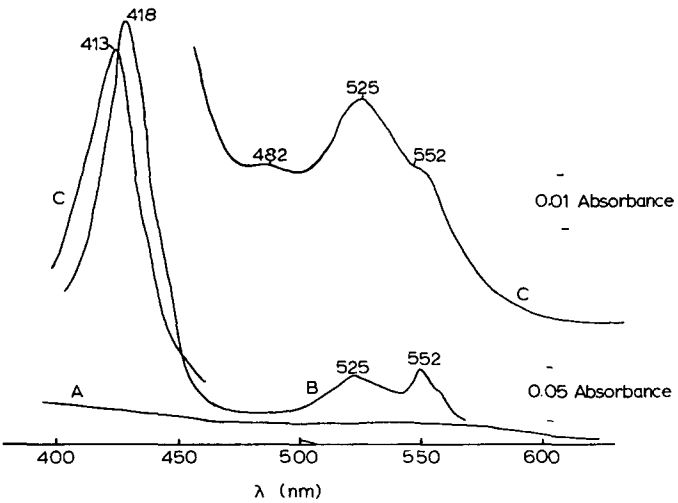


Fig. 1. Spectra of whole cells of mutant O_1. Cells were suspended (approximately 6 mg protein per ml) in 20 mM Tris buffer, pH 7.2, and spectra recorded both in the anaerobic state (Trace B) and in the aerobic state (Trace C). The base line (Trace A) was obtained using dilute milk in both cuvettes; the concentration of milk was adjusted to give light scattering at 630 nm equal to that of the suspension of O_1. For clarity, Trace C has been displaced upwards.

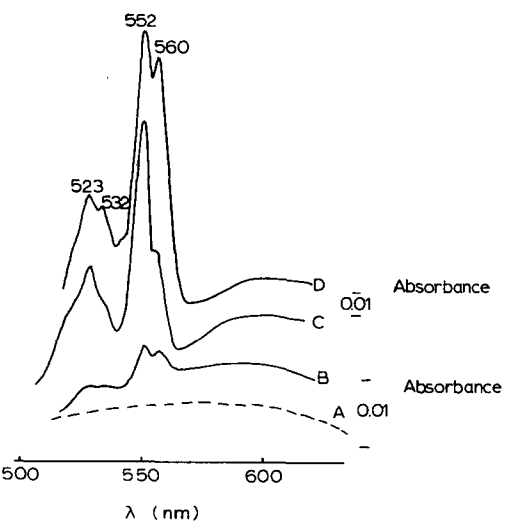


Fig. 2. Reduced minus oxidised difference spectra of O_1 particles. The particles, suspended in 20 mM Tris buffer, pH 7.2, at a concentration of 1.9 mg protein per ml were divided between the two cuvettes and a base line drawn (Trace A). Succinate (1 mM) was addded to the sample cuvette and the spectrum recorded in the aerobic steady state (Trace B) and after anaerobiosis was achieved by respiratory activity (Trace C). Dithionite was added to the sample and 0.5 mM $K_3Fe(CN)_6$ to the reference before recording Trace D.

can be demonstrated in membranes of *Rps. spheroides* by difference spectra of extent of reduction at varying time intervals following the addition of dithionite. This is demonstrated for mutant O_1 in Fig. 3. These difference spectra are similar in every respect to spectra obtained from wild-type cell membranes. We are not sure of the explanation for the time-dependent reduction of two of these *b*-cytochromes: it may be due to the inaccessibility of the haem prosthetic groups to external reductant

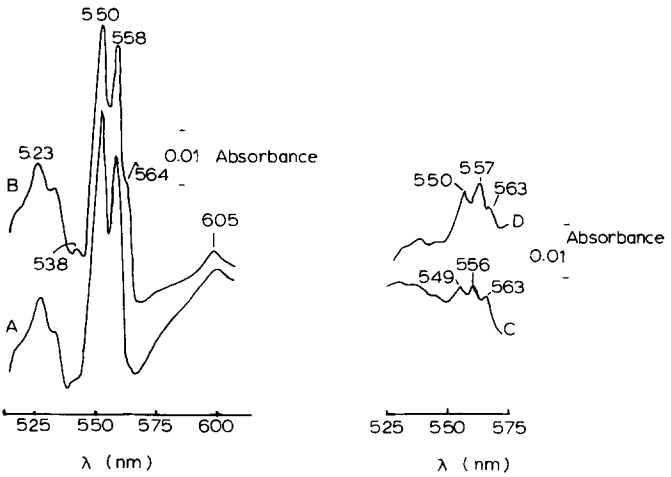


Fig. 3. Time-dependent difference spectra at 77 °K following dithionite reduction of O_1 particles. Particles (3 mg protein per ml) were suspended 20 mM Tris buffer, pH 7.2. Dithionite was added to the sample and spectrum recorded after 25 s reduction before freezing (Trace A) and after 7 min reduction (Trace B). To obtain Trace C the sample was reduced for 11 min before freezing and the reference for 3 min. Dithionite was added to the sample and 0.5 mM $K_3Fe(CN)_6$ to the reference before recording Trace D.

Membrane preparations from mutant O_1 can perform oxidative phosphorylation. The P : O ratios for NADH and succinate of 0.7 and 0.29 respectively are in good agreement with those published for other varieties of photosynthetic bacteria such as *R. rubrum* [23], *Rhodopseudomonas capsulata* (Melandri, B. A., personal communication) and *Rhodopseudomonas virdis* [20]. P : O ratios for preparations from aerobically grown wild-type cells of *Rps. spheroides* were similar to those from O_1 as were respiratory rates and effects of inhibitors.

Our results lead us to believe that the aerobic electron transport system of membranes from mutant O_1 is the same as that of membranes from wild-type cells. Since reversion of O_1 to wild-type occurs on prolonged culture under anaerobic light conditions and since the mutant excretes plant chlorophyll-like molecules into the medium we think it likely that the only mutation in O_1 is one affecting the later stages in bacteriochlorophyll synthesis. This makes it an appropriate organism for our attempts to reconstitute photosynthetic electron reactions by the addition of purified reaction centres. Membranes from aerobically grown wild-type cells are unsuitable for this since they always contain some bacteriochlorophyll, and this chlorophyll has reaction centre properties (Saunders, V. A. and Jones, O. T. G., unpublished).

Method of addition of purified reaction centre to membranes from mutant O_1 with subsequent removal of detergent

The reaction centre preparations in lauryldimethylamine oxide (2 %) was mixed, under the conditions described below, with a suspension of membranes prepared from mutant O_1, at pH 7.2 in 20 mM Tris buffer. The final reaction centre concentration was approximately 0.8 μM and the concentration of membrane protein was approximately 7 mg/ml. The suspension of O_1 membranes was placed in a closed redox titration vessel, sealed with a bung fitted with platinum and calomel electrodes, and gassed with O_2-free N_2. The following dyes were added to facilitate electron equilibration between the platinum electrode and the membrane-bound electron transport carriers: $K_3Fe(CN)_6$ (100 μM); diaminodurol (40 μM); phenazine methosulphate (20 μM); phenazine ethosulphate (20 μM); pyocyanine (50 μM); 2-hydroxy-1,4-naphthoquinone (20 μM) and the potential allowed to drift down. The reaction centre preparation was injected into the vessel, followed by the injection of an aliquot of dithionite solution sufficient to bring the electrode potential to about −150 mV, where it was maintained for about 40 min. This was sufficient to cause the reduction of the lauryldimethylamine oxide, with resulting loss of detergent activity. (We are grateful to Dr P. L. Dutton for advising us of this method for removing the detergent.) The suspension was then centrifuged at 38 000 × *g* for 45 min, yielding a pellet with a small, blue, lower layer of precipitated unbound reaction centre, and an upper layer of membrane fraction complexed with reaction centre. This upper layer was collected, resuspended in 20 mM Tris, pH 7.2, and recentrifuged at 38 000 × *g* for 45 min. This centrifuging removes dithionite and degradation products together with a little uncomplexed cytochrome *c*. The pellet was finally suspended in 20 mM Tris, pH 7.2. The spectra of the membranes obtained after mixing varying

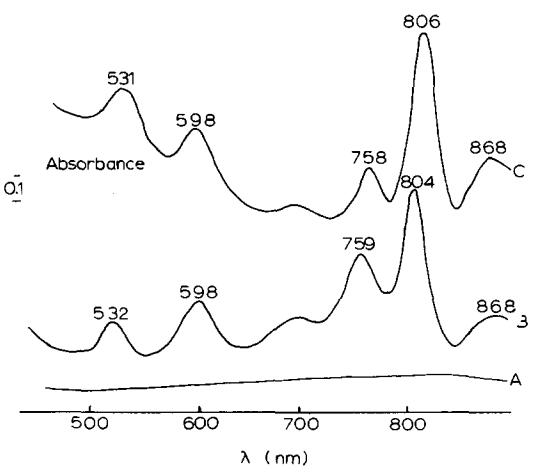


Fig. 4. Spectra of the purified reaction centre suspension and of complex between O_1 particles and the reaction centre suspension. Details for the formation of the complex are given in the text. Reconstituted particles were suspended in 20 mM Tris buffer, pH 7.2, to a concentration of 4 mg protein per ml reaction centre of 3.9 mM. Trace A, base line (buffer only); Trace B, reaction centre suspension (3.3 μM); Trace C, reconstituted reaction centre O_1 membrane complex. For trace C the reference cuvette contained diluted milk suspension as a light-scattering blank.

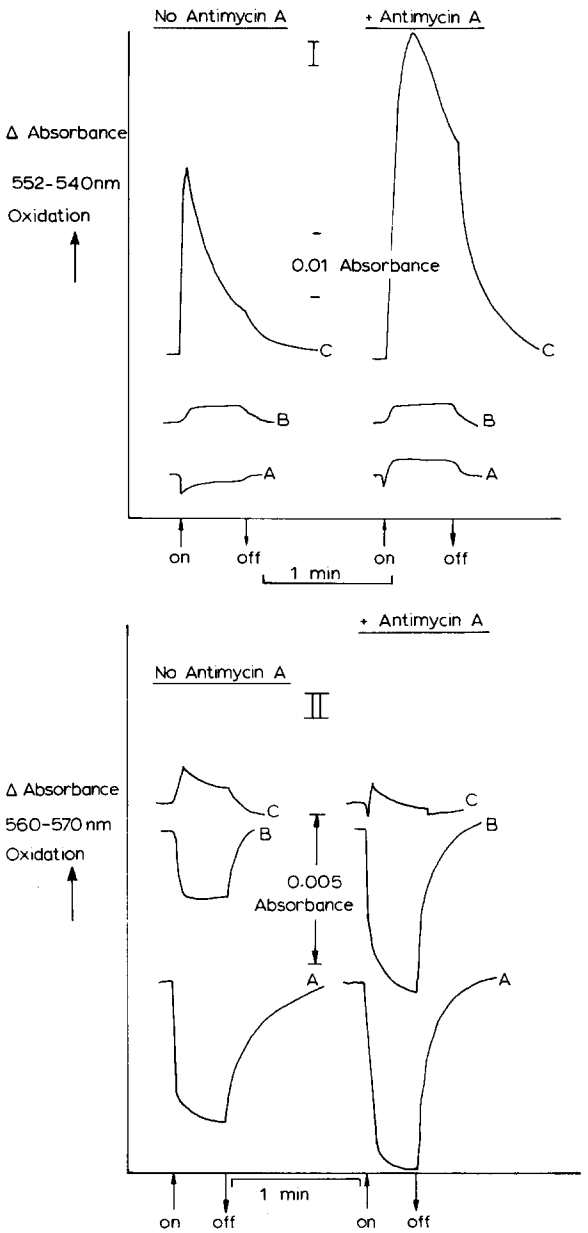


Fig. 5. Light induced oxidation–reduction reaction of cytochromes *b* and *c* of the O_1 particle–reaction centre complex. The reconstituted particles (5 mg protein per ml) were suspended in 20 mM Tris buffer, pH 7.2, in the cuvette of a dual-wavelength spectrophotometer. Cytochrome *c* concentration of the particle suspension was 0.7 nmole/mg protein; cytochrome *b* was 0.6 nmole/mg protein; reaction centre concentration was 3.6 μM. In Expt I measurements were made at 552 minus 540 nm, in Expt II measurements were made at 560 minus 570 nm. Antimycin A (5 μg/ml) was present where indicated. In each case Trace A records changes following illumination in the aerobic state; Trace B shows changes on illumination when 1 mM succinate was present, but conditions were still aerobic; Trace C shows illumination induced changes after the sample had become anaerobic by respiratory activity.

amounts of O_1 membrane with a fixed concentration of reaction centre suggested that a complex was formed with a ratio of 1 reaction centre to 2 molecules of cytochrome *b*. A spectrum of the complex is shown in Fig. 4. Slight changes in the absorption maxima of bands at 759 and 804 nm are apparent on binding reaction centre to the membrane, and the ratio of the absorptions of these two bands also appeared to change; the 758 nm band became relatively less intense.

Properties of the reconstituted membranes

Illumination of the suspension of reconstituted membranes, under aerobic conditions, caused a reduction of cytochrome *b*, measured at 560 nm, with little effect upon the cytochrome *c*, measured at 552 nm. The extent of reduction of cytochrome *b* was increased by the addition of antimycin A (Fig. 5). After succinate was added to the cuvette, the cytochrome *c* was slightly oxidised on illumination. The endogenous cytochrome became reduced after the consumption of dissolved oxygen by respiration; illumination then caused an oxidation of cytochrome *c* which was stimulated by the addition of antimycin A, causing 67 % of the total cytochrome *c* to become oxidised (Fig. 5). Under these anaerobic conditions some oxidation of cytochrome *b* took place on illumination. Control experiments (Fig. 6) showed only small, very fast changes at 552 and 560 nm when reaction centres alone were illuminated although large typical changes were obtained at 598 nm. There were no changes when O_1 membranes alone were illuminated. No reaction centre changes (at 598 nm) were detected in the O_1 membranes before reconstitution.

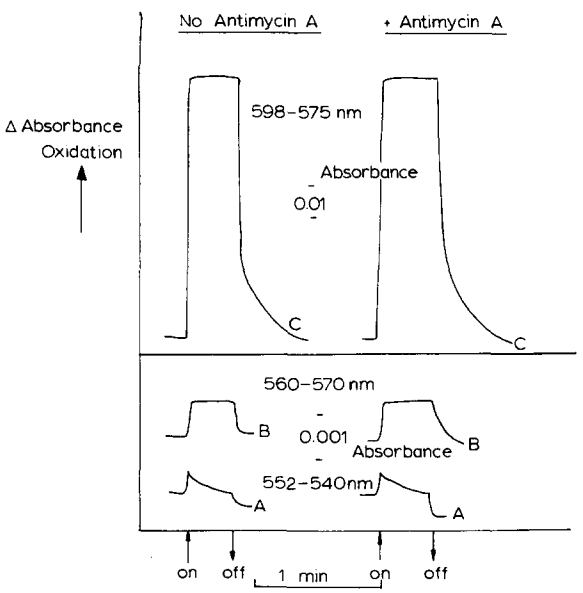


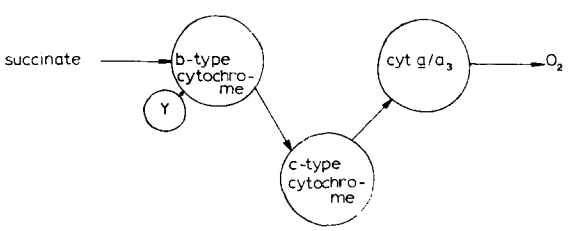
Fig. 6. Light-induced changes in purified reaction centres measured at various wavelengths. A 3.3-μM suspension of reaction centres in 20 mM Tris buffer, pH 7.2, was illuminated in the dual wavelength spectrophotometer. Trace A was recorded at 552 minus 540 nm, Trace B at 560 minus 570 nm and Trace C at 598 minus 575 nm (as a measure of "true" reaction centre change). Note the change in sensitivity for Trace C. Experiments were repeated in the presence of antimycin A (5 μg/ml).

The addition of antimycin A increased the extent of the 598 nm bleaching by approximately 15 % in steady state illumination in aerobic or anaerobic conditions. This suggests that some of the reaction centre must be in close proximity to the membrane cytochrome *c*. Under similar conditions the addition of antimycin A to intact chromatophores of *Rps. spheroides* increased the steady state oxidation of reaction centre by 2- or 3-fold.

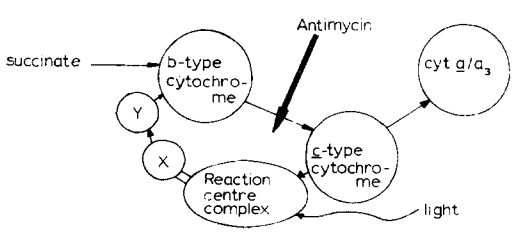
DISCUSSION

In the introduction we have summarised evidence that suggests that the chromatophore membranes of *Rps. spheroides* are not synthesized de novo but originate as outgrowths from the cytoplasmic membrane. Since the *b*- and *c*-type cytochromes of the membranes of aerobic cells appear to be same as the cytochromes of photosynthetic cells [9], it appeared possible that an early event in the assembly of a chromatophore was the incorporation of the special reaction centre bacteriochlorophyll into the cytoplasmic membrane that already contains the respiratory chain assembly. The results of our present work strongly suggest that the respiratory cytochromes have the same spatial organisation as those used in photosynthesis.

The reaction centre preparation that we have used in our works contains the primary acceptor, X [24, 25], so we are uncertain whether X, possibly an iron–sulphur compound, is present even in the aerobic membrane. A simple electron transport system for the respiratory chain and the reconstituted photosynthetic electron transport system is shown in Scheme 1, where Y is the secondary acceptor, a H^+-binding carrier, possibly a quinone [26]. Support for the reconstitution of a cyclic system as shown in Scheme 1 is given by the illumination experiments where antimycin A is shown to cause increased reduction of *b* and oxidation of cytochrome *c* (Fig. 5). Such complex effects would be unlikely if reaction centre was merely reacting with



Scheme 1. Simplified representation of aerobic electron transport system of *Rps. spheroides* present in O_1 membranes. Y is the secondary acceptor in photosynthetic reaction, possibly a quinone.



Postulated reconstituted cyclic photosynthetic electron transport system of *Rps. spheroides* formed by addition centres to O_1 membranes. X is the primary acceptor in photosynthetic reactions.

cytochrome *c* loosely attached to the outside of the membrane. This reconstituted photosynthetic system appears very similar in electron transport properties to that of the normal chromatophore [27, 28].

Membranes from bacteriochlorophyll-less single mutants of *Rps. spheroides* lack not only bacteriochlorophyll but also the three proteins that appear to be associated with the reaction centre form of bacterial chlorophyll [29]. Thus it appears possible that in normal wild type cells the reaction centre complex is synthesised and inserted at some pre-existing site in the cytoplasmic membrane at the onset of adaptation to anaerobic conditions. Our results show that such a mechanism is possible. The ATPase required for photophosphorylation is likely to be the same as that used in oxidative phosphorylation [30] and already present in the aerobic membrane. We have evidence that our reconstituted system is capable of light-dependent energy conservation (Plewis, K. M. and Jones, O. T. G., unpublished).

ACKNOWLEDGEMENTS

We are grateful to the Science Research Council for a Research Studentship and for financial support. The cytochrome *c* antibody was a kind gift from Dr G. Hauska and Dr R. C. Prince.

REFERENCES

1 Cohen-Bazire, G., Sistrom, W. R. and Stanier, R. Y. (1957) J. Cell Comp. Physiol. 49, 25–68
2 Cohen-Bazire, G. in Gest, H., San Pietro, A. and Vernon, L. P. (1963) Bacterial Photosynthesis 89–114, Antioch Press, Yellow Springs, Ohio
3 Vatter, A. E. and Wolfe, R. S. (1958) J. Bacteriol. 74, 480–483
4 Gibson, K. D. (1965) J. Bacteriol. 90, 1059–1072
5 Oelze, J. and Drews, G. (1972) Biochim. Biophys. Acta 265, 209–239
6 Peters, G. A. and Cellarius, R. A. (1972) J. Bioenerg. 3, 345–359
7 Gibson, K. D., Segen, B. J. and Niederman, R. A. (1972) Arch. Biochem. Biophys. 152, 561–568
8 Brown, A. E., Eiserling, F. A. and Lascelles, J. (1972) Plant Physiol. 50, 743–746
9 Connelly, J. L., Jones, O. T. G., Saunders, V. A. and Yates, D. W. (1973) Biochim. Biophys. Acta 292, 644–652
10 Dutton, P. L. and Jackson, J. B. (1972) Proc. 2nd Int. Congr. Photosynth., Stresa, 1971, pp. 995–1008, Junk N.V., The Hague
11 Saunders, V. A. and Jones, O. T. G. (1974) Biochim. Biophys. Acta 333, 439–445
12 Adelberg, E. A., Mandel, M. and Chein Ching Chen, G. (1965) Biochem. Biophys. Res. Commun. 18, 788–795
13 Lederberg, J. (1950) in Methods in Medical Research (Gerard, R. W., ed.), Vol. 3, pp. 5–22, Year Book Publishers, Chicago
14 Sistrom, W. R. (1960) J. Gen. Microbiol. 22, 778–785
15 Griffiths, M. and Stanier, R. Y. (1956) J. Gen. Microbiol. 14, 698–715
16 Whale, F. R. and Jones, O. T. G. (1970) Biochim. Biophys. Acta 223, 146–157
17 Clayton, R. K. and Wang, R. T. (1971) in Methods in Enzymology (San Pietro, A., ed.), Vol. XXIII, pp. 696–704, Academic Press
18 Avron, M. (1960) Biochim. Biophys. Acta, 40, 257–272
19 Nielson, S. O. and Lehninger, A. L. (1955) J. Biol. Chem. 215, 555–570
20 Saunders, V. A. and Jones, O. T. G. (1973) Biochim. Biophys. Acta 305, 581–589
21 Baccarini-Melandri, A. and Melandri, B. A. (1971) in Methods in Enzymology (San Pietro, A., ed.), Vol. XXIII, pp. 556–561, Academic Press
22 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193. 265–275

23 Geller, D. M. (1962) J. Biol. Chem. 237, 2947–2954
24 Prince, R. C., Cogdell, R. J. and Crofts, A. R. (1974) Biochem. Soc. Trans. 2, 162–164
25 Dutton, P. L., Leigh, J. S. and Wraight, C. A. (1973) FEBS Lett. 36, 169–173
26 Cogdell, R. J., Jackson, J. B. and Crofts, A. R. (1972) J. Bioenerg. 4, 413–429
27 Nishimura, M. and Chance, B. (1963) Biochim. Biophys. Acta 66, 1–16
28 Jones, O. T. G. (1969) Biochem. J. 114, 793–799
29 Takemoto, J. and Lascelles, J. (1973) Proc. Natl. Acad. Sci. U.S. 70, 799–803
30 Baccarini–Melandri, A. and Melandri, B. A. (1971) 2nd Int. Congr. Photosynth., Stresa, pp. 1185–1193, Junk, N. V., The Hague