Biochimica et Biophysica Acta, 387 (1975) 212–227

BBA 46912

ASYMMETRY OF AN ENERGY TRANSDUCING MEMBRANE THE LOCATION OF CYTOCHROME c_2 IN *RHODOPSEUDOMONAS SPHEROIDES* AND *RHODOPSEUDOMONAS CAPSULATA*

R. C. PRINCE*, A. BACCARINI-MELANDRI[a], G. A. HAUSKA[b], B. A. MELANDRI[a] and A. R. CROFTS

Department of Biochemistry, University of Bristol, Bristol BS8 1TD (U.K.), [a]Instituto Botanico, Universita di Bologna, Bologna (Italy) and [b]Lehrstuhl für Biochemie der Pflanzen, Ruhr-Universität-Bochum, (G.F.R.)

(Received November 11th, 1974)

SUMMARY

Monospecific antibodies have been prepared against cytochrome c_2 from *Rhodopseudomonas spheroides* and *Rhodopseudomonas capsulata*, and against cytochrome c' from *Rps. capsulata*. These antibodies precipitated their respective antigens, but did not cross react with a wide range of procaryotic or eucaryotic cytochromes, or with other bacterial proteins. The cytochromes produced during aerobic growth were immunologically indistinguishable from those produced during photosynthetic growth.

Cytochrome c_2 is located in vivo in the periplasmic space between the cell wall and the cell membrane, and when chromatophores are prepared from whole cells the cytochrome becomes trapped inside these vesicles. The implications of these results to energy coupling in the photosynthetic bacteria are discussed.

INTRODUCTION

During the primary light reaction of photosynthetic electron flow in *Rhodopseudomonas spheroides* and *Rhodopseudomonas capsulata*, the excited reaction centre bacteriochlorophyll is oxidised, and the primary electron acceptor is reduced. The reaction centre is subsequently re-reduced by a *c*-type cytochrome [1, 2]. In the work reported in this paper, we have used monospecific antibodies prepared against cytochrome c_2 from both *Rps. spheroides* and *Rps. capsulata* to determine the location of these proteins in the bacterial cell.

Abbreviations: Anti-c_2, antibodies precipitating cytochromes c_2; anti-c', antibodies precipitating cytochrome c'; TES, *N*-tris(hydroxymethyl)methyl 2-aminoethane sulphonic acid; Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid.

* Present address, to which all correspondence should be sent: Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa. 19174, U.S.A.

The very specific interactions between antibodies and antigens have been widely used in the elucidation of the functional roles of the proteins used as antigens, and their intracellular location. This latter approach has been of great interest in the study of energy conserving organelles. Racker et al. [3] have used antibodies prepared against mammalian cytochrome *c* to show that in mitochondria this protein is outside the inner membrane, while in sub-mitochondrial particles the antigen is inaccessible to the antibody. Similarly, antibodies have been used to demonstrate that spinach plastocyanin is situated inside the chloroplast thylakoids [4], as is *Euglena gracilis* cytochrome c_{552} [5].

The results presented here show that in both *Rps. spheroides* and *Rps. capsulata* cytochrome c_2 is located between the cell wall and the cell membrane in the periplasmic space. When chromatophores are prepared from whole cells, this cytochrome becomes trapped inside the vesicles.

METHODS

Bacteria

The bacterial strains used were routinely grown anaerobically in the light, and chromatophores prepared by breaking the cells in a French pressure cell as previously described [6]. Spheroplasts were prepared from freshly harvested cells which were in the middle of the exponential growth phase. Lysozyme (Sigma) and ethylenediamine tetra-acetic acid (EDTA, British Drug Houses) were used to digest the cell wall, using the method of Karunairatnam et al. [7]. Digestion was carried out in a medium containing 10 % (w/v) sucrose, but the spheroplasts were kept in a 20 % sucrose solution after preparation, to minimise rupture due to osmotic stress. The yield of spheroplasts was estimated by osmotic shock under the microscope. Lysed spheroplasts could be readily distinguished from non-lysed whole cells.

Oxygen-limited, aerobically grown cells were grown in the dark, and bubbled with air. Cells were also grown in a vigorously stirred fermenter under conditions where the oxygen tension was always saturated. Cells grown in this way produced very little bacteriochlorophyll.

Cytochromes

The bacterial cytochromes were purified from the supernatants obtained during the preparation of chromatophores, using the data of Bartsch [8]. This involved chromatography on DEAE-cellulose (Whatman DE 52) and gel filtration on various grades of Sephadex (Pharmacia). In the case of the cytochromes c_2, a trace of sodium ascorbate was added between purification steps to keep them in the fully reduced from, and they were eventually stored at −20 °C in this state. Cytochromes c' were also stored at −20 °C in the oxidised state.

Other bacterial proteins

Reaction centres were prepared from the blue-green mutants of the bacteria using the zwitterionic detergent dodecyldimethylamine oxide as previously described [9, 10], and the bulk bacteriochlorophyll complexes obtained in a similar way [11]. The coupling factor from *Rps. capsulata* was prepared as described previously [12].

Antibodies

Antibodies were prepared against cytochrome c_2 from *Rps. spheroides* Ga and *Rps. capsulata* St. Louis, and against cytochrome c' from the latter organism. These proteins were all obtained from bacteria grown anaerobically in the light. The antisera were prepared by immunising two eight-week-old rabbits per antigen with multiple injections subcutaneously into the foot pads and intradermally into the back, with 1 mg of antigen in 1 ml of saline emulsified with 1.5 ml of complete Freunds adjuvant (Difco). After 28 days each rabbit was boostered with 1 mg of antigen in saline into the ear vein. Blood was taken from the ear vein one week after the booster, and every following fortnight for a period of two months. Control sera from each rabbit were taken before the immunisation schedule was begun. All the rabbits innoculated with these antigens produced antibodies.

Standard procedures for ring tests and double diffusion were applied [13], and immunoelectrophoresis on agarose [14] was performed on Camag-Gelman equipment. The antisera were shown to be pure by immunoelectrophoresis with crude extracts of the respective bacteria. The antibody prepared against *Rps. spheroides* Ga cytochrome c_2 (anti-c_2) reacted with the homologous protein from strains R_{26} and 2. 4. 1 when these were grown either photosynthetically or aerobically. It did not cross react with cytochrome c', or with reaction centres or the bulk bacteriochlorophyll-protein complex isolated from this species. Similarly the antibody prepared against *Rps. capsulata* cytochrome c_2 (anti-c_2) reacted with the cytochrome isolated from *Ala pho*$^+$ and Drews' strain of the wild type when these were grown either photosynthetically or aerobically. It did not cross react with cytochrome c', reaction centres, bulk bacteriochlorophyll-protein complex or the ATPase isolated from this species. Likewise anti-c' reacted only with this protein from the various strains of *Rps. capsulata*, and not with any of the other proteins listed above. It should be noted that the two anti-c_2 antisera did not cross react, so throughout this paper it must be borne in mind that experiments with *Rps. spheroides* involved anti-c_2 prepared against the cytochrome from this organism, while those involving *Rps. capsulata* used an antiserum prepared against *Rps. capsulata* cytochrome c_2.

None of the antisera cross-reacted with any of the following proteins: cytochrome c_2 from *Rhs. rubrum*, *Rps. viridis*, *Rps. gelatinosa* or *Rps. palustris*; cytochrome c' from *Rps. spheroides*, *Rhs. rubrum* or *Chromatium D*; cytochromes c_3 and c_{554} from *Rps. spheroides*; cytochromes c_{552} from *Anabaena cylindrica*, *Anacystis nidulans*, *Scenedesmus obliquus* or *Euglena gracilis*; equine or bovine cytochrome *c*. However, these tests only assay for precipitating antibodies. Non-precipitating, complement-requiring antibodies may also have been present which might have cross-reacted with some of these cytochromes, but these would not be expected to be functional in the ammonium sulphate fractionated serum, since complement is destroyed by such treatment.

Quantitative precipitation was carried out according to the method of Heidelberger [5, 13]. Precipitation titres of 2–3 nmol antigen/ml were found for each serum. In the range of equivalence, a ratio of about two antibodies per cytochrome was found, indicating that each antigen possessed four determinants. These are similar to the results obtained with other small redox proteins [4, 5].

Immunoglobulins were isolated by precipitation with ammonium sulphate (45 % saturation) after dilution of the sera 1:3 with physiological saline. The preci-

pitate was washed twice with 40 % ammonium sulphate, and finally dissolved in saline to a concentrated solution. After dialysis against saline, with many changes of the external solution, the solution of immunoglobulins was adjusted to about 30 mg protein per ml. This preparation was used instead of whole sera, since haemoglobin in the sera would interfere with spectrophotometric analyses.

Agglutination tests were carried out essentially as described by Berzborn [15]. Chromatophores or spheroplasts were suspended to about 1 mg bacteriochlorophyll/ml in saline, and 20 μl of this was mixed with an equal volume of antiserum in a dilution series from 1:1 to 1:32, on a micro-slide. For indirect agglutination, 20 μl of an antigen dilution series (0.5 to 50 μM cytochrome) was added to the above mixture after 5 min.

Spectroscopy

The photo-oxidation of cytochrome c_2 and the reduction of cytochrome *b* were monitored in the double beam spectrophotometer previously described [16]. The photo-oxidation of *c*-type cytochromes by isolated photochemical reaction centres was monitored on a rapidly responding double beam, double photo-multiplier spectrophotometer as described earlier [17]. Transmembrane pH gradients were measured using the fluorescent amine 9-amino acridine in the fluorimeter described previously [18] equipped with a facility for illuminating the chromatophores with saturating actinic light.

Assays

Bacteriochlorophyll was estimated after extraction into acetone: methanol (7.2, v:v) using an extinction co-efficient of 75 $mM^{-1} \cdot cm^{-1}$ at 775 nm [19]. Cytochrome c_2 was estimated from ascorbate reduced minus ferricyanide oxidised difference spectra, assuming an extinction coefficient of 20 $mM^{-1} \cdot cm^{-1}$ at the wavelength pair 551-540 [20]. The spectra for these assays were recorded on the computer-linked spectrophotometer described by Evans and Crofts [2]. Cytochrome *c'* was estimated from the maximum Soret absorption peak for the reduced form at 423 nm; ε (mg/ml) = 4.0 [21]. Photophosphorylation was assayed by the method of Baccarini-Melandri and Melandri [22].

RESULTS

The cytochrome c_2 content of Rps. spheroides and Rps. capsulata.

When chromatophores are prepared from whole cells, considerable amounts of soluble proteins are liberated into solution, and remain in the supernatant when the chromatophores are sedimented at 144000 $\times g$. Among these soluble proteins are cytochromes c_2 and *c'* [8]. However, as Table I indicates, although a large amount of cytochrome c_2 is liberated into solution, the mole ratio of bacteriochlorophyll :cytochrome c_2 in the chromatophores is very similar to that of whole cells. Thus chromatophores are not depleted in cytochrome c_2 with respect to whole cells, but rather the soluble cytochrome has escaped from the partially broken cellular debris and unliberated or broken chromatophores removed by sedimentation at 20 000 $\times g$. Table II shows that when spheroplasts are prepared from *Rps. spheroides* and *Rps. capsulata*, cytochrome c_2 is liberated into free solution. In some preparations from

TABLE I

THE MOL RATIOS OF CYTOCHROME c_2: BACTERIOCHLOROPHYLL IN *Rps. spheroides* Ga AND *Rps. capsulata Ala pho*$^+$

The data are expressed as mol of cytochrome c_2 per 100 mol of bacteriochlorophyll. In each case the chromatophores were prepared from the whole cells opposite them in the table.

Rps. spheroides Ga*		*Rps. capsulata Ala pho*$^+$ **	
Whole cells	Chromatophores	Whole cells	Chromatophores
1.03	1.15	1.53	1.49
1.32	1.20	1.32	1.52
0.87	0.93	0.97	1.01
0.65	0.72	1.26	0.98
1.03	0.98	1.01	1.07
1.21	0.97	0.88	0.93
0.92	1.03	0.75	0.91
0.80	0.78	1.31	0.99
0.76	0.92	1.46	1.33
1.31	1.08	1.08	1.21

* mean 0.98
** mean 1.15

Rps. spheroides as much as 85 % of the *c*-type cytochrome (from ascorbate reduced minus ferricyanide oxidised difference spectra) was lost, with the concomittant loss of more than 90 % of cytochrome photo-oxidation. Both *Rps. spheroides* and *Rps. capsulata* possess other *c*-type cytochromes in addition to c_2. In *Rps. spheroides* there is a *c*-type cytochrome with $E_{m7} = +120$ mV [23] which may account for up to 18 % of the total. Both this and cytochrome c_2 ($E_{m7} = 346$ mV [8]) would be reduced by ascorbate, and so both would contribute to the ascorbate reducible *c*-type cytochrome measured in Tables I and II. Similarly, in *Rps. capsulata* there are three *c*-type cytochromes, with midpoint potentials at pH 7 of +340 mV (cytochrome c_2), +120 mV and 0 mV [2], and the latter two may comprise up to 50 % of the total, although this is somewhat variable. [2]. In addition, both *Rps. spheroides* [8] and *Rps. capsulata*

TABLE II

THE AMOUNT OF CYTOCHROME c_2, AND PHOTO-OXIDISABLE *c*-TYPE CYTOCHROME REMAINING IN SPHEROPLASTS OF *Rps spheroides* Ga AND *Rps. capsulata Ala pho*$^+$

The data are expressed as moles of cytochrome c_2 per 100 mol of bacteriochlorophyll. The data presented are the average of six preparations which showed greated than 90 % lysis when distilled water was added.

	Cytochrome c_2	*c*-type cytochrome photo-oxidation
Rps. spheroides Ga		
Whole cells	0.93	0.84
Spheroplasts	0.12	0.05
Rps. capsulata Ala pho$^+$		
Whole cells	1.23	0.88
Spheroplasts	0.43	0.13

(Prince, R. C. and Hauska, G. A., unpublished observation) liberate cytochromes c_3 and c_{554} into solution when broken in a French pressure cell, and although these have not yet been detected in chromatophores, these too might be reduced by ascorbate, and hence be included in the ascorbate reducible *c*-type cytochrome measured in Tables I and II. However, there is no evidence that any cytochrome other than c_2 can be directly photooxidised by the reaction centre bacteriochlorophyll in either organism, so the amount of *c*-type cytochrome photo-oxidised (Table II) is perhaps a better estimate of the amount of cytochrome c_2 remaining in spheroplasts. As can be seen from Table II, a large proportion of the cytochrome c_2 was lost during the removal of the cell wall, although there was negligible cell rupture as observed under the microscope.

The effects of the antisera on sub-cellular fractions

i) Photochemical reaction centres

The reaction centres used in this work contained no endogenous cytochromes [9, 10]. Nevertheless these reaction centres will rapidly photooxidise added *c*-type cytochromes [17]. Fig. 1 shows that the photo-oxidation of added cytochromes c_2 can be totally inhibited by the addition of the appropriate antiserum. The control sera had little effect, and neither the control nor immune sera had any effect on the photo-oxidation of mammalian cytochrome *c*.

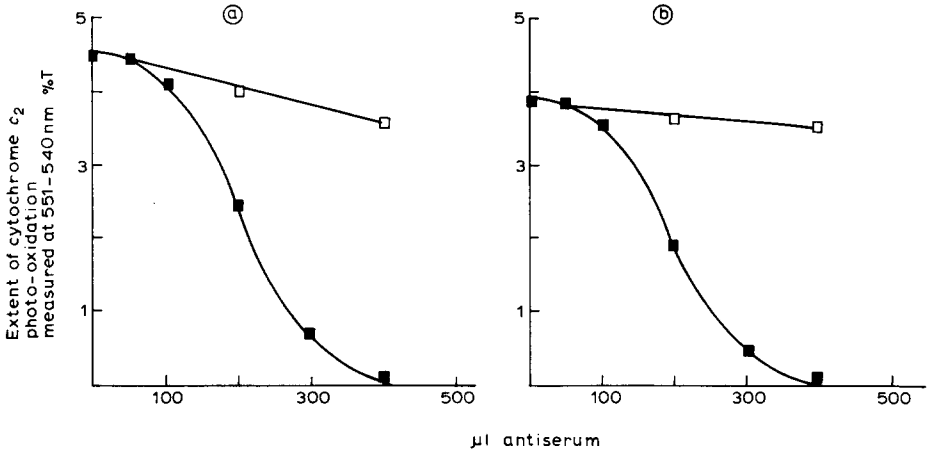

Fig. 1. The effect of anti-c_2 on the photo-oxidation of cytochrome c_2 by isolated photochemical reaction centres. (a) The reaction mixture contained 2.5 ml 10 mM Tris · Cl, 100 mM KCl, pH 7.5, 2 % dodecyldimethylamine oxide, 200 μM 1,4-naphthaquinone [17] 0.7 μM reaction centres from *Rps. spheroides* R_{26} and 1 μM *Rps. spheroides* Ga cytochrome c_2. (b) As (a) except the reaction centres and cytochrome were replaced by 0.7 μM reaction centres from *Rps. capsulata Ala pho*$^+$ and 1 μM St. Louis cytochrome c_2. The appropriate antiserum was added as indicated. The solid symbols represent the immune serum; the open, the control serum.

Reaction centres would not photo-oxidise added reduced cytochromes *c'*, even in the presence of catalytic amounts of cytochrome c_2.

ii) Chromatophores

Chromatophores possess a cyclic photosynthetic electron transport chain,

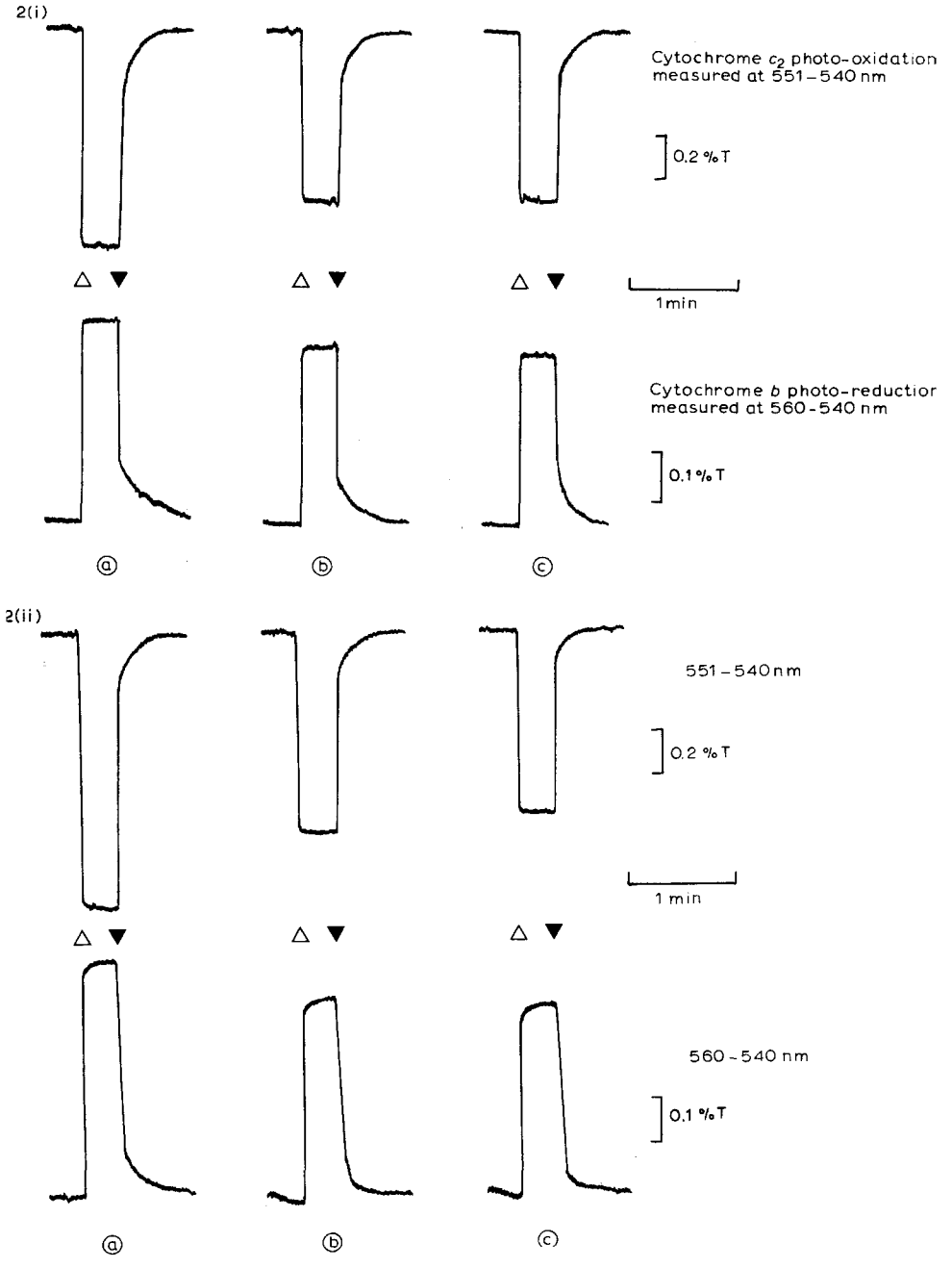

Fig. 2. The effects of the antisera on chromatophores. The reaction mixture contained 3 ml 10 mM Tris · Cl, 100 mM KCl, pH 7.5, 2 μM antimycin A and 2 μM valinomycin. (i) *Rps. spheroides* Ga. The cuvette also contained 0.1 mg bacteriochlorophyll/ml of chromatophores: (a) No further additions; (b) plus 500 μl control serum, and (c) plus 500 μl immune serum. (ii) *Rps. capsulata Ala pho*$^+$. The cuvette also contained 0.13 mg bacteriochlorophyll/ml of chromatophores: (a) no further additions, (b) plus 500 μl control serum, and (c) plus 500 μl immune serum.

but in the presence of Antimycin A this becomes an essentially linear pathway; illumination causes an oxidation of *c*-type cytochrome, and a reduction of *b*-type cytochrome (Fig. 2). We have used the Antimycin inhibited system to test the effects of the antisera on photosynthetic electron flow.

When anti-c_2 was added to chromatophores it had little effect (Fig. 2) and sonication in the presence of antiserum had no effect either. However, in the presence of 1 % sodium cholate, anti-c_2 had a marked inhibitory effect on cytochrome c_2 photo-oxidation (Figs 3 and 4). Sodium cholate is a mild detergent, and at concen-

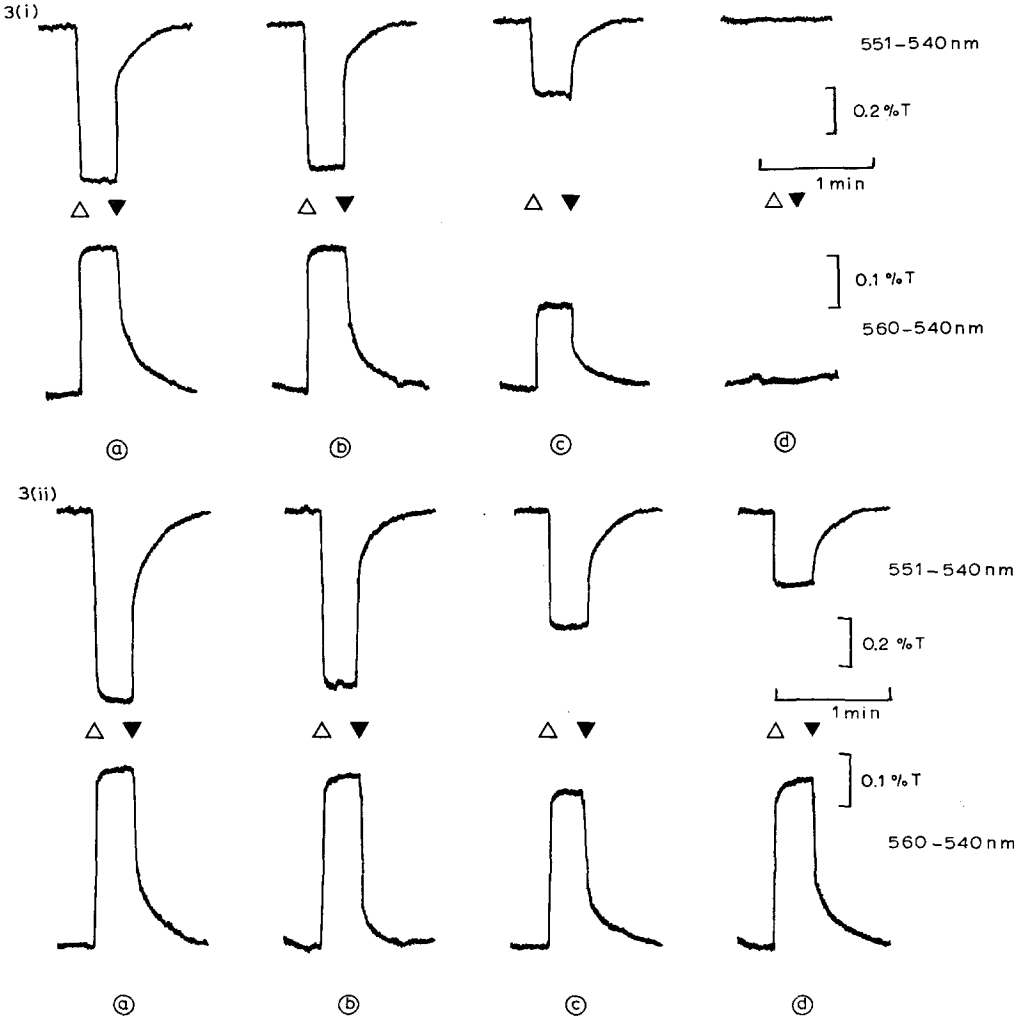

Fig. 3. The effects of the antisera on chromatophores in the presence of 1 % sodium cholate. The reaction mixture contained 3 ml 10 mM Tris · Cl, 100 mM KCl, pH 7.5, 1 % sodium cholate, 100 μM sodium ascorbate, 2 μM Antimycin A plus 2 μM Valinomycin. (i) *Rps. spheroides* Ga. The cuvette also contained 0.1 mg bacteriochlorophyll/ml of chromatophores: (a) no further additions; (b) plus 500 μl control serum; (c) plus 200 μl immune serum, and (d) plus 500 μl immune serum. (ii) *Rps. capsulata Ala pho*$^+$. The cuvette also contained 0.13 mg bacteriochlorophyll/ml of chromatophores: (a) no further additions; (b) plus 500 μl control serum; (c) plus 200 μl immune serum, and (d) plus 500 μl immune serum.

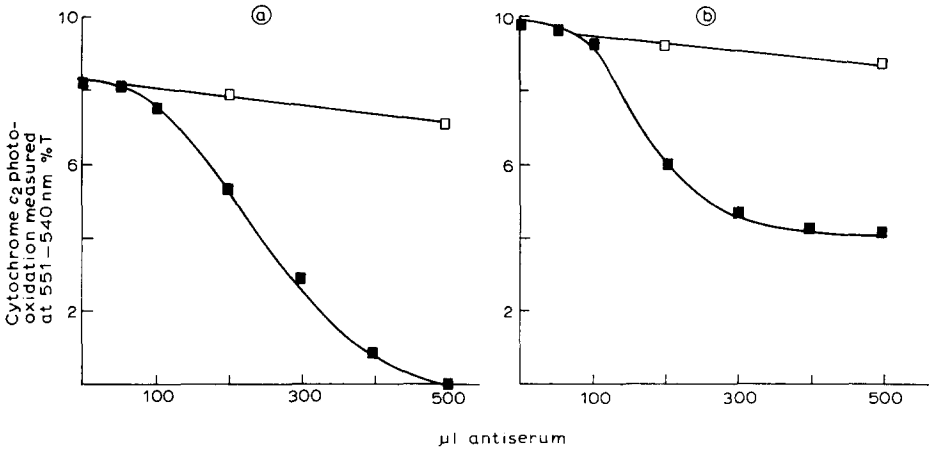

Fig. 4. Titration of the antisera effects in the presence of 1 % sodium cholate. (a) *Rps. spheroides* Ga. Conditions as Fig. 3 (i), and (b) *Rps. capsulata Ala pho*$^+$. Conditions as Fig. 3 (ii). The solid symbols represent immune sera; the open ones, control sera.

trations of up to 1 % it solubilises only a negligible amount of the bacteriochlorophyll. This is in marked contrast to detergents such as Triton X-100 or dodecyldimethylamine oxide, which at such concentrations completely solubilise the membrane. Nevertheless, 1 % sodium cholate completely inhibits the generation of a transmembrane pH gradient, as shown by the inhibition of light induced quenching of 9-amino acridine fluorescence [18] (Fig. 5).

When chromatophores from *Rps. spheroides* Ga were washed five times in 50 mM HEPES, 150 mM KCl, 1 % sodium cholate, pH 7, all the light induced *c*-type

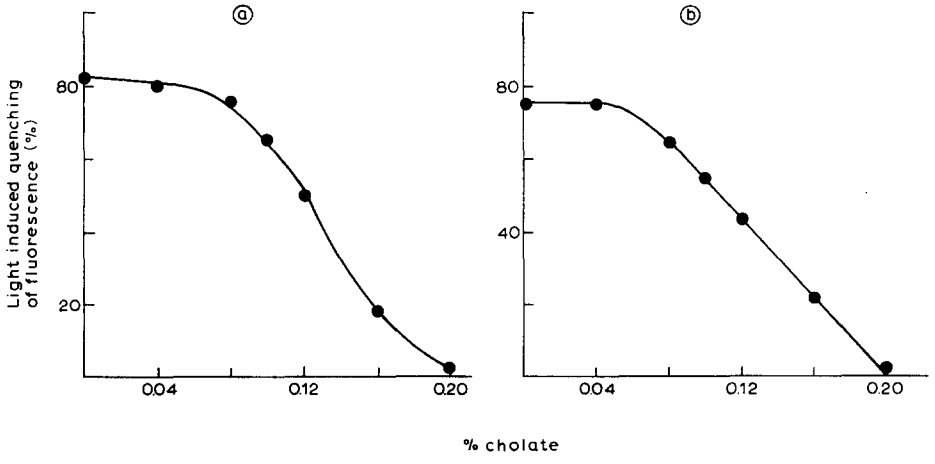

Fig. 5. The effect of cholate on the ability of chromatophores to generate a transmembrane pH gradient. The reaction mixture contained 2.5 ml sodium phosphate/potassium pyrophosphate buffer (0.1–0.1 M) pH 8.5, 2 μM Valinomycin, 0.4 μM 9-aminoacridine and cholate as indicated: (a) contained in addition, chromatophores of *Rps. spheroides* Ga (0.25 mg bacteriochlorophyll/ml), and (b) contained in addition, chromatophores of *Rps. capsulata Ala pho*$^+$ (0.2 mg bacteriochlorophyll/ml).

cytochrome photo-oxidation was lost, as was most of the *c*-type cytochrome. The solubilised cytochrome was precipitated by anti-c_2 prepared against the cytochrome liberated by French pressure cell treatment of whole cells.

As shown in Fig. 4, while in *Rps. spheroides* Ga the light-induced cytochrome changes could be completely inhibited by anti-c_2, this was not the case in *Rps. capsulata Ala pho*$^+$, or in *Rps. capsulata* St. Louis. In this species, the *c*-type cytochrome photo-oxidation was never completely inhibited; the residuum varied from 10 % to 35 % in different preparations, and cytochrome *b* reduction was either slightly inhibited or unaffected. In addition, not all the *c*-type cytochrome photo-oxidation was lost after repeated washing of the chromatophores in 50 mM HEPES, 150 mM KCl, 1 % sodium cholate, pH 7. However, a redox titration of the cytochrome photo-oxidation occurring in the presence of 1 % sodium cholate and excess anti-c_2, indicated that the remaining cytochrome photo-oxidation was indistinguishable by its redox characteristics from the total cytochrome photo-oxidation occurring in the presence of 1 % cholate (Crowther D. and Prince, R. C. unpublished observation). This suggests that 1 % cholate does not render the chromatophores of *Rps. capsulata* as permeable to large molecules as it does in *Rps. spheroides*.

Anti-*c'* had no effect on electron flow in chromatophores, either in the presence or absence of 1 % sodium cholate.

Chromatophores will also photo-oxidise added cytochrome c_2 or cytochrome *c* and the former reaction can be inhibited by anti-c_2 in the absence of cholate. However, this photo-oxidation is not coupled to photophosphorylation (Table III), in

TABLE III

THE EFFECT OF EXTERNAL CYTOCHROME c_2 ON PHOTOPHOSPHORYLATION BY CHROMATOPHORES OF *Rps. capsulata* ST. LOUIS

Photophosphorylation was measured using P^{32}-labeled phosphate, by the method of Baccarini-Melandri and Melandri [22] in a total volume of 1.5 ml.

Concentration of *Rps. capsulata* cytochrome c_2 added (μM)	μmol P_i esterified per mg bacteriochlorophyll/h
0	150
1.1	129
5.5	143
11.0	158

contrast to the earlier work of Horio and Yamashita (24) on *Rhodospirillum rubrum*. Our results are similar to those of Racker et al. [3] with sub-mitochondrial particles, and will be discussed later.

iii) *Spheroplasts*

As shown in Table II, during the preparation of spheroplasts cytochrome c_2 is liberated into solution, and this cytochrome is precipitated by the appropriate anti-c_2. Since the cytochrome is liberated without the rupture of the cell membrane or the liberation of the cell contents, this indicates that the cytochrome in vivo is

localised in the periplasmic space, between the cell membrane and cell wall. The small amount of cytochrome c_2 photo-oxidation remaining was totally insensitive to anti-c_2, suggesting that it was still protected by undigested cell wall.

Spheroplasts will photo-oxidise both added cytochrome c and cytochrome c_2, although the rate of their reduction is slow in an aerobic cuvette in the absence of added reducing agents. As in the case of reaction centres (Fig. 1), anti-c_2 inhibited the photo-oxidation of cytochrome c_2 (Fig. 6) but not of cytochrome c.

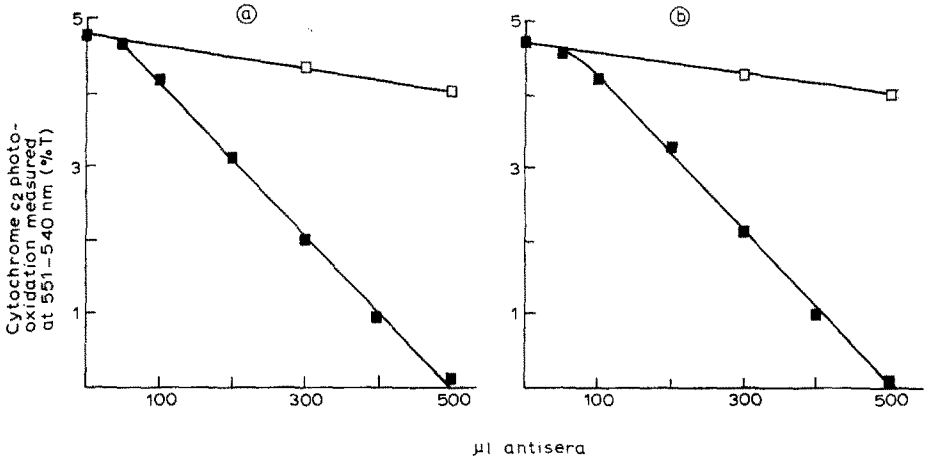

Fig. 6. The effects of the antisera on the photo-oxidation of cytochrome c_2 by spheroplasts. The reaction mixture contained 2.5 ml 10 mM Tris, 150 mM KCl, 20 % (w : v) sucrose, 4 μM Valinomycin, 4 μM Antimycin. (a) *Rps. spheroides* Ga spheroplasts added (120 μM bacteriochlorophyll) plus 1 μM *Rps. spheroides* Ga cytochrome c_2. (b) *Rps. capsulata Ala pho*$^+$ spheroplasts added (100 μM bacteriochlorophyll) plus 1 μM *Rps. capsulata* St. Louis cytochrome c_2. The appropriate antiserum was added as indicated. The amount of cytochrome photo-oxidation measured was that due only to the added cytochrome; any residual cytochrome c_2 photo-oxidation (see Table II) was subtracted from all points.

During the preparation of spheroplasts, cytochrome c' is also liberated into solution. However, adding it back had no effect on the photo-oxidation of added cytochrome c_2, nor did the addition of anti-c'.

Neither anti-c_2 nor anti-c' agglutinated reaction centres, chromatophores or spheroplasts, either directly or indirectly. This is in contrast to the activity of an antiserum prepared against the coupling factor from *Rps. capsulata* (12), which agglutinated chromatophores, but not spheroplasts. This indicates that the coupling factor is located exclusively outside the chromatophore membrane, and is relatively tightly bound with its antigenic sites accessible to soluble antibodies, while under the conditions used here, neither cytochrome c_2 nor c' is tightly bound to the membrane.

The secondary electron donor of Rps. spheroides

An interesting anomaly of *Rps. spheroides* is the fact that the c-type cytochrome which donates electrons to the oxidised reaction centre has an $E_{m7} = +295$ mV [1], while that of the soluble cytochrome c_2 liberated into solution during the preparation of chromatophores has an E_{m7} of 346 mV [8]. As shown in Fig. 7, we

have been able to confirm these results, but in addition, Fig 7 shows that in the presence of 1 % cholate, the midpoint potential of the *c*-type cytochrome in the chromatophores rises to +340 mV. Since this cytochrome, when solubilised by cholate treatment, cross reacts with anti-c_2 prepared against the cytochrome liberated during the preparation of chromatophores, and it has the same redox potential, the two

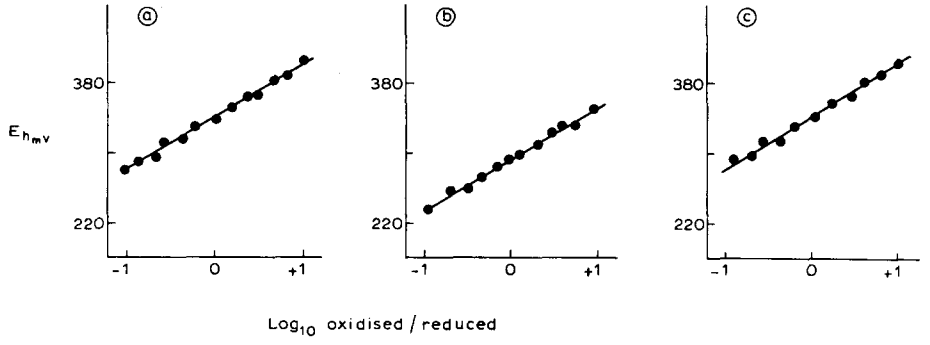

Fig. 7. Redox titrations of cytochrome c_2 from *Rps. spheroides* Ga. (a) Pure cytochrome c_2, (b) chromatophores and (c) chromatophores plus 1 % cholate. The reaction mixture contained 200 μM $K_3Fe(CN)_6$ and 20 μM each of *N*-methyl phenazonium methosulfate, *N*-ethyl phenazonium ethosulphate and 2,3,5,6-tetramethyl-*p*-phenylenediamine in 3 ml of 50 mM TES, 150 mM KCl pH 7.0. In (a) this was supplemented with 20 μM *Rps. spheroides* Ga cytochrome c_2, (b) and (c) with chromatophores (145 μg bacteriochlorophyll). 1 % sodium cholate was added in (c).

forms must both be the same protein species, and the difference in midpoint potential when bound to the chromatophore must be due to this binding. The midpoint shift observed indicates that the oxidised form is bound approximately six times more tightly than the reduced form. The midpoint potential of both the soluble and the chromatophore associated cytochrome c_2 of *Rps. capsulata* are the same; +340 mV [2].

DISCUSSION

Immunological relationships

Several workers [21, 25, 26] have produced antibodies against cytochromes c_2 and c' from *Rhs. rubrum*, but these were not used to localise the antigen in vivo. Orlando et al. [21] found that neither *Chromatium* cytochrome c' nor *Rhs. rubrum* c_2 had antigenic sites in common with *Rhs. rubrum* c'; these results are similar to ours, which suggest that *Rps. spheroides* cytochrome c_2 and *Rps. capsulata* cytochromes c_2 and c' do not share antigenic determinants. Interestingly, the anti-c_2 of Smith et al. [26] prepared against the protein from *Rhs. rubrum*, cross reacted with a variety of other bacterial cytochromes, while none of our antisera did, suggesting that the various cytochromes may share antigenic determinants.

We have found that the proteins produced during anaerobic, photosynthetic growth are immunologically indentical to those produced under oxygen-limited or oxygen-saturated aerobic growth. Similar results have been obtained for *Rhs. rubrum* by Taniguchi and Kamen [25].

The location of cytochrome c_2

The results presented in this paper indicate that in both *Rps. spheroides* and *Rps. capsulata*, the cytochrome c_2 released during the French pressure cell treatment of whole cells is identical to that retained in the chromatophores, and that in vivo the cytochrome is trapped in the periplasmic space. Since the cytochrome is lost during the removal of the cell wall to form spheroplasts, the contents of the chromatophores must be a continuum of the periplasmic fluid, which implies that the chromatophores are not sealed vesicles in vivo. This is shown diagrammatically in Fig. 8 (c.f. Fig. 1 of ref. 27). Further evidence for the reversal of polarity of the photosynthetic membrane in these organisms during the preparation of chromatophores comes from the work of Scholes et al. [28]. They demonstrated that in whole cells proton translocation coupled to photo-oxidoreduction is directed outwards, while in chromatophores it is directed inwards.

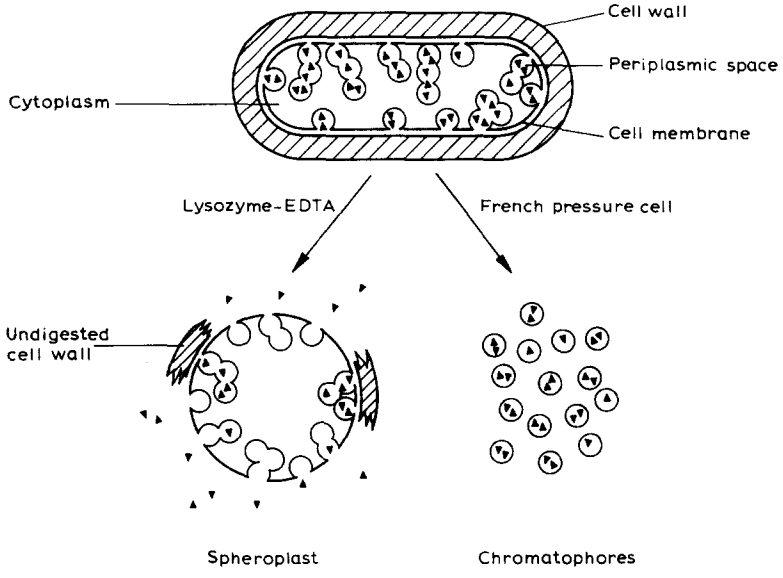

Fig. 8. Diagrammatic representation of the localisation of cytochrome c_2 in *Rps. spheroides* and *Rps. capsulata*. The small solid triangles represent cytochrome c_2, but do not represent the number of molecules inside each chromatophore.

Our results are in agreement with those of Orlando et al., [21], who concluded that cytochromes c' and c_2 were associated with the chromatophores, not the cytoplasm, but not with the results of Kakuno et al., [29] who concluded that 75–90 % of the cytochrome c_2 was present in the cytoplasm of *Rhs. rubrum*. In view of the similarity of *Rps. spheroides* and *Rps. capsulata* in our experiments, it is surprising that *Rhs. rubrum* should be so markedly different, and this anomaly deserves further investigation.

The photo-oxidation of external *c*-type cytochromes by chromatophores of both *Rps. spheroides* and *Rps. capsulata* is probably due to the presence of non-vesicular membrane fragments [30], although it might be due to "membrane scrambling" [31]. The fact that this photo-oxidation is uncoupled, leads us to believe that it has no physiological significance in vivo.

The topography of the light reaction

The experiments reported in this paper have demonstrated that cytochrome c_2 is located inside the chromatophores. Rapid proton uptake occurs at the outer face of chromatophores [32], and we have recently shown that part of this is due to the reduction of ubiquinone [33], indicating that the site of reduction of this component must be at the outer face of the chromatophore.

These results show that the secondary donor (Cytochrome c_2) and the secondary acceptor (H^+ and ubiquinone) react with the primary photochemical reactants on opposite side of the membrane, implying that the photochemical reaction centre is so arranged that the primary oxidising equivalents ("holes") and reducing equivalents (electrons) are available to the secondary reactants on opposite sides of the membrane. The photochemical reaction must therefore occur effectively across the membrane, and involve the performance of electrical work in transporting charge across the membrane dielectric; this charge separation must be effective in the aqueous phases on either side of the membrane at least as rapidly as the secondary reactions occur.

Recent work by Jackson and Dutton [34] and Dutton et al. [35] has indicated that in fact there are two cytochrome c_2 molecules closely associated with each reaction centre, and that these two cytochrome molecules are functionally identical.

Our conclusions are summarised in Fig. 9. We had previously suggested (following Mitchell [36, 37] and Junge and Witt [38]), on the basis of the carotenoid

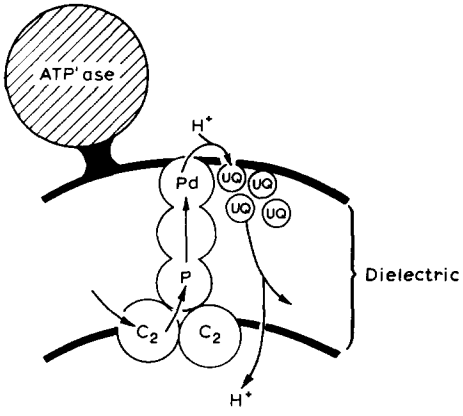

Fig. 9. The topography of the light reaction in *Rps. spheroides* and *Rps. capsulata*. This figure is explained in the text.

change [39, 40] and delayed fluorescence [41] that the photochemical reactions involved charge transfer across the membrane. Our present results show that the vectorial transport of charge occurs across the full width of the membrane, and is not localised within it [42]. Analysis of the carotenoid change by Jackson and Dutton [34] indicated that both the photochemical reaction, and the oxidation of cytochrome c_2 by P^+, contributed to charge separation across the membrane. Using their spherical dielectric model, this indicates that P must be located some way into the dielectric, as shown in Fig. 9. The photochemical reaction and the subsequent re-reduction of the light generated P^+ by cytochrome c_2 may thus be regarded as the electrogenic

arm of a chemiosmotic loop, in which the secondary acceptor (ubiquinone) is an H-carrier potentially completing the loop (Fig. 9).

The role of cytochrome c'

Cytochrome *c'* was first detected in extracts of photosynthetically grown *Rhs. rubrum* [43] and since then has been isolated from several species of photosynthetic bacteria [8]. In *Rps. spheroides* this haem protein is produced in large quantities (cytochrome c_2: cytochrome *c'* approx. 1:1) only under growth conditions where the photosynthetic pigments are also produced, i.e. when grown anaerobically in the light or aerobically in the dark under oxygen-limited conditions. Only a trace of the protein is produced during oxygen-saturated aerobic growth. In contrast, in *Rps. capsulata*, quite large amounts are produced even during oxygen-saturated aerobic growth, and the protein is immunologically indistinguishable when produced either in the light or the dark.

Kakuno et al. [29] suggested that when cytochrome *c'* was bound to the membrane its spectrum was similar to that of a *b*-type cytochrome, although there was little evidence to support this contention. We have recently shown that the purified protein has a large e.s.r. signal [44] which cannot be detected in chromatophores or whole cells, and the results presented here, that anti-*c'* had no effect on any light-induced reaction by any subcellular fraction, are further evidence that a change in the conformation of this protein occurs on solubilisation. That the protein is not bound very firmly to the membrane is illustrated by the fact that cytochrome *c'* is liberated with cytochrome c_2 during the preparation of spheroplasts. The functional role of the bound protein thus remains obscure, and awaits further clarification.

ADDENDUM

Cytochrome *c'* has in the past been known as cytochrome *cc'* and as *Rhodospirillum* haem protein. Recent work [45] suggests that the protein as isolated is a dimer of two monohaem subunits.

Brief preliminary reports of part of this work have been published [46, 47].

ACKNOWLEDGEMENTS

We are grateful to NATO for travel grants to allow this collaboration between our laboratories, and to the Science Research Council, the Deutsche Forschungsgemeinschaft and the Consiglio Nationale delle Ricerche of Italy for general support. We are also grateful to Mrs J. Fielding, Mr G. Tierney and Miss A. Hellmich for expert and cheerful technical assistance.

REFERENCES

1 Dutton, P. L. and Jackson, J. B. (1972) Eur. J. Biochem. 30, 495–510
2 Evans, E. H. and Crofts, A. R. (1974) Biochim. Biophys. Acta 357, 78–88
3 Racker, E., Burstein, C., Loyter, A. and Christiansen, R. O. (1970) in Electron Transport and Energy Conservation (Tager, J. M., Papa, S., Quagliariello, E. and Slater, E. C., eds), pp. 235–252, Adriatica Editrice Bari
4 Hauska, G., McCarty, R. E., Berzborn, R. and Racker, E. (1971) J. Biol. Chem. 246, 3524–3531

5 Wildner, G. F. and Hauska, G. A. (1974) Arch. Biochem. Biophys. 164, 136–144
6 Jackson, J. B., Crofts, A. R. and von Stedingk, L. V. (1968) Eur. J. Biochem. 6, 41–54
7 Karunairatnam, M. C., Spizizen, J. and Gest, H. (1958) Biochim. Biophys. Acta 29, 649–650
8 Bartsch, R. G. (1971) in Methods in Enzymology (San Pietro, A. ed.), 23A, 344–363
9 Clayton, R. K. and Wang, R. T. (1971) in Methods in Enzymology (San Pietro, A. ed.), 23A, pp. 696–704
10 Prince, R. C. and Crofts, A. R. (1973) FEBS Lett. 35, 213–216
11 Clayton, R. K. and Clayton, B. J. (1972) Biochim. Biophys. Acta 283, 492–504
12 Baccarini-Melandri, A., Gest, H. and San Pietro, A. (1970) J. Biol. Chem. 245, 1224–1230
13 Williams, C. A. and Chase, M. W. (1967) in Methods in Immunology and Immunochemistry, 1. Academic Press, New York
14 Ouchterlony, O. (1968) in Handbook of Immunodiffusion and Immunoelectrophoresis, Ann Arbor-Humphrey Science Publishers, Ann Arbor
15 Berzborn, R. J. (1968) Z. Naturforsch. 23B, 1096–1104
16 Jackson, J. B. and Crofts, A. R. (1969) Eur. J. Biochem. 10, 226–237
17 Prince, R. C., Cogdell, R. J. and Crofts, A. R. (1974) Biochim. Biophys. Acta 347, 1–13
18 Deamer, D. W., Prince, R. C. and Crofts, A. R. (1972) Biochim. Biophys. Acta 274, 323–335
19 Clayton, R. K. (1963) in Bacterial Photosynthesis (Gest, H., San Pietro, A. and Vernon, L. P. eds), p. 498, Antioch Press, Yellow Springs, Ohio
20 Reed, D. W. (1969) J. Biol. Chem. 244, 4936–4941
21 Orlando, J. A., Levine, L. and Kamen, M. D., (1961) Biochim. Biophys. Acta 46, 126–133
22 Baccarini-Melandri, A. and Melandri, B. A. (1971) Methods in Enzymology (San Pietro, A. ed.), 23A, pp. 556–561
23 Saunders, V. A. and Jones, O. T. G. (1974) Biochim. Biophys. Acta 333, 439–445
24 Horio, T. and Yamashita, J. (1964) Biochim. Biophys. Acta 88, 237–250
25 Taniguchi, S. and Kamen, M. D. (1965) Biochim. Biophys. Acta 96, 395–428
26 Smith, W. R., Sybesma, C., Litchfield, W. J. and Dus, K. (1973) Biochemistry 12, 2665–2671
27 Oelze, J. and Drews, G. (1972) Biochim. Biophys. Acta 265, 201–239
28 Scholes, P., Mitchell, P. and Moyle, J. (1969) Eur. J. Biochem. 8, 450–454
29 Kakuno, T., Bartsch, R. G., Nishikawa, K. and Horio, T. (1971) J. Biochem. 70, 79–94
30 Crofts, A. R. (1970) in Electron Transport and Energy Conservation (Tager, J. M., Papa, S., Quagliariello, E. and Slater, E. C., eds), pp. 221–228, Adriatica Editrice, Bari
31 Chance, B., Erecinska, M. and Lee, C. P. (1970) Proc. Natl. Acad. Sci. U.S. 66, 928–935
32 Cogdell, R. J. and Crofts, A. R. (1974) Biochim. Biophys. Acta 347, 264–272
33 Prince, R. C., Cogdell, R. J. and Crofts, A. R. (1974) Biochem. Soc. Trans. 2, 162–164
34 Jackson, J. B. and Dutton, P. L. (1973) Biochim. Biophys. Acta 325, 102–113
35 Dutton, P. L., Morse, S. D., Petty, K. M. and Bonner, H. S. (1975) Biochim. Biophys. Acta, in the press
36 Mitchell, P. (1966) in Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation, Glynn Research Ltd. Bodmin, Cornwall
37 Mitchell, P. (1968) Chemiosmotic coupling and Energy Transduction, Glynn Research Ltd., Bodmin, Cornwall
38 Junge, W. and Witt, H. T. (1968) Z. Naturforsch. 23, 244–254
39 Crofts, A. R. and Jackson, J. B. (1970) in Electron transport and Energy Conservation (Tager, J. M., Papa, S., Quagliariello, E. and Slater, E. C., eds), pp. 383–410, Adriatica Editrice, Bari
40 Jackson, J. B. and Crofts, A. R. (1971) Eur. J. Biochem. 18, 120–130
41 Crofts, A. R., Wraight, C. A. and Fleischmann, D. E. (1971) FEBS Lett. 15, 89–99
42 Williams, R. J. P. (1969) in Current Topics in Bioenergetics (Sanadi, D. R. ed.), 3, pp. 79–156, Academic Press, New York
43 Vernon, L. P. and Kamen, M. D. (1954) J. Biol. Chem. 211, 643–662
44 Prince, R. C., Leigh, J. S. and Dutton, P. L. (1974) Biochem. Soc. Trans. 2, 950–953
45 Cusanovich, M. A. (1971) Biochim. Biophys. Acta 236, 238–241
46 Prince, R. C., Hauska, G. A. and Crofts, A. R. (1974) Biochem. Soc. Trans. 2, 534–537
47 Prince, R. C., Hauska, G. A., Crofts, A. R., Melandri, A. and Melandri, B. A. (1974) Proc. 3rd Int. Cong. Photosynthesis, Israel, in press