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A ROLE FOR CYTOCHROME c_2 IN *RHODOPSEUDOMONAS VIRIDIS*

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A membrane fraction was isolated from the purple photosynthetic bacterium *Rhodopseudomonas viridis*. It contained the membrane cytochromes *c*-552 and *c*-557. A limit on the concentration of the soluble cytochrome c_2 was set at 1% of total *c*-type haem by a technique involving fluorescent gels. Cytochrome *c*-557 had extremely slow kinetics of re-reduction after oxidation by a flash of light: $\tau = 40$ s. The rate of re-reduction was considerably accelerated by adding back purified cytochrome c_2. Cytochromes *c*-557 and c_2 were monitored individually by appropriate wavelength pairs. Their kinetics subsequent to a flash of light fitted a linear mechanism with cytochrome c_2 as the donor to cytochrome *c*-557:

$$X \xrightarrow{k_1} \text{cytochrome } c_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{cytochrome } c\text{-}557 \xrightarrow{\text{fast}} P960$$

X being an uncharacterised donor and P960 the photoreactive bacteriochlorophyll. Approximate rate constants were obtained: $k_1 = 2 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ and $k_2 = 1 \cdot 10^6$ $M^{-1} \cdot s^{-1}$. The presence of 5 μM antimycin slowed k_1 by 30% but had no effect on k_2. Dark reduction of oxidised cytochrome c_2, added to membranes plus succinate, showed kinetics similar to those observed after a flash of light, and antimycin had the same effect in both cases.

Introduction

The purple photosynthetic bacteria fall into two main classes as far as *c*-type cytochromes are concerned. In one class, typified by *Rhodopseudomonas sphaeroides* and *Rhodopseudomonas capsulata,* a pair of cytochromes is found, analogous in many ways to mitochondrial cytochromes c_1 and *c* [1,2]. The soluble member of the pair, cytochrome c_2, is the donor to the photosynthetic reaction centre. Its donor is a membrane cytochrome, denoted c_1 or c_B, and electron flow from the membrane cytochrome to the reaction centre is very slow in the absence of the soluble cytochrome [2,3]. This paper concerns a member of the second class, *Rhodopseudomonas viridis.* Here two membrane-bound *c*-type cytochromes are closely connected to the reaction centre, the redox potentials of their haems being close to +300 and 0 mV [4]. Other species with this property include *Chromatium vinosum* and all other purple sulphur bacteria investigated, and *Rhodopseudomonas gelatinosa* [5,6]. In all cases, the low-potential member of the pair is photooxidisable at 77 K [5,7]. At room temperature both can be efficiently oxidised, with preferential oxidation of the low-potential cytochrome if both are initially reduced [8–10]. However, re-reduction of the low-potential cytochrome is always a slow process, leading to a

* To whom all correspondence should be addressed.
Abbreviation: Mops, 4-morpholinepropanesulphonic acid.

belief that it is not a component of the cyclic electron-transport chain, but instead is involved in a minor pathway of non-cyclic electron flow [8,9]. For *C. vinosum,* Van Grondelle et al. [11] have characterised a soluble cytochrome, *c*-551, which appears to act as principal donor to the high-potential bound cytochrome. However, studies with *C. vinosum* are complicated by the fact that several other soluble cytochromes *c* are present, only a small proportion being cytochrome *c*-551 [6].

Rhodopseudomonas viridis has a number of advantages for study in this respect. Firstly, cytochrome c_2 is the only soluble *c*-type cytochrome to have been isolated from this organism [6]. It has been sequenced, and among bacterial cytochromes is particularly close to mitochondrial cytochrome *c* [12]. Secondly, no carotenoid spectral changes have ever been observed [13]. This can be contrasted with the red shift in response to a light-induced membrane potential in *Rps. sphaeroides* and *Rps. capsulata,* and the blue shift in *C. vinosum* [14]. Thirdly, the high-potential membrane cytochrome *c* has an α-band maximum at an exceptionally long wavelength (557 or 558 nm) making for easy spectroscopic distinction from cytochrome c_2 [4]. Two further points deserve mention. Firstly, *b*-type cytochromes have not been detected in photosynthetically grown *Rps. viridis* [15]. However, the fact that antimycin has its standard inhibitory effect suggests that, as in *C. vinosum,* they may well be present at a low stoichiometry relative to the *c*-type cytochromes, making them difficult to detect [9,16,17]. Secondly, the membrane organisation in intact cells is in flat lamellae or thylakoids, very different from the vesicular structures of *Rps. sphaeroides* and *C. vinosum* [17]. Membranes isolated from broken cells are in the form of slabs; there is little evidence for closed vesicles and photophosphorylation activity is very low [18–20].

Materials and Methods

Rps. viridis (NCIB 10028) was grown anaerobically in medium as used by Rutherford et al. [21], except that ammonium malate was omitted and the following were added: 17 mM succinate, 0.3 mM aspartate, 8.6 mM NaCl and 3.8 mM ammonium sulphate. Illumination was provided by 40 W tungsten filament light bulbs. The cells were harvested, washed and resuspended in 20 mM Mops/NaOH/80 mM NaCl/20 mM KCl (pH 7.0). They were disrupted by two passages through a French pressure cell at 0.12 GPa, 0.5 mM $MgCl_2$ and 100 μg DNAase being added before the first passage. Unbroken cells and large debris were removed by centrifugation ($17000 \times g$, 12 min). The membranes were pelleted by centrifugation at $130000 \times g$ for 2.5 h and resuspended in the same buffer.

For purification of cytochrome c_2 from the supernatant, $(NH_4)_2SO_4$ was added to 50% saturation and the precipitate was removed. Further $(NH_4)_2SO_4$ was added to 100% saturation. The resultant precipitate was redissolved in 50 mM sodium phosphate (pH 7.5), followed by gel filtration on Sephacryl S-200 equilibrated with the same buffer. This yielded a preparation in which cytochrome c_2 was the major protein and only cytochrome, as determined by dodecyl sulphate gel electrophoresis coupled with cytochrome visualisation techniques [22,23]. Further purification could be achieved by methods recommended by Bartsch [6], but was not found to affect the results. For experiments requiring oxidised or reduced cytochrome c_2, ferricyanide or ascorbate, respectively, was added and the cytochrome separated by passage through a Sephadex G-25 column.

Protein was assayed by the method of Bramhall et al. [24]. Bacteriochlorophyll *b* was determined spectrophotometrically at 790 nm after extraction into acetone/methanol (7:2), assuming $\epsilon = 75$ $mM^{-1} \cdot cm^{-1}$ [15].

SDS-polyacrylamide gel electrophoresis and subsequent visualisation of *c*-type cytochromes was conducted as described by Wood [1,23]. The photographic negative was scanned with a Joyce Loebl Chromoscan 3 (Vickers Inst., York, U.K.).

Difference spectra were taken at room temperature on a Pye Unicam SP8-200 spectrophotometer. A dual-wavelength spectrophotometer was used for cytochrome reactions resultant from a flash of light [25], the flash being produced by discharge of a 2.5 μF capacitor at 2 kV through a snaked xenon tube (Wingent, Milton, Cambridge, U.K.). The light-pulse had a half-width of 120 μs. The actinic light was passed through two Kodak Wratten 88A filters (infrared transmitting), while the photomultiplier was protected by a Corning 4-96 blue glass

filter (10% transmittance at 585 nm). The wavelength pairs used for monitoring cytochromes c_2 and *c*-557 were 549 vs. 562.5 nm and 556 vs. 545 nm, respectively. The corresponding extinction coefficients ($\Delta\epsilon$((reduced − oxidised)) were estimated as 23 and 25 $mM^{-1} \cdot cm^{-1}$. The former was calculated from a difference spectrum for cytochrome c_2, assuming $\Delta\epsilon_{550-541\,nm} = 23$ $mM^{-1} \cdot cm^{-1}$ for the pyridine haemochrome [1]. The latter was calculated from a difference spectrum for membranes in the dark (succinate reduced vs. ferricyanide oxidised; cf. Fig. 1 below), assuming $\Delta\epsilon_{557\,nm}$(reduced minus oxidised) = 20 $mM^{-1} \cdot cm^{-1}$ [26].

Results

Fig. 1 shows that for membranes incubated with succinate in the dark the cytochrome *c*-552 was in the oxidised state and the cytochrome *c*-557 was reduced, at least to 80–90% in each case. Photooxidation experiments (see below) also showed no evidence for the presence of any reduced cytochrome *c*-552. Cytochrome c_2 was not spectroscopically detectable in such preparations,

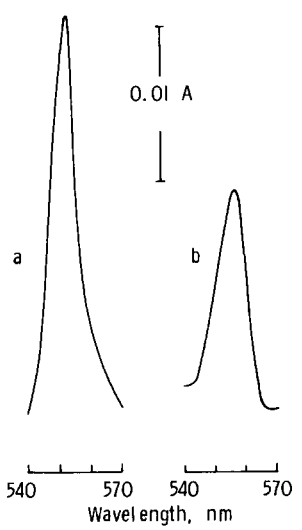

Fig. 1. Redox state of dark-adapted membranes. The membranes were diluted with medium as for resuspension, to give 11 $\mu g \cdot ml^{-1}$ bacteriochlorophyll *b*. Difference spectra were taken 5 min after addition of the reagents: (a) dithionite reduced minus succinate (5 mM) reduced; (b) succinate (5 mM) reduced minus ferricyanide oxidised.

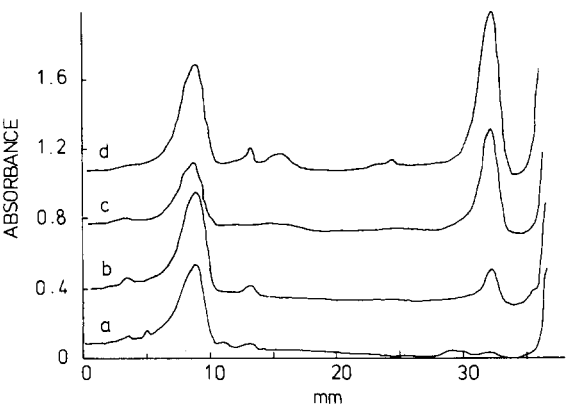

Fig. 2. Setting a limit on the cytochrome c_2 in the membrane fraction. The figure shows densitometer scans of a photographic negative exposed to fluorescence from dodecyl sulphate/polyacrylamide gels illuminated with near-ultraviolet light. Each tube was loaded with membranes containing 620 pmol *c*-type haem, plus varying amounts of cytochrome c_2: (a) none; (b) 22 pmol; (c) 110 pmol; (d) 220 pmol.

as was first reported by Olson and Nadler [13]. A more sensitive test for its presence is provided by the fact that *c*-type cytochromes give rise to fluorescent bands when subjected to polyacrylamide gel electrophoresis in the presence of dodecyl sulphate. Fig. 2 shows densitometer scans of a photographic negative exposed to fluorescence from gels loaded with membranes plus varying amounts of purified cytochrome c_2. With membranes alone, no fluorescence corresponding to cytochrome c_2 could definitely be detected. Since added cytochrome c_2 at 3.5% of the loading of membrane *c*-type haem gave a well-defined band, it can be concluded that any cytochrome c_2 in the membrane preparation constituted less than 1% of total *c*-type haem. Despite the clear spectroscopic evidence for cytochromes *c*-552 and *c*-557, the membranes gave rise only to a single fluorescent band, at an apparent molecular weight of 37000. This is as found by other workers for both *Rps. viridis* and *C. vinosum* and points to both haems being on the same polypeptide [27,28]. (However, detergent treatment of *C. vinosum* enables cytochrome *c*-553 to be extracted in a state largely free from *c*-555 [29].)

With a view to reconstitutions, wavelength pairs were selected for monitoring either cytochrome *c*-557 or added cytochrome c_2 individually. The

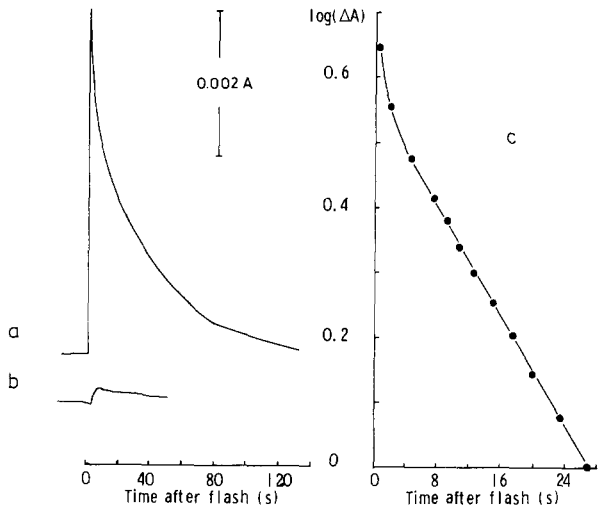

Fig. 3. Cytochrome *c*-557 kinetics after excitation by a flash of light. Membranes were diluted to 10.5 $\mu g \cdot ml^{-1}$ bacteriochlorophyll *b* in medium as for resuspension plus 5 mM succinate, and were excited with a flash of light. (a) Monitoring 556 minus 545 nm, for cytochrome *c*-557; (b) control, with wavelengths appropriate for cytochrome c_2, 549 minus 562.5 nm; (c) log plot of (a). Temperature, 25°C.

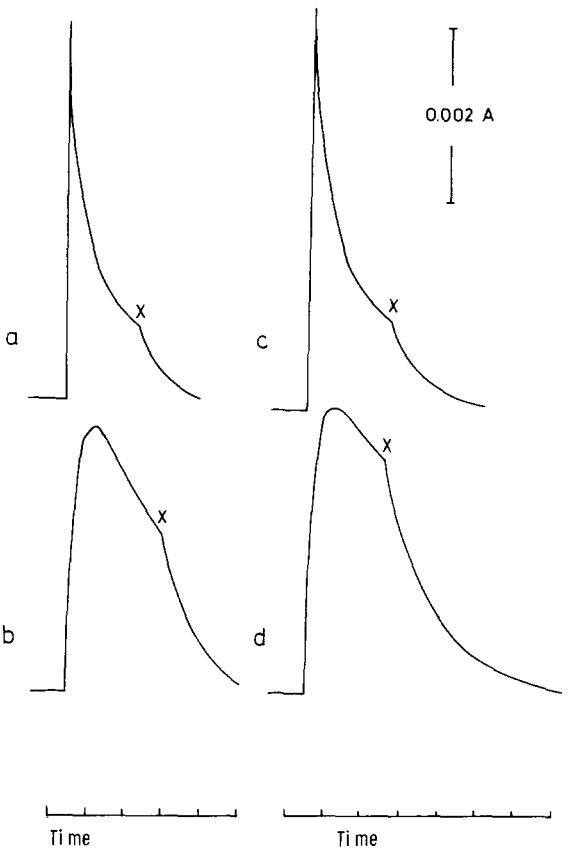

Fig. 4. Cytochrome kinetics with added cytochrome c_2. Conditions were as in Fig. 3, but with reduced cytochrome c_2 added to a final concentration of 0.37 μM. (a) Monitoring 556 minus 545 nm, for cytochrome *c*-557; (b) monitoring 549 minus 562.5 nm, for cytochrome c_2; (c) and (d): as (a) and (b), respectively, but with 5 μM antimycin present. Temperature, 25°C. In each case at (X) the chart speed was slowed by a factor of 4. The intervals on the time-scale correspond to 5 s before (X) and 20 s after (X).

wavelength pair chosen for cytochrome *c*-557 gave negligible absorbance change on addition of ascorbate to oxidised cytochrome c_2, while the wavelengths selected for cytochrome c_2 gave little change on flash excitation of isolated membranes (Fig. 3b). Fig. 3a shows that a flash of light caused rapid photooxidation of cytochrome *c*-557, as expected from the literature [10,30], but that its reduction was very slow. The difference spectrum of the effect was as expected for cytochrome *c*-557. A log plot of the time-course shown in Fig. 3a is provided by Fig. 3c; after a short faster phase the kinetics fitted a slow first-order decay, $\tau = 40$ s.

Fig. 4a and b show similar profiles after addition of a low concentration of reduced cytochrome c_2. It can be seen that the re-reduction of cytochrome *c*-557 was greatly accelerated. Cytochrome c_2 became oxidised, initially with kinetics matching those for re-reduction of cytochrome *c*-557, and was subsequently re-reduced. The inhibitory effect of antimycin on the re-reduction of cytochrome c_2 is shown by comparing these traces with Fig. 4c and d.

The simplest scheme to explain these results is a linear one:

$$X \rightarrow \text{cytochrome } c_2 \rightleftharpoons \text{cytochrome } c\text{-}557 \xrightarrow{\text{fast}} P960 \xrightarrow{h\nu} \text{acceptor}$$

Here X is the donor to cytochrome c_2, identity unknown, and P960 is the photoreactive bacteriochlorophyll. The back reaction between cytochromes c_2 and *c*-557 cannot be ignored, because their redox potentials differ only by about 34 mV [6,31]. Oxidation of cytochrome *c*-557 by flash-oxidised P960 is so fast ($\tau = 2$ μs [10]), that it is complete within the time span of the flash. The

subsequent reactions are:

$$\text{cytochrome } c\text{-}557_{ox} + \text{cytochrome } c_{2\,red} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{cytochrome } c\text{-}557_{red} + \text{cytochrome } c_{2\,ox} \quad (1)$$

$$\text{cytochrome } c_{2\,ox} + X \xrightarrow{k_1} \text{cytochrome } c_{2\,red} + X^+ \quad (2)$$

During the initial stages of cytochrome *c*-557 re-reduction the concentration of oxidised cytochrome c_2 is low. Moreover, since $k_{-2} < k_2$, the back reaction can be ignored for longer than would otherwise be the case. The kinetics then approximate to:

$$d[\text{cytochrome } c\text{-}557_{ox}]/dt = -k_2[\text{cytochrome } c_{2\,red}] \times [\text{cytochrome } c\text{-}557_{ox}],$$

or, on integration,

$$\ln[\text{cytochrome } c\text{-}557_{ox}] = -k_2 \cdot [\text{cytochrome } c_{2\,red}]\,t + \text{constant} \quad (3)$$

A second simple relationship concerns the total amount of oxidised *c*-type cytochrome:

$$d([\text{cytochrome } c\text{-}557_{ox}] + [\text{cytochrome } c_{2\,ox}])/dt = -k_1 \cdot [X] \cdot [\text{cytochrome } c_{2\,ox}] \quad (4)$$

Fig. 5 shows plots based on Eqn. 3 for two concentrations of cytochrome c_2, including experiments with antimycin present. Good straight lines are obtained for the initial stages in all cases, and it can be seen that antimycin had no effect. Doubling the concentration of cytochrome c_2 gave rather less than a doubled slope, probably because the rate tends to a maximal velocity (V_{max}) as cytochrome c_2 is increased. The corresponding values of k_2 are $1.1 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ and $0.8 \cdot 10^6$ $M^{-1} \cdot s^{-1}$, for 0.37 and 0.74 μM cytochrome c_2, respectively.

Fig. 6 shows plots testing the constancy with time of the relationship given in Eqn. 4. In accordance with the kinetic model, the doubled concentration of cytochrome c_2 made little difference. As another contrast with Fig. 5, about a 30% inhibition can be seen with antimycin. The value

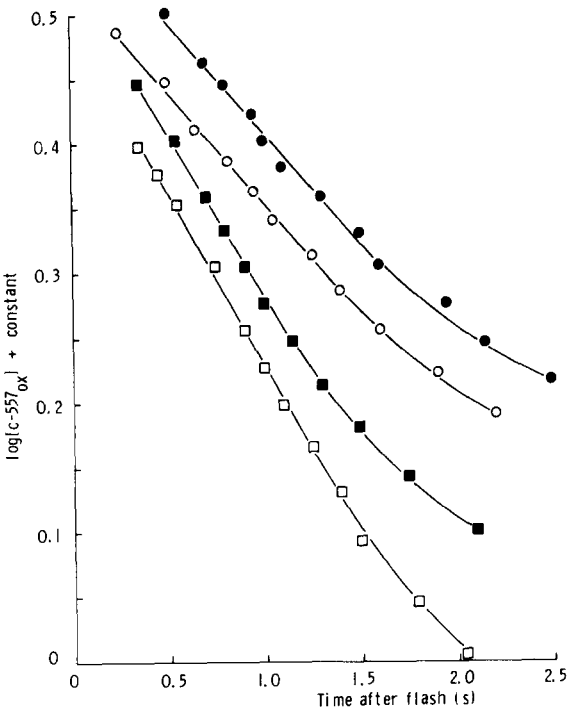

Fig. 5. First-order plots for initial stage of cytochrome *c*-557 re-reduction. (○) 0.37 μM reduced cytochrome c_2; (□) 0.74 μM reduced cytochrome c_2; (●) 0.37 μM reduced cytochrome c_2 and 5 μM antimycin; (■) 0.74 μM reduced cytochrome c_2 and 5 μM antimycin. Other conditions as for Fig. 4.

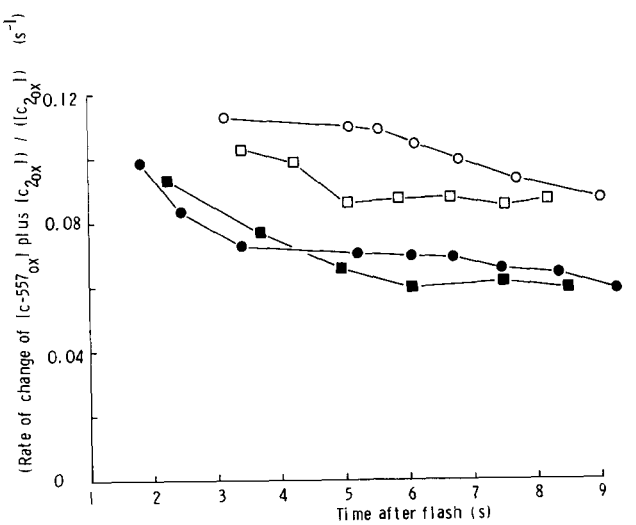

Fig. 6. Plots for the evaluation of k_1. The ordinate shows the rate of decline in concentration of total oxidised cytochrome, divided by the concentration of oxidised cytochrome c_2. This is plotted as a function of time. Symbols used for the same conditions as in Fig. 5.

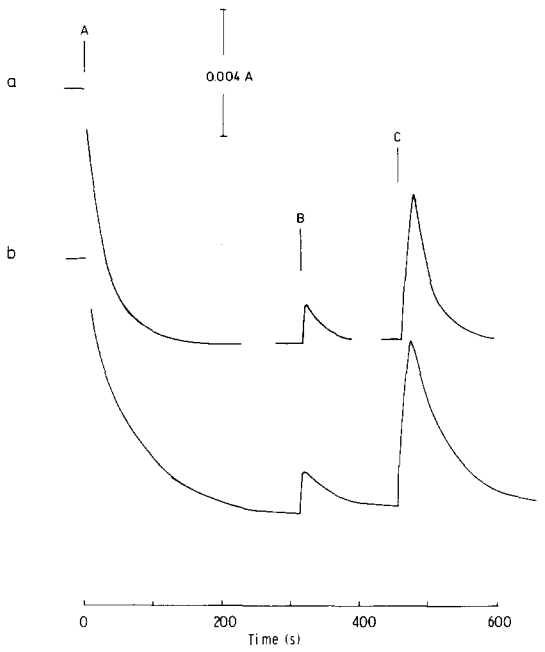

Fig. 7. Reduction kinetics for preoxidised and photooxidised cytochrome c_2. Membranes were diluted to 3 $\mu g \cdot ml^{-1}$ bacteriochlorophyll *b* in medium as for resuspension, plus 5 mM succinate. The traces show time-courses for absorbance at 549 relative to 562.5 nm. At point (A) 0.37 μM oxidised cytochrome c_2 was added with stirring. At (B) a single flash was given, and at (C) a train of 11 flashes at 1 s intervals. Temperature, 25°C. (a) No antimycin; (b) plus 5 μM antimycin.

for $k_1 \cdot [X]$ without antimycin is 0.095 s^{-1}; if the concentration of X is assumed to be as for cytochrome *c*-557, then $k_1 = 2 \cdot 10^5\ M^{-1} \cdot s^{-1}$.

Finally, Fig. 7 shows results for experiments in which oxidised cytochrome c_2 was added to membrane plus succinate in the dark. The time-course for its reduction was compared with the kinetics subsequent to a flash of light. It can be seen that the dark reduction showed exactly the same kinetics as the later stages of the flash-initiated reaction, and antimycin had the same effect in both cases.

Discussion

A number of studies have commented on the low level of photosynthetic activity in membranes from *Rps. viridis,* by contrast with the highly active chromatophores from other species [15,20,32]. The present results show that what is missing is cytochrome c_2, evidently lost because the membranes are not in the form of sealed vesicles. Olson and Nadler, in one of the earliest studies on *Rps. viridis* [13], commented 'in view of its presence in the soluble fraction rather than the chromatophore fraction of extracts, it would seem not to be associated with photosynthetic energy transfer'. This statement has been cited ever since (e.g., Ref. 6), but so far as we are aware, no one has previously tried reconstituting with the cytochrome. In our experiments with added cytochrome c_2, recovery after a flash still took a matter of seconds, but the concentration of the donor X (less than 1 μM) was far lower than is likely to prevail in vivo. The only previous report of experiments involving reconstitution of membranes with a soluble cytochrome *c* is that of Saunders [25]. She studied NADH-horse cytochrome *c* reductase activity in the dark and found it to be partially inhibited by antimycin. This agrees well with the present results.

The conclusion is that the interrelation of the two high-potential cytochromes *c* in *Rps. viridis* is the inverse of that in *Rps. sphaeroides,* or for instance in algal photosynthesis or mitochondria: the soluble cytochrome *c* is donor to the bound one. A conventional Rieske-type iron-sulphur centre is present as in *C. vinosum* [33] (Rutherford, W.A., personal communication). Whether this is the donor to the soluble cytochrome remains to be seen.

An intriguing parallel is found in certain thermophilic bacteria, for which cytochrome aa_3 as isolated includes a membrane-bound cytochrome *c*. This has been found for *Thermus thermophilus* and 'PS 3' [34,35]. In both cases the *c*-type haem was attached to a protein of molecular weight 38 000, far larger than any member of the mitochondrial cytochrome *c* family. Electron transfer from *c*- to *a*-type haem was possible down to −80°C, showing that bound cytochrome *c* was efficiently connected to the oxidase in the absence of any soluble cytochrome *c* [34]. The complex oxidised soluble cytochromes *c* from PS 3 and certain other bacteria, while yeast cytochrome *c* was oxidised at a rather slower rate. The complete pathway from the ubiquinone-cytochrome *b* region to the oxidase remains to be characterised.

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