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The interrelation of the two *c*-type cytochromes in *Rhodopseudomonas sphaeroides* photosynthesis

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Photosynthetic electron flow in the bacterium *Rhodopseudomonas sphaeroides* involves two *c*-type cytochromes, one membrane-bound and the other a soluble protein, cytochrome c_2. Membranes deficient in cytochrome c_2 were used for photo-oxidation studies, with and without the addition of purified cytochrome c_2. The results favour a series interrelation, membrane cytochrome *c*–cytochrome c_2–reaction centre.

A recent paper has presented evidence that the light-driven cyclic electron-transport chain of the photosynthetic bacterium *Rhodopseudomonas sphaeroides* involves two *c*-type cytochromes, and not merely cytochrome c_2 as had previously been supposed (Wood, 1980). The line of argument was based on a combination of spectrophotometric measurements and studies of gel fluorescence. With these complementary techniques it was shown that chromatophores prepared from this organism contained two *c*-type cytochromes in roughly equal amounts, and that the magnitude of cytochrome oxidation brought about by a succession of flashes of light could only be explained by contributions from both of them. One of these cytochromes was cytochrome c_2, and the other was a membrane protein having a molecular weight of 30000 as determined by SDS/polyacrylamide-gel electrophoresis.

In chromatophores the equilibration between these two cytochromes appears to be very rapid, and I have not as yet been able to design experiments showing their relative positions in the electron-transport chain. A simpler approach, described in the present paper, is to make use of a membrane preparation containing negligible amounts of cytochrome c_2 (lysed membranes), and to study its properties with and without this cytochrome added back.

Methods

Rps. sphaeroides strain 2.4.1 Ga (Cohen-Bazire *et al.*, 1956) was grown anaerobically in the light as described by Sistrom (1960). Lysed membranes were prepared by treatment with lysozyme followed by a severe osmotic shock, as described by Wood (1980). The methods for assay of *c*-type cytochromes and purification of cytochrome c_2 were also as described by Wood (1980).

Abbreviation used: SDS, sodium dodecyl sulphate.

Experiments with flash illumination made use of the apparatus described by Hunter & Jones (1979). The membranes were placed in the sample compartment of a dual-wavelength spectrophotometer interfaced to a transient recorder, and illuminated with a light-flash having a half-width of 30μs. The actinic light was passed through a Kodak Wratten 88A filter (i.r.-transmitting), and the spectrophotometer photomultipliers were protected by blue-glass filters (Corning 4–96, 10% transmittance at 585nm).

SDS/polyacrylamide-gel electrophoresis was conducted as described by Wood (1980) except for the following improvements: in experiments with gels prepared in phosphate buffer the SDS concentration in the denaturing medium was 1% and the SDS/protein ratio was 5:1; and during photography of the fluorescence the camera was shielded by a Corning 2–61 filter (red-transmitting, 50% transmittance at 620nm).

Results

Wood (1980) showed that the predominant *c*-type cytochrome in the lysed membrane preparation was one having an α-band maximum at 552nm, redox potential ($E_{m,7}$) +290mV and molecular weight on SDS/polyacrylamide-gel electrophoresis of 30000. It was stated that the cytochrome c_2 concentration was so low that it could not be detected by gel fluorescence. For the present work it was necessary to quantify this statement. Gels were run for membranes with and without the addition of low concentrations of cytochrome c_2. The gels were photographed and the negatives were scanned by

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densitometry, as shown in Fig. 1. This Figure shows that added cytochrome c_2 at 16% of the concentration of the *c*-type cytochrome already present gave a clear band of red fluorescence, whereas no such band was visible with the membranes alone. From the signal-to-noise level it is fair to conclude that the concentration of contaminating cytochrome c_2 was less than 4% of the total content of cytochrome *c*.

To study the interrelation of the membrane-bound and soluble *c*-type cytochromes, the membranes were subjected to a flash of light either without added cytochrome c_2 (Fig. 2) or with purified cytochrome c_2 added back (Fig. 3). Ascorbate was included in these experiments, plus a low concentration of a redox mediator, so that the cytochromes would relax to a reduced state in the dark. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (an uncoupler) was added to prevent complications arising from proton movements, and cyanide to inhibit any dark respiration. A low-salt medium was found preferable, as explained below.

Fig. 2 shows that flash illumination of the membranes led to a very sluggish *c*-type cytochrome oxidation. The use of different wavelength pairs confirmed that this effect had a spectrum similar to that of the membrane cytochrome *c*, and its magnitude was much greater than the possible contribution from residual cytochrome c_2. Antimycin A, an inhibitor of cyclic electron flow (Dutton & Prince, 1978), had a marked effect on the kinetics of re-reduction. In its absence (Fig. 2*b*) the photo-oxidation was maximal at about 80 ms after the flash, and had decayed completely within 1 s. By contrast, if antimycin A was included (Fig. 2*a*), photo-oxidation was still near its maximum extent at 1 s after the flash, and took about 10 s to decay.

Fig. 3 shows the effect of addition of a very small amount of cytochrome c_2. As shown in Fig. 3(*b*), the presence of a mere 150 nM-cytochrome c_2 led to *c*-type cytochrome photo-oxidation being largely complete within 1 ms of the flash. Fig. 3(*c*) shows that the rate of re-reduction was still sensitive to antimycin A (cf. Fig. 2). It was also found that with

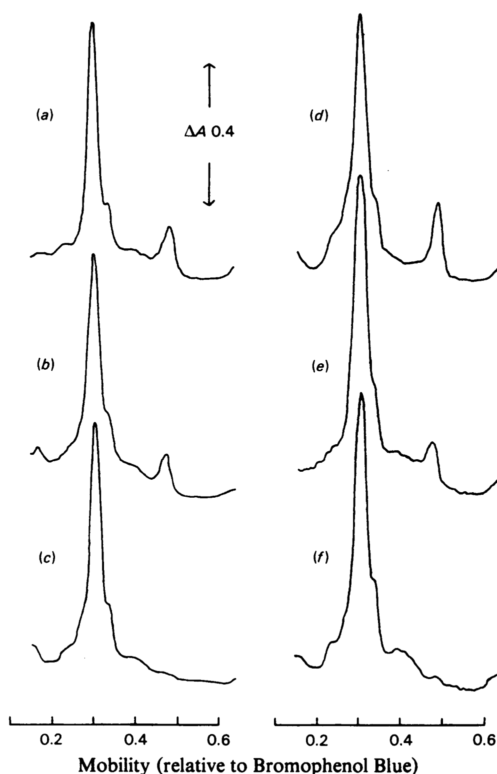


Fig. 1. *Effects of cytochrome c_2 on gel fluorescence*
Polyacrylamide gels (15%) and 0.2 M-sodium phosphate buffer, pH 7.2, were used (Weber & Osborn, 1975). The traces shown are densitometer scans of a photographic negative. (*a*) Lysed membranes at a loading of 135 pmol of *c*-type cytochrome per tube, plus 44 pmol of cytochrome c_2; (*b*) as (*a*), but with only 22 pmol of added cytochrome c_2; (*c*) as (*a*), but without added cytochrome c_2; (*d*), (*e*) and (*f*) a similar series, with membranes containing 200 pmol of *c*-type cytochrome per tube, and 66, 33 and 0 pmol of cytochrome c_2 respectively.

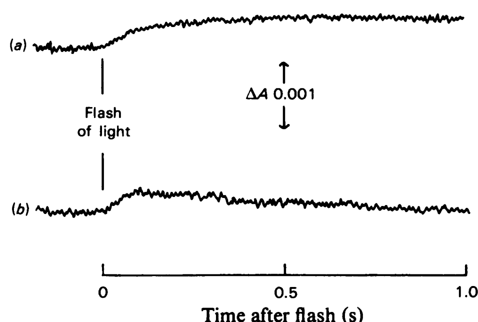
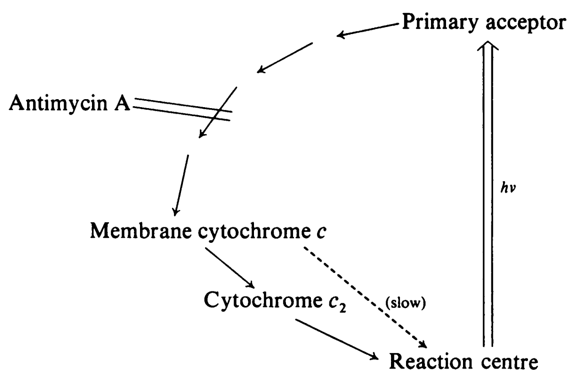


Fig. 2. *c-type cytochrome photo-oxidation without added cytochrome c_2*
For details of the apparatus see the Methods section. The buffer consisted of 7 mM-3-morpholinepropanesulphonic acid adjusted to pH 7.0 with NaOH. Lysed membranes were present at 16 μM-bacteriochlorophyll, plus 1 mM-ascorbate, 1 μM-2,6-dichlorophenol-indophenol, 5 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and 1 mM-KCN. Absorbance was monitored at 551 nm minus 542 nm. The time constant was 1 ms. The temperature was 19°C. (*a*) In the presence of 1.5 μM-antimycin A; (*b*) without antimycin A.

added cytochrome c_2 the rate of photo-oxidation was markedly slowed by raising the ionic strength; Fig. 3(*d*) shows a time profile obtained under conditions identical with those for Fig. 3(*b*) except for the presence of an additional 0.1 M-KCl. This salt effect parallels the findings obtained by Prince *et al.* (1974) on the kinetics of cytochrome c_2 photo-oxidation by purified reaction centres. Similar results to all these experiments were obtained with use of mediators other than 2,6-dichlorophenol-indophenol (e.g. diaminodurene), and indeed without any reducing agent other than ascorbate, although then the dark reduction tended to be incomplete and slow to reach equilibrium.

Discussion

The results presented above rule out the possibility that the membrane cytochrome *c* acts between cytochrome c_2 and the reaction centre. The antimycin-sensitivity of re-reduction both with and without added cytochrome c_2 supports a simple linear scheme, with interaction in series as shown below:



This ordering is consistent with the redox potentials measured for the two cytochromes, +290 mV for the membrane one, and +360 mV for cytochrome c_2 (Wood, 1980). For a discussion of the rest of the chain, see Dutton & Prince (1978).

The naming of the membrane cytochrome raises some problems, as a result of the central position of the purple non-sulphur bacteria in the study of bioenergetics. The name 'cytochrome *f*', originally applied by Hill & Scarisbrick (1951) to the *c*-type cytochrome in higher-plant chloroplasts, has since been extended to the similar protein in eukaryotic algae and cyanobacteria (Wood, 1977; Ho *et al.*, 1979). Cytochrome *f* has a molecular weight on SDS/polyacrylamide-gel electrophoresis similar to that of the cytochrome described here (Gray, 1978; Krogmann & Ho, 1980). Most algae contain in addition a soluble *c*-type cytochrome (usually referred to as cytochrome *c*-552, *c*-553 or *c*-554, depending on the α-band maximum), which shows sequence homology with cytochrome c_2, and functions in a linear chain, cytochrome *f*–cytochrome *c*-553–Photosystem I reaction centre (Crofts & Wood, 1978). Therefore a case could be made for calling the *Rps. sphaeroides* cytochrome 'cytochrome *f*'. However, it could be argued that this term should be reserved for organisms containing plastoquinone. An alternative name would then be 'cytochrome c_1'. The justification for this is that the protein is also present in cells grown aerobically in the dark (P. M. Wood, unpublished work), and there is good evidence that under these conditions the ubiquinone–cytochrome c_2 part of the chain is common to both photosynthesis and respiration (Baccarini-Melandri *et al.*, 1978). Membrane cytochromes of similar molecular weight and redox potential in many non-photosynthetic bacteria have been named cytochrome c_1, e.g. *Paracoccus dentrificans* (Jones, 1977), *Pseudomonas aeruginosa* (Matsushita *et al.*, 1980; Wood & Willey, 1980) and *Thermus thermophilus* (Fee *at al.*, 1980). A third solution, perhaps preferable, pending sequence studies, is to refer to it merely as 'membrane cytochrome *c*-552', although this is cumbersome and uninformative. The simpler form 'cytochrome *c*-552' could lead to confusion with unrelated cytochromes of the cytochrome c_2–mitochondrial cytochrome *c* family.

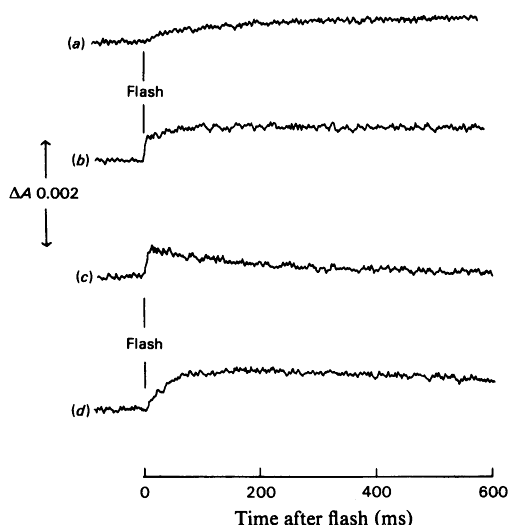


Fig. 3. *c-type cytochrome photo-oxidation with added cytochrome* c_2

The conditions were as indicated in Fig. 2 except as noted below. (*a*) As Fig. 2(*a*), but displayed at higher time resolution; (*b*) as (*a*), plus 150 nM-cytochrome c_2; (*c*) as (*b*), but without antimycin A; (*d*) as (*b*), but with KCl added to 0.1 M.

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