Biochimica et Biophysica Acta, 545 (1979) 339—351

BBA 47610

THE KINETICS OF FLASH-INDUCED ELECTRON FLOW IN BACTERIOCHLOROPHYLL-LESS MEMBRANES OF *RHODOPSEUDOMONAS SPHAEROIDES* RECONSTITUTED WITH REACTION CENTRES

C. NEIL HUNTER * and OWEN T.G. JONES

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

(Received May 16th, 1978)

Key words: Bacterial photosynthesis; Reaction center; Membrane reconstitution; Electron flow; (Rhodopseudomonas, Kinetics)

Summary

Membranes isolated from aerobically grown mutants 01 and PM8bg II-15 of *Rhodopseudomonas sphaeroides* lack reaction centres. Incorporation of purified reaction centres into these membranes can be achieved by mixing the protein and membranes in 1% sodium cholate with added soybean phospholipid and removing the cholate by dialysis.

The kinetics of light-stimulated electron flow in these reconstituted membranes have been examined and compared with those observed in chromatophore membranes isolated from photosynthetically grown *R. sphaeroides*. Following a single saturating flash, reconstituted reaction centres become photo-oxidised, and about 60% are re-reduced within about 200 ms by cytochrome c_2 in the 01 membrane. Cytochrome c_2 photo-oxidation is biphasic, the half-time of the first fase being faster than 20 μs. The second phase is variable and can be as slow as 60 ms. A cytochrome *b* in the membrane becomes photoreduced with a half-time of 27 ms. Electron flow between cytochromes *b* and c_2 is slow and appears only partially sensitive to antimycin A.

Using membranes from the reaction centre-less mutant PM8bg II-15 similar reconstitution measurements were performed. The resulting kinetic measurements showed that fast cytochrome *b* photoreduction and cytochrome c_2 photo-oxidation occurred.

The absorbance change at 560 minus 570 nm induced by steady-state

* To whom correspondence should be addressed. Present address: Department of Microbiology, Rutgers University, New Brunswick, NJ 08903, U.S.A.

Abbreviations: MOPS, 3-(*N*-morpholino) propane sulphonic acid; *P*-605, absorption maxima of photo-oxidisable reaction centre in the visible region of the spectrum; P^+, the photo-oxidised form of *P*-605; *P*, *P*-605 in its fully reduced state; Tricine, *N*-tris(hydroxymethyl)methyl glycine.

illumination of 01 membranes reconstituted with reaction centres was measured at a range of ambient potentials; the reaction was abolished at oxidation-reduction potentials below 0 mV. The change was approximately halved at +50 mV, indicating that cytochrome b_{+50} is the recipient of electrons from the reconstituted reaction centres.

Introduction

Adaptation of aerobically growing *Rhodopseudomonas sphaeroides* to anaerobic photosynthetic conditions may involve the incorporation of photoreactive pigment proteins into a membrane containing the aerobic respiratory chain [1]. The similarity of the properties of membranes isolated from aerobically growing cells and photosynthetically growing cells has already been summarised in a previous paper [2]. If no rearrangement of existing *b* and *c*-type cytochromes is necessary, insertion of the appropriate light-sensitive pigment proteins into the unpigmented membrane should result in the formation of a cyclic light driven electron transport system.

It has proved possible to perform the insertion in vitro by mixing membranes prepared from the aerobically grown mutant 01 of *R. sphaeroides* with purified reaction centres, prepared from photosynthetically grown cells, in the presence of the detergent sodium cholate and added soybean phospholipid. Subsequent removal of cholate by dialysis or dilution yields a vesicular membrane preparation which contains bound reaction centres and exhibits some of the properties of chromatophores [3]. It has been possible to include light-harvesting pigment-protein complex in our reconstitutions and demonstrate energy transfer from it to reaction centre [4]. Since the kinetic properties of chromatophores of the photosynthetic system from *R. sphaeroides* strain Ga have been established, these may be used as a standard of photosynthetic competence [5]. We have made similar measurements on our reconstituted membranes and these were compared with those obtained from chromatophores from the photosynthetic carotenoid-less strain R-26 of *R. sphaeroides*. The implications of the results to the development of the photosynthetic apparatus are discussed.

Methods

Growth of cells. All bacteria were grown in the medium of Sistrom [6].

Preparation of reaction centres. Reaction centres from the blue-green mutant R-26 of *R. sphaeroides* were prepared as described by Clayton and Wang [7] using the detergent lauryldimethylamine oxide. The final levitate containing purified reaction centres was dissolved in 10 mM Tris-HCl (pH 7.5) containing 2% cholate. The estimation of the concentration of reaction centres in solution was as described previously [8].

Spectrophotometry. This was performed using an unchopped dual wavelength spectrophotometer with two photomultipliers each masked with a Corning blue glass filter (no. 9782). Signals were stored and averaged in an LS1-11 microprocessor. Flashes from a xenon lamp of approx. 40 μs duration, passed through a far-red filter (Kodak Wratten 88A), activated photosynthetic reactions.

Preparation of chromatophores from the blue-green mutant R-26. After harvesting, cells were washed and resuspended in 20 mM MOPS/100 mM KCl

buffer, pH 7.0. After one passage through a French pressure cell the material was centrifuged at 30 000 × *g* for 25 min to remove unbroken cells and aggregated material. The supernatant was centrifuged at 104 000 × *g* for 90 min and chromatophores recovered as a translucent dark blue-green pellet.

Reconstitution of reaction centres into membranes from aerobically grown cells. Aerobically grown cells of the bacteriochlorophyll-less mutant 01 of *R. sphaeroides* were harvested at late log phase, washed and then suspended in 10 mM Tris/100 mM KCl, pH 8.0. After passage through a French pressure cell at 18 000 lb/inch^2 the mixture was centrifuged at 30 000 × *g* for 25 min to sediment unbroken cells and other heavy material. The supernatant was centrifuged at 104 000 × *g* for 90 min and the resulting brown membranous pellet resuspended in 10 mM Tris/100 mM KCl buffer (pH 8.0) at a protein concentration of approx. 10 mg/ml. For reconstitution, 20 mg membrane protein, 14 nmol reaction centres and 40 mg sonicated soybean phospholipid suspension were mixed with sodium cholate at a final concentration of 1% and the mixture dialysed at 4°C for 48 h against two changes of 4 l 10 mM Tris/100 mM KCl buffer (pH 8.0). The dialysis residue was then assayed for reconstituted photosynthetic reactions.

All manipulations involved in the reconstitution were carried out in the dark using a Tungsten safe light filtered through a green Kodak Wratten filter (No. 38).

Results

The light minus dark difference spectrum of a dialysed reconstituted membrane system (Fig. 1) shows that following a single saturating light flash there

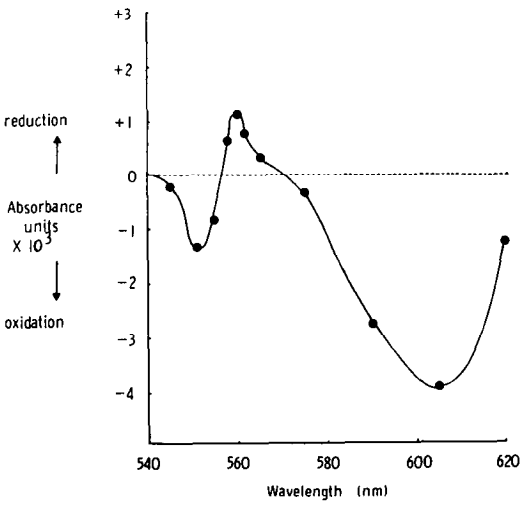


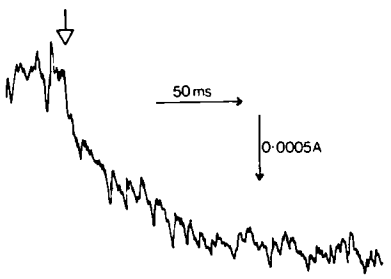
Fig. 1. Flash induced spectrum of a dialysed 01 membrane-reaction centre preparation poised at a potential of about +50 mV. The maximum absorbance change at each wavelength was recorded on a storage oscilloscope linked to an unchopped dual wavelength spectrophotometer using 540 nm as reference wavelength throughout. The anaerobic cuvette contained a total volume of 2.5 ml: 2 mg protein, 1.5 ml 20 mM MOPS/100 mM KCl (pH 7.0), 10 mM sodium fumarate, 1 mM sodium succinate, 1 mM potassium cyanide, 20 μg antimycin A.

are absorbance changes with maxima at 551, 560 and 605 nm, corresponding to the wavelengths of absorption bands of cytochrome c_2, cytochrome *b* and oxidised reaction centre bacteriochlorophyll, respectively. We suggest that cytochrome c_2 becomes photo-oxidised and cytochrome *b* becomes reduced as a result of illumination in the presence of reaction centres in the membrane. Such changes were absent in membranes that had not been mixed with reaction centres.

The kinetics of each of these reconstituted flash-induced absorbance changes were examined. Following a flash, cytochrome *b* becomes reduced, with a half-time of about 27 ms (Fig. 2). Similar results were obtained when cytochrome *b* was assayed using either 560—540 nm or 560—570 nm wavelength pairs or by measuring the absorbance change at 560 nm and subtracting the contribution of reaction centre bleaching at 560 nm. (We are grateful to Mr. J. Bowyer for supplying this last correction data).

The kinetics of cytochrome *b* reduction were found to be rather variable; the fastest kinetics obtained for R-26 chromatophores indicated that a cytochrome *b* became reduced with a half-time of 2 ms in the presence of antimycin A (Table I). However, in some experiments the half-time of cytochrome *b* reduction was slower, in the range of 20—30 ms. The same parameter in reconstituted membranes was never faster than 27 ms; possible explanations will be discussed later but it appears that faster kinetics of cytochrome *b* reduction are possible in reconstituted membranes if the ambient potential is controlled precisely.

In the above experiments the response of the apparatus was limited by using a 100 μs time constant filtering the output from the differential amplifier connected to the photomultipliers; this meant that sub-millisecond reactions could not be accurately measured. R-26 chromatophores used as controls yielded the same fast phase kinetics of cytochrome c_2 photo-oxidation in such experiments. In another experiment the light induced absorbance decrease at



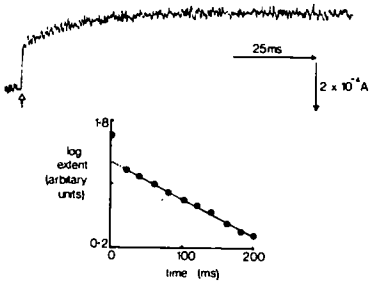


Fig. 2. The flash induced absorbance increase in a reconstituted membrane preparation measured at 560 minus 540 nm. Eight signals were stored and averaged using an LSI-11 microprocessor linked to a DL901 transient recorder. Assay conditions were the same as for Fig. 1.

Fig. 3. The flash-induced absorbance decrease in a reconstituted membrane preparation measured at 551-540 nm. Signals were stored and averaged using a PDP-11 minicomputer linked to a Datalab DL905 transient recorder. The time constant on the differential amplifier was 10 μs full scale. A semi-logarithmic plot of the absorbance is shown; similar plots were obtained for chromatophore membrane preparations. The anaerobic cuvette contained in a total volume of 2.8 ml: 2.2 mg protein, 2.5 ml 20 mM Tris/100 mM KCl (pH 8.0), 10 mM sodium fumarate, 1 mM sodium succinate, 20 μg antimycin A.

TABLE I

A COMPARISON OF THE KINETICS OF FLASH INDUCED ABSORBANCE CHANGES OBSERVED IN RECONSTITUTED AND CHROMATOPHORE MEMBRANES

Signals were stored and averaged in a LS1-11 microprocessor linked to a DL901 transient recorder. Assay conditions as for Fig. 1 except for cytochrome c_2 photo-oxidation measurements as for Fig. 2.

Assay	Dialysed preparation of Ol membranes reaction centres	R-26 chromatophores	R-26 chromatophores treated with cholate and dialysed
Cytochrome c_2 photo-oxidation (551—540 nm)			
$t1/2$ first phase (μs) *	—	—	—
$t1/2$ second phase (ms)	53	1.1	1.8
% first phase	35	86	52
$t1/2$ cytochrome b photoreduction (ms) (Δ560 nm)	47	29	33
P-605 re-reduction (605—540 nm)			
$t1/2$ first phase (A) (ms)	27	32	26
$t1/2$ second phase (A) (ms)	639	1000	727

* Values not shown because of limitations imposed by instrument used.

TABLE II

A COMPARISON OF THE KINETICS OF FLASH INDUCED ABSORBANCE CHANGES IN RECONSTITUTED AND CHROMATOPHORE MEMBRANES WITH A FAST TIME RESOLUTION

Signals were stored and averaged in a PDP-11 minicomputer linked to a DL905 transient recorder. Assay conditions as for Fig. 3. Half-times were obtained from pseudo first order kinetic plots as in Fig. 3. Separate kinetic traces on a faster time scale were used to obtain approximate values for first phase half-times.

Assay	Dialysed preparation of Ol membranes reaction centres	R-26 chromatophores	R-26 chromatophores treated with cholate and dialysed
Cytochrome c_2 photo-oxidation (551—540 nm)			
(nmol per mg protein)	0.052	0.148	0.41
$t1/2$ first phase (μs)	≈20 **	≈20 **	≈20 **
$t1/2$ second phase (ms)	62	0.49	13
$t1/2$ cytochrome b photoreduction (ms) (560—570 nm)	n.d.	2.0	n.d.
% of P-605 change * re-reduced by cytochrome c_2	22	57	39

* Measured by the incremental increase of the P-605 absorbance decrease measured at 598-575 nm produced by the addition of 2 mM ferricyanide/2 mM ferrocyanide. We have assumed that the redox potential of the cuvette was around +440 mV. (E_m ferricyanide/ferrocyanide +440 mV) rendering half the reaction centres oxidised before the flash.

**These values were close to the instrumental limit.

n.d., not determined.

551 minus 540 nm was measured using a 10 μs time constant and the signal from 64 flashes averaged (Fig. 3 and Table II). Both the reconstituted membranes and R-26 chromatophores exhibited a biphasic absorbance decrease with a first phase half-time near 20 μs. These microsecond values were close to the instrumental limit but are less than 20 μs. The effect of cholate treatment on the cytochrome c_2 reactivity is manifested in the variable kinetics of the second phase; dialysed chromatophores behave in a manner intermediate between that of untreated chromatophores and dialysed reconstituted membranes (see Table II).

The kinetics of re-reduction of photo-oxidised reaction centres in reconstituted membanes (Fig. 4) suggest that about 30% of the reaction centres were not directly re-reduced by cytochrome c_2 even on the slow time scale shown. The ratio of total cytochrome c_2 to cytochrome b calculated from difference spectra shows that some c_2 is lost from membranes when cholate is initially added to the reconstitution mixture. The fast phase of reaction centre re-reduc-

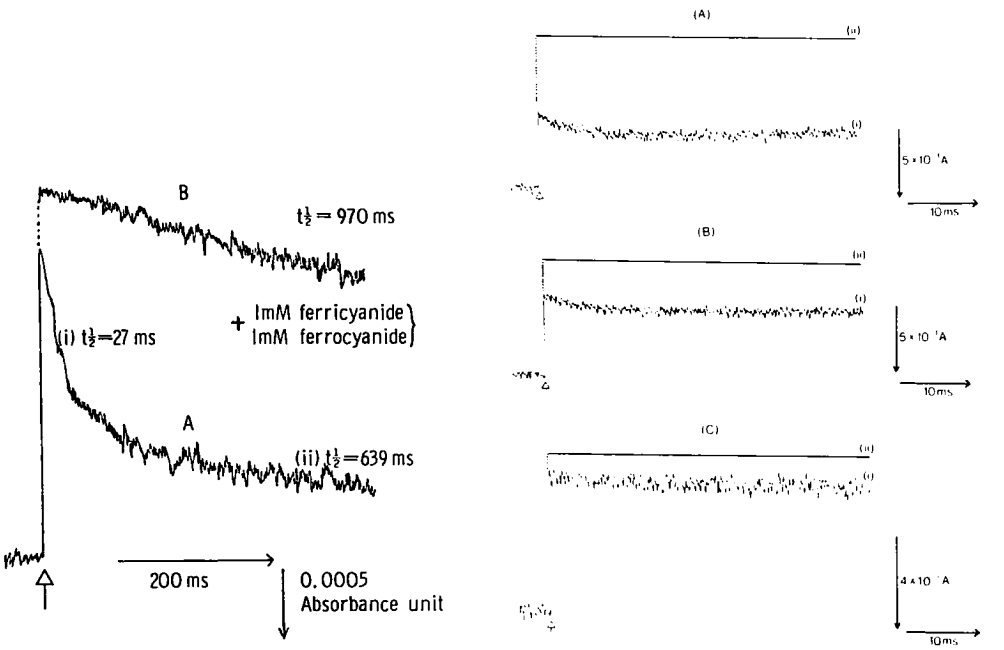


Fig. 4 The flash induced absorbance decrease and its recovery in a reconstituted membrane preparation measured at 605 minus 540 nm. Eight signals were stored and averaged using an LSI-11 microprocessor linked to a DL901 transient recorder. Trace B differs from Trace A in that 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide were included in the final cuvette mixture. The kinetics of trace A were resolved into two pseudo first-order kinetic traces using semi-logarithmic plots. Assay conditions are the same as for Fig. 1.

Fig. 5. Flash induced *P*-605 absorbance decreases measured at 598-575 nm. A, R-26 chromatophores. B, R-26 chromatophores treated with 1% sodium cholate and dialysed as in usual reconstitution procedure. C, dialysed preparation of 01 membranes and reaction centres. The membranes were assayed in 10 mM Tris/100 mM KCl (pH 8.0) containing: (i) 10 mM sodium fumarate/1 mM sodium succinate; (ii) 2 mM potassium ferricyanide/2 mM potassium ferrocyanide added; corrected for *P*-605 being 50% oxidised before the flash. Assay conditions and averaging of signals same as for Fig. 3.

tion shows similar kinetics to the second phase of cytochrome c_2 photo-oxidation; a third, slow component presumably reflects the re-reduction of reaction centres by components other than cytochrome c_2. The level of P-605 photo-oxidation increased upon addition of ferri/ferrocyanide to oxidise all the c_2 in the membrane before the flash, suggesting that a fast phase of electron transfer between c_2 and P^+ ($t_{1/2} < 1$ ms) has been missed by using a long time scale to measure the kinetics of the second and third phases of P^+ re-reduction. Cholate treated R-26 chromatophores give quantitatively similar results suggesting some dislocation or removal of cytochrome c_2 by cholate. Untreated chromatophores, too, always showed a variable amount of this third phase suggesting that the method of preparation produced some disrupted c_2 reaction centre units.

In Fig. 5, each solid line represents the absorbance decrease at 605 minus 540 nm calculated from a trace obtained with the cuvette contents poised at a redox potential of +430 mV using equimolar potassium ferro- and ferricyanide.

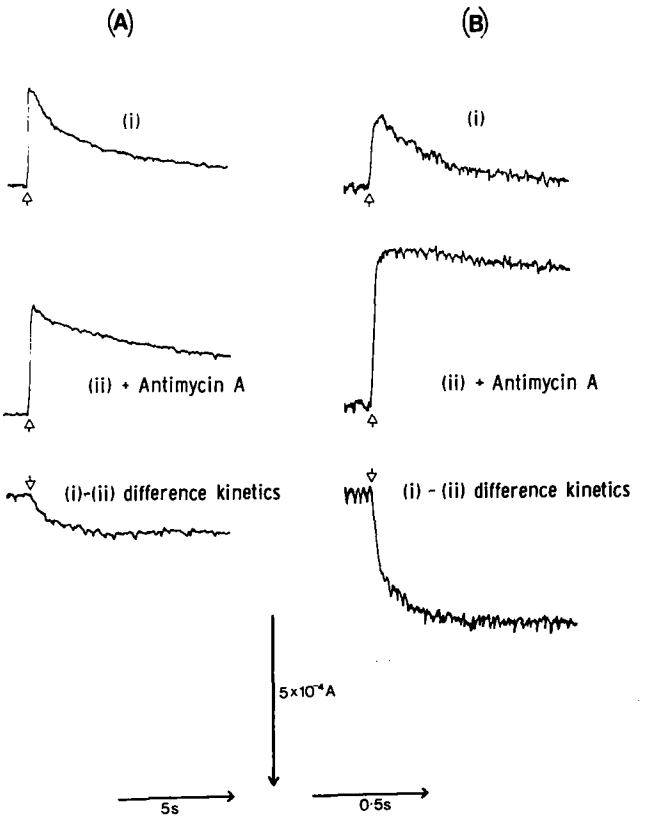


Fig. 6. Flash-induced absorbance changes at 551—540 nm in (A) 01 membranes reconstituted with reaction centres and (B) R-26 chromatophores. The signals from an unchopped dual wavelength spectrophotometer were stored, averaged and subtracted in an LSI-11 microprocessor linked to a DL901 transient recorder. In each case the anaerobic cuvette contained approx. 4 mg protein, 3.0 ml 20 mM MOPS/100 mM KCl buffer (pH 7.0), 4 mM sodium fumarate, 1 mM sodium succinate. 20 μg antimycin A were added where indicated.

We have assumed that at this potential 50% of the reaction centres were oxidised before the flash. Each trace below the solid line was obtained in the absence of the ferro/ferricyanide couple. The traces show that membranes reconstituted with reaction centres have quantitative differences in properties when compared with either cholate-treated dialysed chromatophores or untreated chromatophores. About 60% of photo-oxidised reaction centres in chromatophore membranes are rapidly re-reduced by an electron from cytochrome c_2; this proportion becomes approx. 40% after treatment with 1% sodium cholate and dialysis to remove the detergent. Only about 20% of reconstituted reaction centres appear to be rapidly re-reduced by 01 membrane cytochrome c_2 according to this criterion. The results of this experiment are summarized in Table II.

The final property to be examined in a cyclic reconstituted system is electron flow between cytochrome b and c, and the effect upon it of antimycin A, which normally greatly slows down electron transfer between cytochrome b_{+50} and cytochrome c_2 [6]. A comparison between the properties of chromatophores and reconstituted membranes can be seen in Fig. 6.

The succinate-fumarate mixture was used to set the redox potential in the

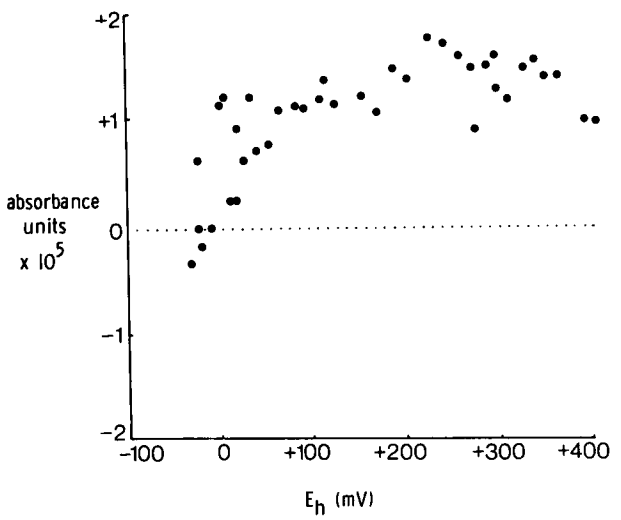


Fig. 7. Variations in light-induced steady-state absorbance changes at 560 minus 570 nm with change in ambient redox potential. The anaerobic redox cuvette contained approx. 16 mg 01 membrane in 4.5 ml 20 mM MOPS/100 mM KCl (pH 7.0). The mediators phenazine methosulphate, phenazine ethosulphate, 2-hydroxy-1,4-naphthoquinone, pyocyanine, potassium ferricyanide, 2,3,5,6-tetramethyl-p-phenylenediamine, all at 3 μM. The ambient potential in the cuvette, monitored using calomel and platinum electrodes, was adjusted by the injection of small aliquots of sodium dithionite or potassium ferricyanide solution. The membrane suspension was illuminated at various E_h values. At the onset of steady-state illumination from a tungsten filament light filtered through a Kodak Wratten far-red filter (No. 88A), any resulting absorbance changes at 560—570 nm were monitored in a dual wavelength spectrophotometer. The 01 membranes had been prepared as described in Methods, except that after resuspension in buffer they were layered onto 30 ml 1.2 M sucrose, 10 mM EDTA (pH 7.5) and centrifuged for 3.5 h at 75 000 × g. A clear brown membranous fraction was collected from the top few millilitres of sucrose, having been freed from contaminating cell envelope material which had sedimented as a colourless pellet. The reconstitution was as described in Methods; membranes and reaction centres were mixed according to the ratio 2 mol cytochrome c_2 per reaction centre.

cuvette at around +50 mV. Under these conditions the half-time of re-reduction of cytochrome c_2 in coupled R-26 chromatophores following a flash was observed to be 65 ms. In the reconstituted system cytochrome c_2 re-reduction was slower, and showed less sensitivity to antimycin A. Again, as found for cytochrome *b* reduction, the kinetics of cytochrome c_2 re-reduction were rather variable, though it appears that there are clear differences between electron flow to cytochrome c_2 in the two preparations. Possible explanations will be discussed later.

The absorbance increase at 560 minus 570 nm following steady-state illumination with far-red light was measured over a range of ambient potential from −40 to +400 mV (Fig. 7). Despite the scatter of points, it can be clearly seen that the photoreduction of a *b*-type cytochrome decreased over the range −20 to +100 mV, which one could expect if reconstituted reaction centres were

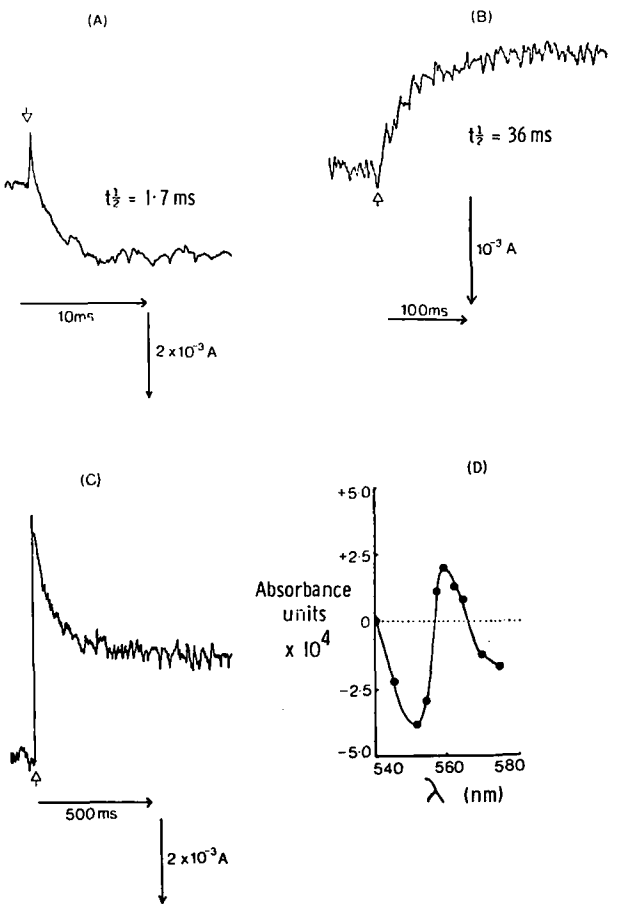


Fig. 8. Flash induced absorbance changes in membranes isolated from aerobically grown PM8bg-II-15 after addition of reaction centres and sodium cholate followed by dialysis to remove the detergent. A, 560 minus 540 nm; B, 551 minus 540 nm; C, 605 minus 540 nm; D, is a light minus dark spectrum, the points having been calculated as for Fig. 1. Signals were stored and averaged in an LSI-11 microprocessor linked to a DL901 transient recorder. The conditions in the cuvette were the same as for Fig. 1.

photoreducing a cytochrome *b* in the 01 membrane with a mid-point potential of around +50 mV.

Membranes were prepared from the reaction centre-less mutant PM8bg II-15 in an attempt to reconstitute the same light driven reactions as in 01 membranes. The results of illuminating a reconstituted preparation obtained by dialysing a mixture of membranes, reaction centres and 1% sodium cholate are shown in Fig. 8.

Following a single flash cytochrome c_2 becomes photo-oxidised rather slowly with a single phase having a half-time of 36 ms (Fig. 8b). Cytochrome *b* is rapidly photoreduced in 1.7 ms (Fig. 8a), which would be expected if the reaction centre has integrated very well into the membrane to pass an electron from the primary acceptor X to the membrane bound cytochrome. The slow electron transfer from c_2 to re-reduce P^+ is also reflected in the slow recovery kinetics of the 605—540 nm absorbance decrease (Fig. 8c). The absorbance changes at a number of wavelengths using 540 nm as reference, are plotted out in Fig. 8d and show that both *b* and *c*-type cytochromes are involved in reconstituted light driven reactions.

Discussion

Our results have shown that unpigmented membranes isolated from aerobically grown cells containing the cytochromes necessary for cyclic photosynthetic electron flow [2] will bind reaction centres so that light induced electron flow is reconstituted. Under conditions of flash illumination the kinetics of the absorbance changes in the reconstituted system exhibit some properties similar to those of chromatophores isolated from photosynthetically grown cells of *R. sphaeroides*. The differences in behaviour of the two systems can be attributed to incomplete reconstitution of reaction centres in the membrane. The kinetics of cytochrome *b* photoreduction were not consistent in different chromatophore preparations. The half-time of this reaction (2 ms in R-26 chromatophores) was sometimes as fast as reported by previous workers [9] but was often much slower. Variability in the kinetics of cytochrome *b* photoreduction can be a consequence of inconsistency in maintaining the ambient redox potential in the cuvette. The half-time of reduction of cytochrome *b* in R-26 chromatophores was often observed to be of the order of 20—30 ms. Petty and Dutton [12] have concluded that this parameter is dependent on the state of oxidation-reduction of P/P^+ following a single flash, being slower when some P^+ is present. In an 'ideal' chromatophore in which efficient electron transfer from cytochrome c_2 to P^+ is manifested as a precise kinetic matching of cytochrome c_2 photo-oxidation and P^+ re-reduction [5], the 1 ms after a flash eliciting a single turnover of the system most of the population of P^+ in the membrane will have reverted to P. 5 ms later the extent of cytochrome b_{+50} reduction ($t_{1/2}$ 1—2 ms) will be at its maximum. If some P^+ persists into this 5 ms period because of slow $c_2 \rightarrow P^+$ electron transfer due to disruption of c_2/p units during French pressing or cholate treatment, one would expect slow cytochrome b_{+50} reduction. This is an explanation for the inconsistency in our chromatophore measurements, and the consistently slow result in the reconstituted system. Fig. 6 illustrates that P^+ units must persist in reconstituted membranes for as

long as 5 s after a flash. Slow kinetics in reconstituted membranes ($t_{1/2}$ 27 ms) may also be a consequence of the reaction centres being incorrectly oriented in the membrane. It is interesting to note however, that 01 membranes contain approx. 6 quinone molecules per total cytochrome *b* compared to 20 quinones per total cytochrome *b* in R-26 chromatophores. It is therefore possible that the relatively low antimycin sensitivity of cytochrome c_2 re-reduction and the slow rate of cytochrome *b* reduction are due to a lack of quinone mediating electron transfer between reaction centres, cytochrome b_{+50} and cytochrome c_2 in the reconstituted system. Studies on the quinone-depleted and quinone-reconstituted membranes in *R. capsulata* by Baccarini-Melandri and Melandri [10] have demonstrated that electron transfer and antimycin sensitivity between cytochrome b_{+50} and cytochrome c_2 require the presence of quinone in the membrane.

Recently, a thorough investigation of electron transfer in the $b_{+50} \rightarrow c_2$ segment of the cyclic system in *R. sphaeroides* [11] has yielded some evidence for ubiquinone participating in electron and proton transfer reactions between cytochromes *b* and c_2.

Another explanation for slow cytochrome c_2 re-reduction in the reconstituted system following a flash is that the cyclic pathway has been incompletely reconstituted and the cytochrome c_2 may be partly reduced by electrons from cytochrome b_{+155} in the linear respiratory chain. This cytochrome can act as a donor to cytochrome c_2 in photosynthetic membranes although its rate of re-reduction from succinate is very slow ($t_{1/2}$ = several seconds) [9]. It has been proposed to act as a non-cyclic substrate-linked supplier of reducing equivalents to the cycle (ref. 12; see also ref. 13 for a review). Electron flow along this pathway can be measured as succinate : cytochrome *c* reductase activity. This has proved to be extremely labile in the presence of 1% sodium cholate (Hunter, C.N. and Jones, O.T.G., unpublished results). After 6 h dialysis to remove the cholate the succinate : cytochrome *c* reductase activity decreases at least 10-fold. Such results make it unlikely that cytochrome *b*+155 re-reduces photo-oxidised cytochrome c_2 in the reconstituted membrane even on the slow time scale employed (10 s full scale).

Photoreduction of cytochrome *b* and cytochrome c_2 photo-oxidation are stimulated in the presence of antimycin A, suggesting that we have reconstituted a cyclic system; lack of quinone may explain the discrepancies between reconstituted and 'natural' chromatophores.

We have experienced some difficulty in preparing chromatophores from the R-26 mutant which are as kinetically competent as those reported by Dutton and co-workers for chromatophores from *R. sphaeroides* Ga [5]. Only about 25% of the cytochrome c_2 becomes photo-oxidised after a flash and the number of functional units as judged by the c_2/*P*-605 ratio which should be >1 (see Table II) is slow. We have taken precautions to guard against the production of disrupted c_2-*P*-605 units by carefully controlling the temperature of the cells while being passed through the French press so that no rise above 15°C was observed, and also pressing once only to avoid overdisruption of chromatophore vesicles. It is therefore not unreasonable to suggest that the controlled addition of reaction centres into membranes prepared from French press extracts of aerobically grown cells will produce reconstituted membranes

which are subject to the same kinetic limitations as chromatophore preparations.

Cytochrome c_2 photo-oxidation was observed to be biphasic with a first phase half-time in good agreement with the kinetics observed with R-26 chromatophores. The second phase was very variable but always considerably slower than in chromatophores. Studies on cytochrome c_2 photo-oxidation by detergent solubilised reaction centres showed monophasic kinetics with a half time of ≈ 25 μs [14,15]. Dutton et al. [16], too, reported biphasic kinetics as externally added cytochrome c_2 was photo-oxidised by reaction centres incorporated into phospholipid vesicles, a system more analogous to our reconstitution. Our attempt to discover which of several cytochromes *b* with different mid-point potentials [15] in the aerobic membrane was being photoreduced by reconstituted reaction centres showed that the chemical reduction of a component between +120 and −20 mV gradually diminished the light-induced reduction. All mediators were 3 μM to minimise any effect of short circuiting the cyclic electron flow. Although Saunders and Jones [17] found five *b*-type cytochromes in aerobically grown *R. sphaeroides*, the two high potential cytochromes *b*-+390 and *b*-+255 could function as oxidases and *b*-90 may transfer electrons to NADH as part of a 'reversed' electron transfer. Cytochromes *b*+155 and *b*+45 appear to be the only two candidates for photoreduction; both have been implicated in light stimulated electron flow in this bacterium [6]. It appears from our potentiometric titration (Fig. 7) that under steady-state illumination at least, *b*+45 is the major *b*-type cytochrome participant in reconstituted electron flow. The slow re-reduction of cytochrome c_2 following a single flash has already been discussed, but the participation of *b*+155 in this reaction cannot be ruled out.

In summary the results suggest that unpigmented membranes from aerobically grown cells of *R. sphaeroides* have the potential for form chromatophore membranes by incorporation of the photochemical apparatus into the membrane structure in the correct orientation.

Our attempts to reconstitute reaction centres into membranes from the mutant PM8bg II-15 were less successful than for 01. However, fast kinetics ($t_{1/2} = 1.7$ ms) of reconstituted cytochrome *b* photoreduction were observed and slow, monophasic kinetics of cytochrome *c* photo-oxidation were reconstituted; such a fast half-time of cytochrome *b* photoreduction was never observed with the 01 membrane reaction centre system. A completely successful interaction of reaction centre and cytochrome *b* in the membrane can explain the fast kinetics in PM8bg II-15 membranes. The mutant PM8bg II-15 possesses one chlorophyll peak only at 860 nm in the far-red region of the spectrum; preliminary results suggest that PM8-type mutants will provide membrane preparations useful for studying energy transfer from the endogenous light-harvesting pigment-protein complexes to reconstituted reaction centres.

Acknowledgements

We are grateful to Dr. A.R. Crofts for the use of his spectrophotometer. C.N.H. was supported by a Science Research Council Studentship. We acknowl-

edge the invaluable technical assistance of Mrs. E. Burd and Mrs. J. Fielding. This work was supported by a Grant from the Science Research Council.

References

1 Peters, G.A. and Cellarius, R.A. (1972) J. Bioenerg. 3, 345—359
2 Connelly, J.L., Jones, O.T.G., Saunders, V.A. and Yates, D.W. (1973) Biochim. Biophys. Acta 292, 644—653
3 Hunter, C.N. and Jones, O.T.G. (1978) Biochim. Biophys. Acta
4 Hunter, C.N. and Jones, O.T.G. (1976) Biochem. Soc. Trans. 4, 669—670
5 Dutton, P.L., Petty, K.M., Bonner, H.S. and Morse, S.D. (1975) Biochim. Biophys. Acta 387, 536—556
6 Sistrom, W.R. (1960) J. Gen. Microbiol. 22, 778—785
7 Clayton, R.K. and Wang, R.T. (1971) in Methods in Enzymology (San Pietro, A., ed.), Vol XXIII, pp. 696—704, Academic Press, New York
8 Jones, O.T.G. and Plewis, K.M. (1974) Biochim. Biophys. Acta 374, 204—214
9 Dutton, P.L. and Jackson, J.B. (1972) Eur. J. Biochem. 30, 495—510
10 Baccarini-Melandri, A. and Melandri, B.A. (1977) FEBS Lett. 80, 459—464
11 Prince, R.C. and Dutton, P.L. (1977) Biochim. Biophys. Acta 462, 731—747
12 Petty, K.M. and Dutton, P.L. (1976) Arch. Biochem. Biophys. 172, 346—353
13 Dutton, P.L. and Prince, R.C. (1978) in The Photosynthetic Bacteria (Clayton, R.C. and Sistrom, W.R., eds.), Plenum Press, New York, in the press
14 Ke, B., Chaney, T.H. and Reed, D.W. (1970) Biochim. Biophys. Acta 216, 373—383
15 Prince, R.C., Cogdell, R.J. and Crofts, A.R. (1974) Biochim. Biophys. Acta 347, 1—13
16 Dutton, P.L., Petty, K.M. and Prince, R.C. (1976) Fed. Proc. 35, 1596
17 Saunders, V.A. and Jones, O.T.G. (1975) Biochim. Biophys. Acta 396, 220—228