Biochem. J. (1980) **189**, 385–391
Printed in Great Britain

Do photosynthetic bacteria contain cytochrome c_1?

Paul M. WOOD
Department of Biochemistry, University of Bristol, Medical School, University Walk, Bristol BS8 1TD, U.K.

(*Received 12 December 1979*)

A method is described for characterizing *c*-type cytochromes in bacterial membrane preparations according to molecular weight on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Applied to the photosynthetic bacterium *Rhodopseudomonas sphaeroides* this technique is used, together with spectroscopic measurements, to demonstrate that a membrane-bound cytochrome *c* of mol.wt. 30000 is active in photosynthetic electron transport in addition to the well-known soluble cytochrome, cytochrome c_2. The membrane cytochrome has a midpoint potential (E'_0) at pH 7 of +290mV, as compared with +360mV for purified cytochrome c_2. Its α-band has a peak near 552nm, as compared with 550nm for cytochrome c_2. Evidence is presented that chromatophores contain roughly equal amounts of the two cytochromes.

Many purple photosynthetic bacteria can derive energy for growth either from aerobic respiration or from anaerobic photosynthesis. The ubiquinone–cytochrome *c* part of the electron-transport chain is then of especial interest, since it is common to both pathways (Baccarini-Melandri *et al.*, 1978), and these organisms are frequently studied as mitochondrial analogues in which electrons can be pulsed at will with flashes of light. Yet there is one important difference: in published schemes for the most widely studied species, the hydrophobic complex formed by ubiquinone, *b*-type cytochromes and iron–sulphur centres is terminated by a single *c*-type cytochrome (cytochrome c_2), homologous with mitochondrial cytochrome *c*, without any component corresponding to mitochondrial cytochrome c_1 (Dutton & Prince, 1978). We have recently shown that the mitochondrial pair of high- and low-molecular-weight *c*-type cytochromes has a close parallel in algal photosynthesis (Wood, 1977; Crofts & Wood, 1978), and the present paper reports a reappraisal of the situation in photosynthetically grown *Rhodopseudomonas sphaeroides*.

Materials and methods

Cells of *Rps. sphaeroides* strain 2.4.1. Ga (Cohen-Bazire *et al.*, 1956) were grown anaerobically in the light as described in Sistrom (1960). Chromatophores were prepared by French-press treatment and differential centrifugation as described by Whale & Jones (1970), except that a medium containing 80mM-NaCl, 20mM-KCl and 20mM-Mops, pH 7.0, was used throughout, to avoid precipitation problems in experiments with SDS.

Lysed membranes (cf. Wood, 1978) were prepared by harvesting the cells at 20°C and resuspending the pellets at 1g/15ml in medium at 30°C containing 0.5M-sucrose and 40mM-Tris/HCl, pH 8.0. An equal volume of the same medium plus 8mM-EDTA (sodium salt) and 0.5mg of lysozyme/ml, at 30°C, was then added. After 6min, $MgCl_2$ was added to 10mM. The suspension was left at 30°C for 30min, then centrifuged (15000*g*, 20min, 5°C) to give a pellet of spheroplasts. The spheroplasts were lysed by resuspension in the same volume of 10mM-Tris/HCl, pH 8.0, plus 2mM-EDTA (sodium salt). A Polytron homogenizer (Kinematica, Luzern, Switzerland) was used to make the suspension homogeneous and of normal viscosity. The lysed membranes were then pelleted by centrifugation (20000*g*, 30min, 5°C). They were washed and resuspended in the medium used for chromatophore preparation.

Protein was assayed by the method of Bramhall *et al.* (1969). Bacteriochlorophyll was assayed as described by Clayton (1963). Redox potentials were measured in a stirred anaerobic cuvette (Dutton, 1978) with a Pt/Ag, AgCl combination electrode (Russell pH, Auchtermuchty, Fife, Scotland, U.K.). Difference spectra were recorded with sensitive split-beam spectrophotometers, calibrated beforehand with standard filters. A specially designed cuvette was used for work at 77K (Bendall *et al.*,

Abbreviations used: SDS, sodium dodecyl sulphate; Mops, 4-morpholinepropanesulphonic acid.

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1971). Experiments with flash illumination made use of the apparatus described by Hunter & Jones (1979). A medium containing 100mM-KCl and 20mM-Mops, pH 7.0, was used for all spectroscopic work.

A standard assay for *c*-type cytochrome was used in all cases. Material containing cytochrome *b* was first treated to remove protohaem, and then *c*-type haem was determined from the pyridine haemochrome. The sample was washed twice with at least a 25-fold excess of acetone/methanol (7:2, v/v) at −10°C, and then washed three times with acetone containing 1% HCl, at −10°C. The final pellet was resuspended in 0.15M-NaOH, and pyridine added to 25% of the total volume. The absorption coefficient used was $\Delta\varepsilon_{550-541} = 23\,mM^{-1}\cdot cm^{-1}$ in a dithionite-reduced-minus-untreated difference spectrum; this value was determined with horse cytochrome *c* by assuming $\Delta\varepsilon_{551-540} = 19.9\,mM^{-1}\cdot cm^{-1}$ for dithionite-reduced-minus-ferricyanide-oxidized, at pH 7 (Margoliash & Frohwirt, 1959).

Cytochrome c_2 was released in the course of spheroplast formation. It was purified by dialysis against distilled water followed by adsorption to DEAE-cellulose equilibrated with 5mM-Tris/HCl, pH 8 (Bartsch, 1971). After elution with a 0–0.4M-NaCl gradient, fractions containing the main coloured band and having an oxidized γ-band with a peak absorption at 410nm were pooled, diluted, and rechromatographed with a 0–0.1M-NaCl gradient. After testing for γ-band absorption, the product was dialysed against water and concentrated with Aquacide (Calbiochem).

Samples for SDS/polyacrylamide-gel electrophoresis were placed in test tubes and twice washed with 10ml of acetone/methanol (7:2, v/v) at −10°C, to remove chlorophyll and carotenoids. The tubes were then placed one at a time in a flask connected to an oil pump, and the organic solvent pumped off for 90s. The medium for denaturation contained 1.5 or 2% SDS, 20mM-sodium phosphate, pH 7.2, 20mM-dithiothreitol (freshly dissolved) and 100μM-phenylmethanesulphonyl fluoride. Sufficient was added to give an SDS/protein ratio of 3:1. On addition of the denaturing medium, the sample was immediately resuspended by means of a vortex mixer and sonication bath (Hilbre Ultrasonics, Hoylake, Wirral, Merseyside, U.K.). It was then placed in boiling water for 3min. The gels were prepared in 6mm-diameter glass tubes and run under standard conditions; the Tris system was preferred for molecular-weight determination because of its superior resolution, and the phosphate system for comparative work because it was found to give less variation from one tube to another (Weber & Osborn, 1975). For fluorescence excitation an 8-lamp transilluminator (Ultraviolet Products, San Gabriel, CA, U.S.A.) served as the long-wavelength u.v. light source. The fluorescence was most conveniently (though not most sensitively) photographed with Polaroid 665 film, with the gels left in the tubes and the camera protected by Chance OR1 and Corning 3-66 glass filters. The negative was then scanned with a Unicam SP. 8-100 densitometer.

Results and discussion

It has been shown that polypeptides with covalently linked haem can be detected on gels run in the presence of SDS by their red fluorescence when illuminated with 360nm light (Katan, 1976). The method relies on loss of the iron atom under these conditions, to give a fluorescent porphyrin. It is specific for *c*-type cytochromes because other haem proteins have only non-covalent links between polypeptide and prosthetic group, leading to dissociation of the haem as the protein unfolds in SDS. Katan (1976) described the use of this technique for mitochondrial cytochromes *c* and c_1, and it has since been used for chloroplast cytochrome *f* (Doherty & Gray, 1979). With the procedure described above, we have been able to use it for *Rps. sphaeroides*, *Pseudomonas aeruginosa* (Wood & Willey, 1980), *Rhodospirillum rubrum* and *Chromatium vinosum* (P. M. Wood, unpublished work). The method promises to be a general one whereby *c*-type cytochromes can be characterized in terms of molecular weight without any purification except removal of lipid and pigment. We have encountered no problems of interference.

The technique gave strikingly different results for the two membrane preparations described above, as shown in Fig. 1. Lysed membranes gave a single band of red fluorescence; chromatophores gave two, of roughly equal intensity. With chromatophores, the band of higher molecular weight corresponded to that found with lysed membranes, whereas the faster-moving band could be matched by purified cytochrome c_2 (mol.wt. 14100; Bartsch, 1978). It was possible to stimulate the fluorescence from chromatophores by mixing lysed membranes with purified cytochrome c_2, as shown in Fig. 1(*c*). The best simulations were obtained at ratios of 1:1 to 1.5:1 for cytochrome c_2 to cytochrome *c* in the lysed membranes. The molecular weight of the membrane cytochrome was determined as 30000 ± 1000 by comparison with standard marker proteins (see Fig. 2). The fact that this value is roughly double the molecular weight of cytochrome c_2 is likely to be fortuitous, since a similar membrane cytochrome in *Pseudomonas aeruginosa* gave a mol.wt. of 32000 ± 1000, whereas the soluble counterpart in that organism (cytochrome *c*-551) has a mol.wt. of only 9000 (Wood & Willey, 1980). In the course of optimizing the conditions for these

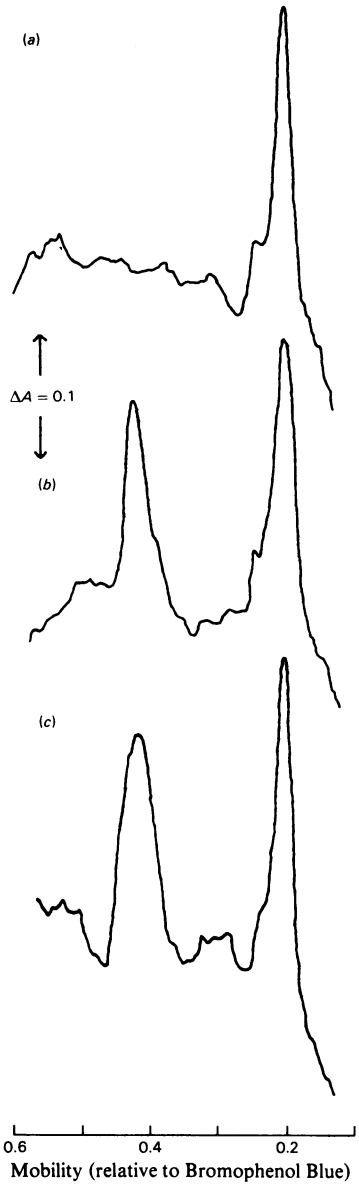

Fig. 1. *Red fluorescence after SDS/polyacrylamide-gel electrophoresis*

Polyacrylamide gels (15%) and phosphate buffer were used (Weber & Osborn, 1975). The traces shown are densitometer scans of a photographic negative. (*a*) Lysed membranes at a loading of 100pmol of *c*-type cytochrome/tube; (*b*) chromatophores at a loading of 250pmol of *c*-type cytochrome/tube; (*c*) lysed membranes plus cytochrome c_2 at loadings of 100 and 150pmol of *c*-type cytochrome (respectively)/tube.

experiments, parameters such as SDS concentration and percentage of acrylamide were varied widely, and similar results were always obtained.

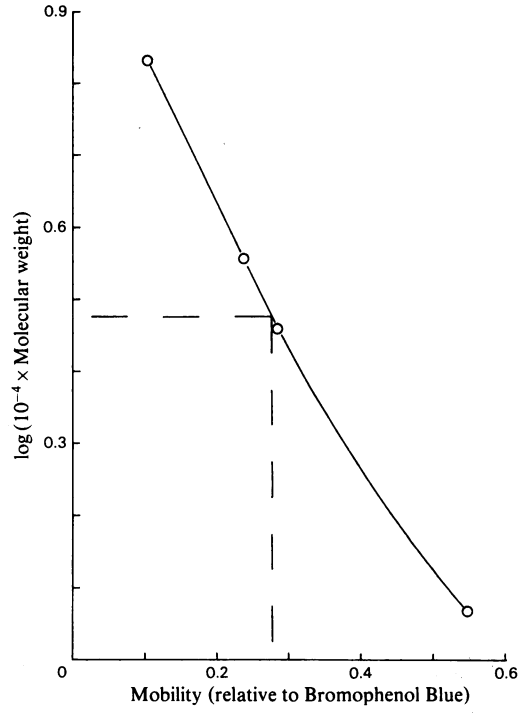

Fig. 2. *Molecular weight of the lysed membrane cytochrome c in SDS*

The graph shows a plot of log (molecular weight) versus relative mobility, when the sample was subjected to electrophoresis through 14% polyacrylamide gels in Tris buffer (Weber & Osborn, 1975). The *c*-type cytochromes were detected by their fluorescence, and the other proteins by staining with Coomassie Blue. The marker proteins (O) were bovine serum albumin (mol.wt. 68000), glyceraldehyde 3-phosphate dehydrogenase (36000), carbonic anhydrase (29000), and horse heart cytochrome *c* (11700) (Weber & Osborn, 1975). The position of the membrane cytochrome *c* is indicated by the intersecting broken lines.

It seemed important to characterize the cytochrome present in lysed membranes by measuring its redox potential. Fig. 3 shows a redox titration, along with one for cytochrome c_2. The results were +290 and +360mV respectively. Similar results were obtained in oxidative and reductive titrations. This value for cytochrome c_2 is close to the +345mV found by Dutton *et al.* (1975), who, in addition, reported that part of the *c*-type cytochrome in chromatophores titrated at +295mV.

Having shown that chromatophores gave results on gels that could be mimicked by lysed membranes plus cytochrome c_2, we undertook a detailed examination of the difference spectra in the α-band range, to see if the same behaviour could be found. As reductant, ascorbate with a low concentration of the mediator 2,6-dichlorophenol-indophenol gave

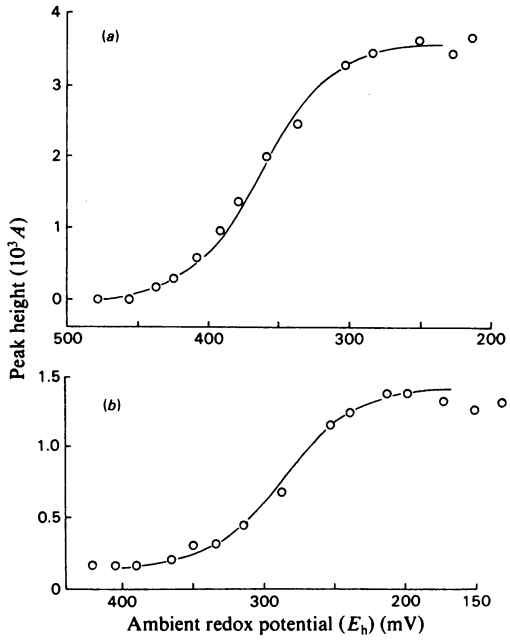

Fig. 3. *Redox titrations at pH 7 for cytochrome c_2 and the lysed membrane cytochrome*

Redox potentials were measured in the presence of 50 μM-*N*-methylphenazonium methosulphate and 70 μM-diaminodurene, as described by Dutton (1978). The potential of the reference electrode (Ag/AgCl/saturated KCl) was assumed to be +198 mV (Bates, 1954). In each case the sigmoid curve is a one-electron plot, fitted by eye. (*a*) Cytochrome c_2 present at 1.5 μM. The reference cuvette contained medium only. A wavelength scan was performed at each redox poise; the ordinate shows A_{550} relative to a line drawn through 539 and 563 nm. (*b*) Lysed membranes present at 118 μM-bacteriochlorophyll. The reference cuvette contained lysed membranes and medium only. The ordinate shows absorption at 552 nm relative to a line drawn through 547 and 557 nm.

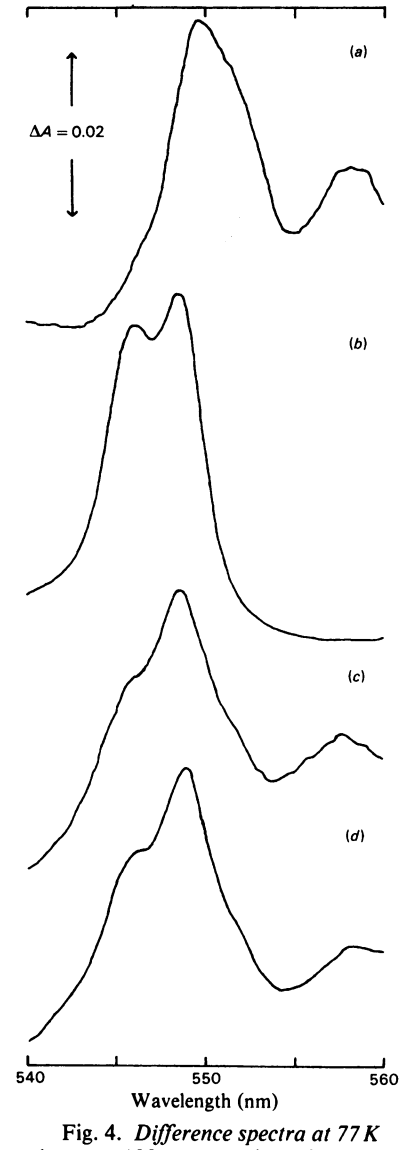

Fig. 4. *Difference spectra at 77 K*

In each case 130 μM-potassium ferricyanide was added to the reference cuvette, and 3 mM-ascorbate plus 10 μM-2,6-dichlorophenol-indophenol to the sample. (*a*) Lysed membranes present at 110 μM-bacteriochlorophyll; (*b*) cytochrome c_2 present at 0.4 μM; (*c*) chromatophores present at 55 μM-bacteriochlorophyll; (*d*) lysed membranes and cytochrome c_2 mixed so that each contributes half of the total cytochrome *c* (64 μM-bacteriochlorophyll and 0.52 μM-cytochrome c_2). Note that the degree of enhancement caused by light-scattering from ice crystals is variable, and hence peak heights cannot be directly compared.

virtually complete reduction of the membrane cytochrome and cytochrome c_2, with very little reduction of the lower-potential *b*-type cytochromes. The bands became more distinct at low temperatures, and Fig. 4 shows results for 77 K. For lysed membranes the *c*-type-haem peak was almost symmetrical, with a slight shoulder to the red side. Cytochrome c_2, by contrast, gave a markedly split peak, having a sub-peak to the violet side. Chromatophores gave an intermediate behaviour. Fig. 4(*d*) shows that, as with the gels, the chromatophore spectrum could be mimicked by mixing lysed membranes with cytochrome c_2. The best simulations were obtained with equal amounts of *c*-type haem from the two sources. Fig. 5 shows similar difference spectra at room temperature; the band shapes are less distinctive, but there is a 2 nm difference between the wavelengths for maximum absorption by lysed membranes (Fig. 5*a*) and

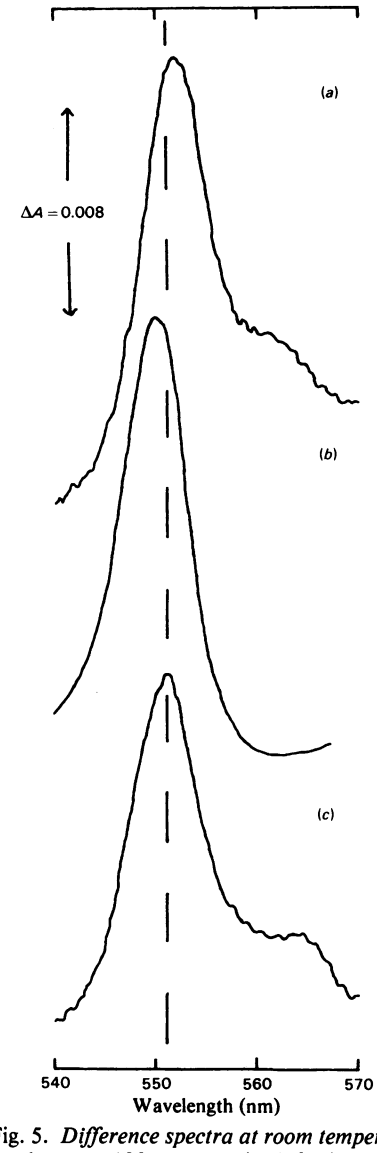

Fig. 5. *Difference spectra at room temperature*
In each case 130μM-potassium ferricyanide was added to the reference cuvette, and 3mM-ascorbate plus 10μM-2,6-dichlorophenol-indophenol to the sample. (*a*) Lysed membranes, present at 110μM-bacteriochlorophyll; (*b*) cytochrome c_2 present at 7.8μM; (*c*) chromatophores present at 55μM-bacteriochlorophyll. A broken line is drawn at 551.0nm.

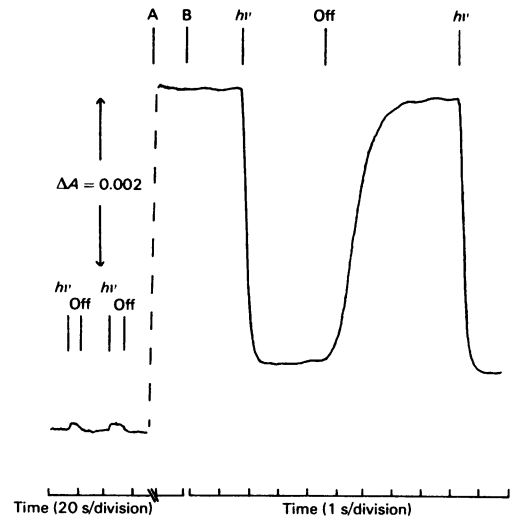

Fig. 6. *Proportion of photo-oxidizable cytochrome c in chromatophores*
The Figure shows the chart-recorder output from a dual-wavelength spectrophotometer set to read $A_{551-540}$. The sample cuvette contained chromatophores at 14μM-bacteriochlorophyll, plus 64μM-potassium ferricyanide, 0.8μM-antimycin A, and 5μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. The actinic light was passed through a Corning 2-59 red filter while the photomultiplier was protected by $CuSO_4$ filter. The light was switched on (*hν*) and off (Off) as indicated. At point A the system was switched off for about 20s while 1.3mM-ascorbate and 2μM-2,6-dichlorophenol-indophenol were added. At point B the chart speed was increased. The temperature was 22°C.

cytochrome c_2 (Fig. 5*b*). The peak for chromatophores (Fig. 5*c*), once again, is intermediate between the other two.

The following results provide evidence that the membrane protein detected on the gels is indeed a *c*-type cytochrome. Firstly, the pyridine haemochrome prepared from the lysed membranes after treatment to remove non-covalently linked haem (see the Materials and methods section) gave a difference spectrum with a peak at 551nm. This is the standard test for a *c*-type cytochrome (Falk, 1964). Secondly, if this difference spectrum was used as an assay for *c*-type haem, as described in the Materials and methods section, the result was consistent with $\Delta\varepsilon_{552-541} \approx 20\,mM^{-1}\cdot cm^{-1}$ for a difference spectrum at pH 7 (cf. Fig. 5*a*). This is the normal value for a *c*-type cytochrome. Thirdly, similar red fluorescence has been found from gels loaded with a wide range of purified *c*-type cytochromes, including cytochromes c_1, c_2, c_3, c', *f*, flavocytochrome *c* and *Pseudomonas* nitrite reductase (EC 1.9.3.2) (P. M. Wood, unpublished work).

Having demonstrated from gels and spectra that two cytochromes are present in chromatophores, the crucial question remained as to whether both have roles in photosynthetic electron transport, or whether one has some other function. This is

answered by the experiments shown in Figs. 6 and 7. Fig. 6 shows that the chemical reduction of *c*-type cytochrome in chromatophores could be reversed to over 80% by continuous illumination. In this experiment, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was present to prevent a protonmotive force building up, and antimycin A was added to slow down greatly the rate of cyclic electron flow. Under the conditions shown, photo-oxidation reached a steady state within 1 s, while re-reduction was complete in 2–3 s. Fig. 7 shows that a similar extent of oxidation could be accomplished by flash illumination, and that it corresponded to approx. 1.2 cytochrome *c* molecules per reaction centre (assuming $\Delta\varepsilon = 19\,\text{mM}^{-1}\cdot\text{cm}^{-1}$ and $\Delta\varepsilon = 10.3\,\text{mM}^{-1}\cdot\text{cm}^{-1}$ respectively; Dutton *et al.*, 1975). The 80% value for photo-oxidation and other features of Figs. 6 and 7 are very similar to those reported in the literature; it has been suggested that the reason why photo-oxidation is incomplete is because some cytochrome becomes dislocated from reaction centres in the course of chromatophore preparation (Prince *et al.*, 1975; Dutton *et al.*, 1975). But in the present study, the important point is that the extent of cytochrome photo-oxidation is considerably greater than the level of cytochrome c_2. It can only be explained by participation of both proteins.

In addition to cytochrome c_2, *Rps. sphaeroides* contains smaller amounts of several other soluble *c*-type cytochromes, the principal one being cytochrome *c'* (Bartsch, 1978). We have found that purified cytochrome *c'* is not separated from cytochrome c_2 on gels run as in Fig. 1; in *Rps. gelatinosa* it contains 129 amino acids, as compared with 124 for *Rps. sphaeroides* cytochrome c_2, which may be present as isoenzymes (Prince *et al.*, 1974; Ambler *et al.*, 1979*a*,*b*). A third cytochrome, 'SHP', also has a similar monomeric molecular weight (Bartsch, 1978). Apart from the very minor component, cytochrome *c*-554, these other cytochromes have negative redox potentials (Bartsch, 1978), and would not be detected in Figs. 4 and 5, since they would be permanently oxidized. They might lead to the gel method of Fig. 1 giving a slight overestimate of the content of cytochrome c_2 in chromatophores, but they do not affect the general interpretation.

The experiments reported above show clearly that two distinct cytochromes are present in whole cells,

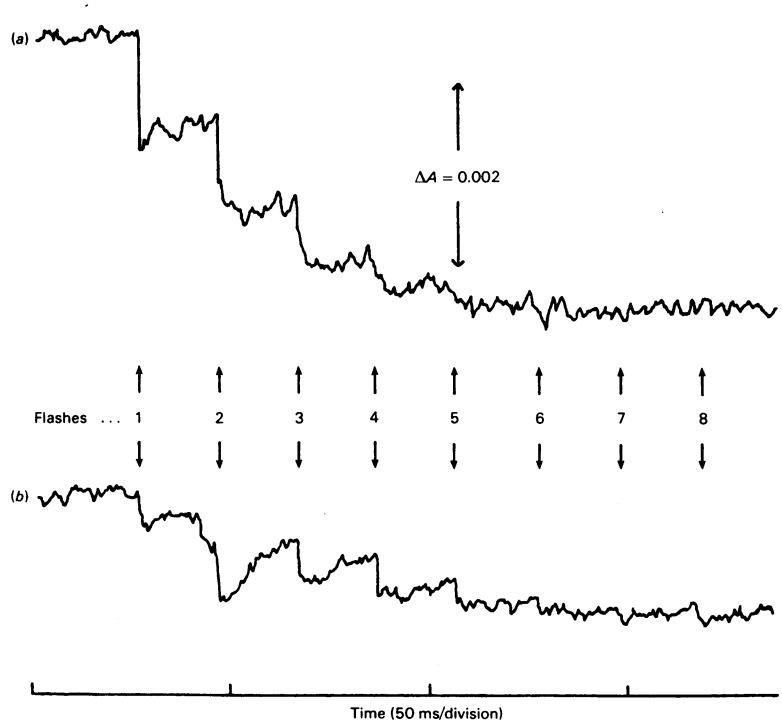

Fig. 7. *Chromatophore cytochrome c and reaction-centre flash photo-oxidation*
The chromatophore preparation was as in Fig. 6, with the ascorbate and 2,6-dichlorophenol-indophenol included. The dual-wavelength spectrophotometer was interfaced to a transient recorder, and a series of eight saturating flashes given at 20 ms intervals, each flash having a half-width of 26 μs. (*a*) Monitoring absorbance at $A_{551-540}$; (*b*) monitoring A_{542} only in order to detect the reaction-centre bacteriochlorophyll.

and both are photochemically important. The fact that chromatophores contain cytochrome c_2, whereas lysed membranes do not, is exactly as predicted from the model of Prince *et al.* (1975). Cytochrome c_2 is a periplasmic protein, loosely adsorbed to the cytoplasmic membrane. In the course of chromatophore preparation it becomes trapped inside the vesicles pinched off during French-press treatment. However, in the lysed-membrane preparation, once the cell wall has been digested away, cytochrome c_2 would be free to diffuse away from the spheroplast membrane, and indeed the supernatant from this step was a convenient starting point for purification. The membrane cytochrome has been reported previously, but has generally been assumed to be a form of the soluble one (Bartsch, 1978). Only with the gel technique reported here is it possible to say positively that it is a distinct entity, with a mol.wt. of about 30000.

Kinetic experiments have been carried out that provide evidence for a series interaction: membrane cytochrome $c \rightarrow$cytochrome $c_2 \rightarrow$reaction centre (P. M. Wood, unpublished work).

I thank the Science Research Council for a research grant, The Royal Society for an equipment grant, Dr. O. T. G. Jones for the use of his spectrophotometers, and Mr. J. A. Tillotson for valuable technical assistance.

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