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THE INCORPORATION OF REACTION CENTRES INTO MEMBRANES FROM A BACTERIOCHLOROPHYLL-LESS MUTANT OF *RHODOPSEUDOMONAS SPHAEROIDES*

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Summary

Reaction centres purified from a blue-green mutant R-26 of *Rhodopseudomonas sphaeroides* can be incorporated into bacteriochlorophyll-less membranes purified from an aerobically-grown bacteriochlorophyll-less mutant 01 of *R. sphaeroides*. This can be accomplished by raising the temperature of the mixture or by addition of the detergent sodium cholate and its subsequent removal by dilution or dialysis. Optimum conditions for the reconstitution are at 4°C in the presence of 1% cholate and soybean phospholipid (2 : 1, w/w, with membrane protein). Isopycnic sucrose density gradient centrifugation of such preparations shows that reaction centres and light-harvesting pigment-protein complex bind to the membranes. Reconstituted membranes exhibit light-induced steady-state cytochrome absorbance changes resembling those observed in chromatophores prepared from the photosynthetically-grown mutant R-26. The effect on these absorbance changes of varying reaction centre content in the membrane has been studied, and the time course of the interaction between 01 membrane cytochrome c_2 and added reaction centre examined.

Cytochrome *b* photoreduction and cytochrome c_2 photo-oxidation were observed in the reconstituted preparation; each increased following the addition of antimycin A, suggesting that a cyclic light-driven system had been reconstituted.

Introduction

The photosynthetic bacterium *Rhodopseudomonas sphaeroides* can grow aerobically in the dark. Membranes from such cells contain the same *b* and

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Abbreviations: MOPS, 3-(*N*-morpholino)propanesulphonic acid; B-875, B-870, B-800 + 850 pigmented components of the photosynthetic apparatus identified by their absorption maxima in the far-red region.

c-type cytochromes found in chromatophores from photosynthetically-grown cells [1] but lack reaction centre and light-harvesting polypeptides [2]. Aerobic cells [3—5] do not contain the pigmented intracytoplasmic membranes found in photosynthetically-grown cells [6,7]; these may arise at the cytoplasmic membrane [8] during adaptation to photosynthetic growth at the onset of anaerobiosis. Studies on the rate of oxidation of reduced cytochromes on oxygenating anaerobic suspensions of membranes prepared from aerobically or photosynthetically-grown cells shows that two different *b*-type cytochromes were oxidised in each membrane sample. The half-times of oxidation correspond closely in the two membranes [1] suggesting that respiratory electron transport in *R. sphaeroides* may have the same components and orientation in both aerobically and photosynthetically grown cells and that unpigmented membranes may carry out photosynthetic reactions if the photoreactive pigment-proteins could be successfully reconstituted into the membranes.

In our experiments the aerobic mutant 01 of *R. sphaeroides* was used; this mutant is incapable of adaptation to photosynthetic growth and excretes chlorophyll-like pigments into the growth medium because of a block in bacteriochlorophyll synthesis [9]. The cytochrome content and respiratory activity of membranes from 01 resemble those of wild-type *R. sphaeroides*. After disruption of 01 cells in a French pressure cell an unpigmented vesicle fraction of similar density to chromatophores is obtained by differential centrifugation. Addition of purified reaction centres to such membranes, with reconstitution of some light-induced steady-state cytochrome absorbance changes has already been reported [9]; reaction centres were solubilised in lauryldimethylamine oxide which was subsequently removed by controlled reduction of the lauryldimethylamine oxide, with resulting loss of detergent activity. The success of Racker and co-workers in reconstituting various mitochndrial enzyme complexes, or bacteriorhodopsin, into phospholipid vesicles suggested that the detergent sodium cholate may be more useful than lauryldimethylamine oxide in our system because of the ease with which its concentration can be reduced by either dialysis [10—14] or dilution [15].

In this paper we present experiments demonstrating the factors affecting the binding of reaction centres to 01 membranes by the addition and subsequent removal of the detergent sodium cholate by dialysis or dilution. We also describe some light-induced steady-state reactions in these reconstituted systems. The following paper deals with the kinetics of flash induced electron flow in such reconstituted preparations.

Methods

Preparation of membranes from the aerobic bacteriochlorophyll-less mutant 01. The mutant 01 was prepared and grown as described previously [9]. 01 cells were washed and resuspended in 20 mM MOPS, 100 mM KCl buffer, pH 7.0. After two passages through a French pressure cell at 18 000 lb/inch^2, the suspension was centrifuged at 30 000 $\times g$ for 25 min. The clear brown supernatant was carefully removed and centrifuged at 104 000 $\times g$ for 90 min. The clear membranous pellet was resuspended in buffer.

Preparation of reaction centres. Reaction centres were purified from mem-

branes prepared from photosynthetically-grown cells of R-26, the blue-green mutant of *R. sphaeroides*, by extraction with the detergent lauryldimethylamine oxide and ammonium sulphate levitation [16]. The final levitate containing purified reaction centres was dissolved in 10 mM Tris-HCl (pH 7.5) containing 2% sodium cholate. The estimation of the concentration of the reaction centres in solution was as described previously [9]. No cytochromes of the *b* or *c*-types were detected in the preparation by difference spectroscopy, and it gave no reaction with antibody to *R. sphaeroides* cytochrome c_2. Sodium dodecyl sulphate-treated reaction centres showed only the Coomassie-staining three bands observed by Clayton, Feher and others [17,18] following electrophoresis on polyacrylamide.

Preparation of light-harvesting pigment protein. A green mutant of *R. sphaeroides*, designated GVP, was grown photosynthetically and the cells disrupted by treatment in a French pressure cell. Chromatophores purified by sucrose density gradient centrifugation were treated with lauryldimethylamine oxide and Triton X-100 to extract light-harvesting complex as described by Clayton and Clayton [19]. The final preparation was dialysed for three days against 4 l 10 mM Tris-HCl, pH 7.5, and dissolved in 10 mM Tris-HCl, pH 7.5, containing 2% sodium cholate.

Spectroscopy. Steady-state light-induced cytochrome changes were measured in a dual wavelength spectrophotometer with the photomultiplier protected by a filter of saturated copper sulphate and the actinic light masked by a Kodak-Wratten far-red filter (No. 88A). The measuring light beams were chopped at 200 cycles/s.

Flash-induced changes were measured in an unchopped dual wavelength spectrophotometer with saturating flashes of about 40 microsecond duration from a xenon lamp passed through a Kodak-Wratten far-red filter (No. 88A). Both multipliers were protected by a Corning blue glass filter (No. 9782).

SDS-polyacrylamide electrophoresis. The gel buffer system used was 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, adjusted to pH 7.1 with glacial acetic acid, and containing 0.1% SDS. A gel was cast as a slab between two glass plates with a gradient of 6—20% polyacrylamide and a stacking gel containing 4% acrylamide. Samples containing about 100 μg protein were treated with SDS to a final concentration of 1% [17] and applied to the gel which was supported vertically with a buffer tank at each end. Gels were stained in 0.25% Coomassie blue dissolved in 7.5% acetic acid/5% methanol and destained 60°C for 16 h by immersion in 7.5% acetic acid/5% methanol [20,21].

Protein assays. Protein concentrations were determined according to the method of Bramhall et al. [22].

Results

SDS-polyacrylamide gel electrophoresis of 01 membranes

SDS-polyacrylamide gel electrophoresis (Fig. 1) yielded no clear evidence for the presence of reaction centre subunits in 01 membranes, especially in view of the fact that the reaction centre-less mutant PM8bg II-15 possesses faint bands in this region of the gel. An interesting observation resulting from the increased resolution of the gradient gel is that the carotenoid-containing light-harvesting

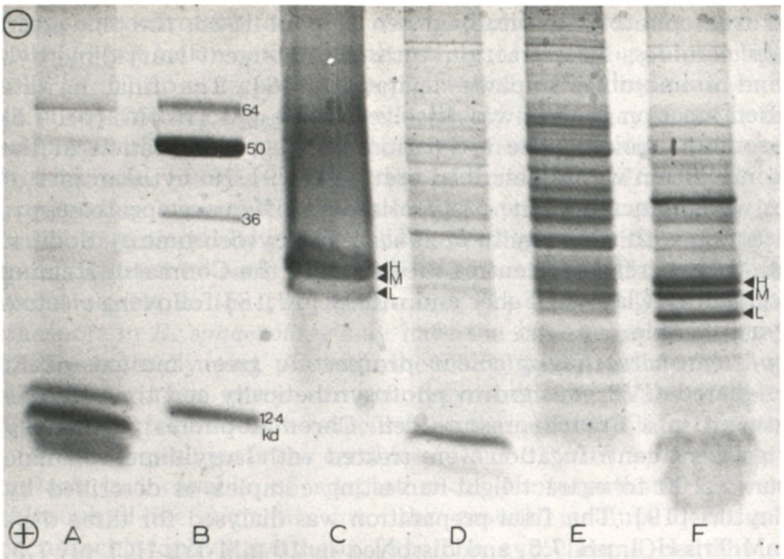

Fig. 1. SDS-polyacrylamide gel electrophoresis of purified membranes and pigment proteins from *R. sphaeroides*. Samples containing approx. 70 μg protein were applied to a 4% acrylamide stacking gel layered on a gel containing a 6—20% continuous gradient of acrylamide. After running at 10 V/cm for 6 h, the gel was stained with 0.25% Coomassie brilliant blue R250 dissolved in destainer and destained with 7.5% glacial acetic acid, 5% methanol. The samples were as follows: A, light harvesting complex (B-800 + 850) purified from photosynthetically-grown GVP. B, standard proteins, bovine plasma albumin (64 000 daltons), glutamate dehydrogenase (subunit 50 000 daltons), lactate dehydrogenase (subunit 36 000 daltons), bovine cytochrome *c* (12 400 daltons). C, reaction centres purified from photosynthetically-grown R-26. D, purified membrane fraction from mutant PM8bg II-15. E, purified membrane fraction from mutant 01. F, purified chromatophore membranes from photosynthetically-grown R-26.

complex prepared from the mutant GVP is found to contain three Coomassie-staining bands whereas the same electrophoretic gel region of the carotenoid less mutant R-26 contains only two clearly distinguishable bands. 01 lacks a light-harvesting complex whereas membranes from the carotenoid-less reaction centre-less mutant PM8bg 11-15 of *R. sphaeroides*, a gift from Dr. W.R. Sistrom, show a light-harvesting pigment-protein region similar to that of R-26. A far-red spectrum of membranes prepared from this mutant showed a single absorption maximum at 870 nm, suggesting that this mutant has the same pigments as R-26 but lacks reaction centres and thus has only the accessory B-870 light-harvesting component. The B-800 + 850 light harvesting complex found in wild type cells is absent. In our experiments there was little contamination of membrane samples by ribosomes, since all samples were purified by centrifugation in a discontinuous gradient and collected from the interface of a 1.0—1.2 M sucrose step after pre-treatment with 10 mM EDTA (pH 7.5).

Binding of reaction centres to 01 membranes

Varying amounts of reaction centres were added to a mixture of 01 membranes and sonicated soya bean lecithin suspension (2 : 1, w/w, lecithin to 01 protein), suspended in 1% sodium cholate. This mixture was incubated at 4°C for 30 min, diluted 25-fold with 20 mM MOPS, 100 mM KCl pH 7.0 and cen-

trifuged at 100 000 X *g* for 90 min. The pellet was resuspended in buffer and assayed for light-induced cytochrome absorbance changes in the chopped dual wavelength spectrophotometer and for the amount of bound reaction centre (Fig. 2a and b). Alternatively the mixture was dialysed overnight against a total of 4 l buffer, the diffusate sedimented as above and assayed as above (Fig. 3a and b). Reaction centre assays showed that neither method of reconstituting reaction centres into membranes resulted in a saturation of the membrane with bound reaction centre (results not shown). A constant percentage of added reaction centre was bound to 01 membranes using the cholate dilution (approx.

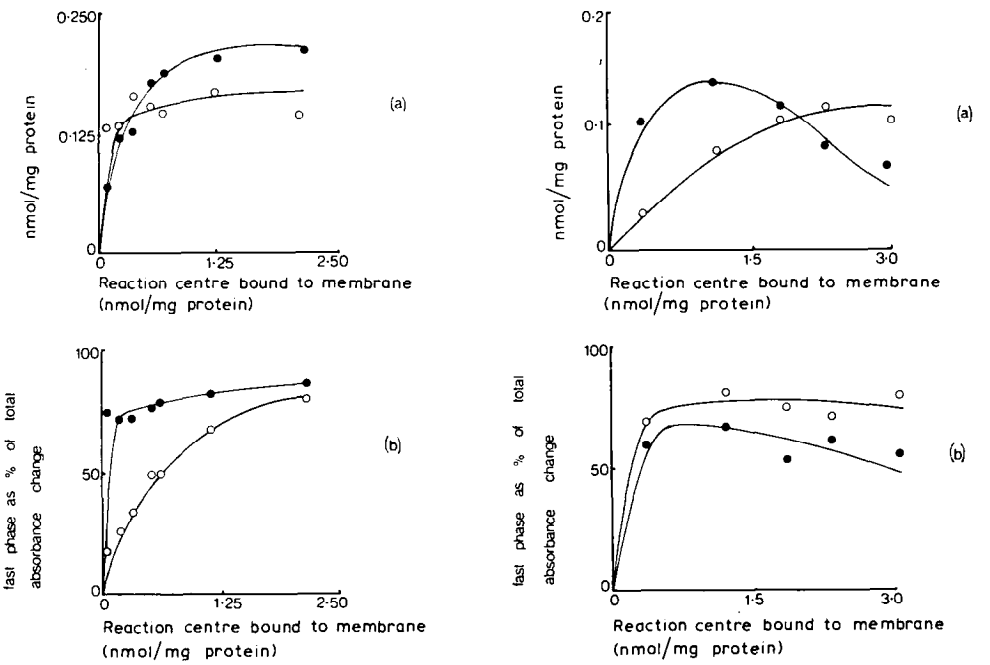

Fig. 2. The effect of the addition of increasing amounts of reaction centre on the properties of 01 membranes. Cholate removed by dilution and sedimentation of membranes followed by resuspension in buffer containing no cholate. Carotenoid-less reaction centres were mixed with 10 mg 01 membrane protein, 20 mg sonicated soybean phospholipid and sodium cholate to a final concentration of 1%. After 30 min incubation at 4° C and 25-fold dilution with 20 mM MOPS/100 mM KCl (pH 7.0), the diluted mixture was centrifuged at 100 000 X *g* for 90 min and the pellet resuspended in buffer. The aerobic suspensions were assayed in a chopped dual wavelength spectrophotometer in a cuvette containing 2.5 ml 20 mM MOPS/ 100 mM KCl (pH 7.0), 2 mM sodium ascorbate and 100 μg antimycin A. (a), the variation of cytochrome *b* photoreduction (○) and cytochrome c_2 photo-oxidation (●) (nmol/mg protein) measured at 560—570 nm and 551—540 nm, respectively, with added membrane-bound reaction centre on continuous illumination. (b), the fast phases from (a) for each absorbance change as a percentage of the total absorbance change at 560—570 nm (○) and 551—540 nm (●) as it varies with membrane-bound reaction centre content (nmol/mg protein).

Fig. 3. The effect of the addition of increasing amounts of reaction centre on the properties of 01 membranes. Cholate removed by dialysis and sedimentation of membranes followed by resuspension in buffer containing no cholate. Carotenoid-less reaction centres were mixed with 10 μg 01 membrane protein, 20 mg sonicated soybean phospholipid and sodium cholate to a final concentration of 1%. The mixture was dialysed for 15 h against a total of 4 l 20 mM MOPS/100 mM KCl (pH 7.0), sedimented at 100 000 X *g* for 90 min and the pellets resuspended in buffer. The aerobic cuvette contents were assayed under the same conditions as for the dilution method in Figs. 2a and b. Details as in Fig. 2a and b.

75%) or dialysis methods (approx. 95%) resulting in a linear increase of membrane-bound reaction centre content. The reaction centre content of photosynthetically-grown R-26 was found to vary between 0.7 and 1.0 nmol/mg protein using our assay. As the amount of reaction centre bound is increased, cytochrome c_2 photo-oxidation and cytochrome *b* photoreduction per mg of 01 membrane protein increase to a level comparable to that found in chromatophores from the photosynthetically-grown R-26 mutant. Addition of more reaction centre causes no further increase in the amount of reconstituted light-driven electron flow observed. On illumination, traces of cytochrome c_2 photo-oxidation and cytochrome *b* photoreduction appeared biphasic, having a fast rise followed by a pronounced slow phase. The separation of these phases was achieved without using logarithmic kinetic plots, so was not precise. However, because a clear difference in kinetics was noticed with increasing reaction centre binding, the fast phase was plotted as a percentage of the total change in Figs. 2b and 3b.

A sample of the dialysed reconstituted membranes was applied to a linear 10—50% sucrose gradient and centrifuged in a swinging bucket rotor (Fig. 4). 01 membranes either alone or reconstituted with reaction centres, showed two bands in the gradient of density 1.0592 and 1.0992 g/ml, respectively; in both reconstituted bands cytochrome c_2 photo-oxidation could be measured. Addition of a light-harvesting pigment-protein complex and reaction centres to 01 membranes in the reconstitution mixture followed by sucrose density gradient centrifugation, resulted in most of the membranous material moving to a region of the gradient at $d = 1.1082$ g/ml. The extent of cytochrome c_2 photo-oxidation per bound reaction centre in this band was observed to be increased when light-harvesting pigment was present in the reconstituted membrane. We have previously briefly reported that such a complex, reconstituted with reaction centres and light-harvesting pigment protein shows enhanced light induced electron flow at low light intensities compared with membranes reconstituted with reaction centre and no light-harvesting complex [23].

Factors affecting reconstitution of reaction centres into 01 membranes

The cholate dilution technique allows the interaction of reaction centres with 01 membranes to be examined under varying conditions. Suspensions of pure photo-oxidised reaction centres in buffer are re-reduced slowly by the return of an electron back from the reduced primary acceptor which is present in such reaction centres [24]. Addition of reduced cytochrome c_2 or mammalian cytochrome *c* shortens the half-time of re-reduction to less than 1 ms [25,26]. We have used measurements of the half-time of reaction centre re-reduction to study the influence of period of incubation, cholate concentration and incubation temperature on the effectiveness of reaction centre binding to 01 membranes.

Table I shows that the rate of recovery of a flash induced *P*-605 absorbance decrease was speeded up by the addition of 01 membranes to reaction centres. The only cholate present was that used to solubilise the reaction centres. The kinetics of the reaction were monophasic with a half-time of 53 ms. No steady-state cytochrome *b* photoreduction was observed under these conditions. In the experiment illustrated in Table II membranes and reaction centres were

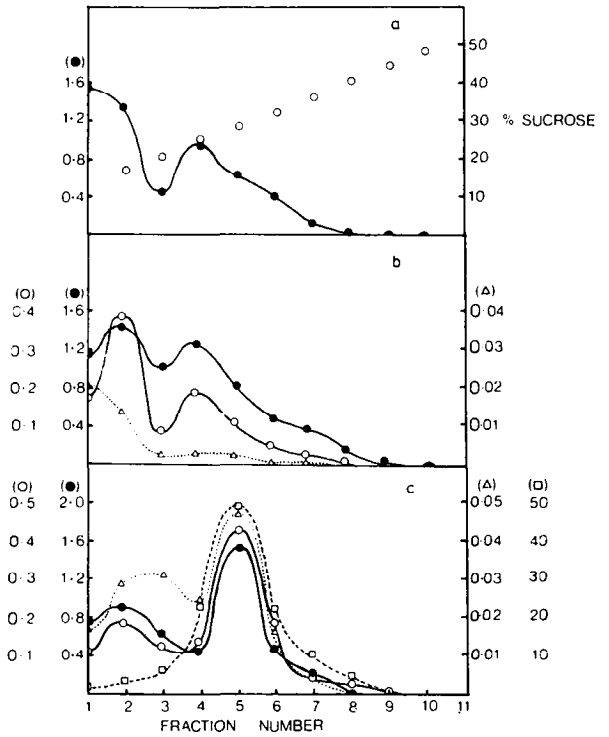

Fig. 4. Isopycnic density gradient centrifugation of reconstituted membranes. 01 membranes were reconstituted with reaction centres (0.7 nmol added per mg 01 membrane protein) as for Fig. 3. Samples containing approx. 5 mg protein were layered on a sucrose gradient (12 ml, 10—50%, w/w) prepared with distilled water and centrifuged in a swing-out rotor for 14 h at 75 000 × *g*. 1-ml samples were carefully removed using a Pasteur-pipette and analysed for protein (●) and bacteriochlorophyll (□). Cytochrome c_2 photo-oxidation (△) and reaction centre content (○) were assayed using an unchopped dual wavelength spectrophotometer, at 551—540 nm and 598—575 nm, respectively. All manipulations were performed under a green safe light (Kodak filter No. 38) to avoid irreversible bleaching of the samples. Sucrose concentrations were determined using a refractometer. Since the sucrose profile was almost identical in each case, the points have been omitted from b and c to increase clarity. The samples were: a, 01 membranes alone. b, dialysed 01 membranes and reaction centre preparation. c, as b except that light-harvesting pigment-protein complex was added (46 mol Bchl/mol reaction centre) before dialysis.

premixed in the presence of 1% cholate and phospholipid for various times before 25-fold dilution into a cuvette containing anaerobic buffer and 2 mM succinate. *P*-605 re-reduction following a flash eventually became triphasic, phases one and two corresponding to re-reduction by reduced cytochrome c_2 in the 01 membrane, the third, slow phase probably corresponded to the re-reduction by a back reaction in reaction centres incorporated in membranes lacking cytochrome c_2. Such membranes may arise as a result of vesicle disruption during detergent treatment. Detergent-treated chromatophores of R-26 showed the same triphasic kinetic behaviour. The membranes and reaction centres appear to require only a short mixing time to induce some reconstitution, although the most effective reconstitution occurred after 60 min mixing before dilution.

Further evidence for the need to 'loosen' membrane structure in order to reconstitute reaction centres came from the following experiment in which the

TABLE I

THE EFFECT OF THE ADDITION OF 01 MEMBRANES TO DILUTED REACTION CENTRES ON THE HALF TIME OF *P*-605 RE-REDUCTION

An anaerobic Thunberg cuvette was prepared containing 3.2 ml 20 mM MOPS/100 mM KCl (pH 7.0) and 0.7 nmol reaction centre. At time zero the contents of the cuvette and 1 mg 01 membrane protein in the bulb were quickly mixed by inversion and the recovery of the *P*-605 absorbance decrease monitored at various times after mixing using an unchopped dual wavelength spectrophotometer linked to a storage oscilloscope. After 67 min the cytochrome c_2 in the cuvette was completely oxidised by the addition of equal concentrations of potassium ferri- and ferrocyanide.

Time after mixing 01 membranes and reaction centres (min)	$t_{1/2}$ *P*-605 re-reduction (ms)
0	1081
0.33	106
2	100
5	91
10	69
22	58
35	53
51	56
67	588

concentration of cholate was varied at 4 and 26°C; at these two temperatures the membranes might be expected to have different fluidities. Biphasic re-reduction of photo-oxidised reaction centres was always observed in this experiment; however, the second phase of around 1 s half-time, showed no correlation with temperature or cholate variation, and probably corresponded

TABLE II

TIME COURSE OF THE CHOLATE DILUTION RECONSTITUTION PROCEDURE

01 membranes, reaction centres, sodium cholate and phospholipid were mixed for various times before dilution in anaerobic buffer. An anaerobic Thunberg cuvette was prepared containing 3.2 ml 20 mM MOPS/100 mM KCl (pH 7.0), 2 mM sodium succinate, 2 mM sodium fumarate, 1 mM potassium cyanide in the main well. The side-arm contained 1 mg 01 membrane protein, 2 mg sonicated soybean phospholipid suspension, 0.7 nmol reaction centre and sodium cholate to a final concentration of 1% at room temperature. The reaction centres were added and mixed and at varying times after the contents of the bulb of the cuvette were mixed by inversion, diluting the bulb contents 25-fold. Following a single saturating xenon flash the recovery of the 605 nm absorbance decrease was measured in a single beam spectrophotometer, and the data stored as for Table I. Columns I, II, and III show the kinetics of *P*-605 re-reduction resolved into constituent phases by plotting points from the traces semi-logarithmically. The half-time of the first phase in column I could not be estimated accurately within the chosen time scale of the experiment.

Time after mixing (min)	$t_{1/2}$ *P*-605 re-reduction (ms)			Phases of total *P*-605 re-reduction (%)		
	I	II	III	I	II	III
0 *			1081			100
2		12	247		80	20
10		8	92		63	37
25		11	218		83	17
60	<1	10	141	27	48	25

* This measurement was made with no 01 membranes present so the behaviour of isolated photo-oxidised reaction centres could be examined in the absence of potential electron donors.

to leakage of an electron back from reduced primary acceptor. The first phase is thought to represent electron transfer between reduced cytochrome c_2 and photo-oxidised reaction centres and is used as an indicator of the efficiency of reconstitution. The results in Fig. 5 showed that when no cholate was added reaction centres appeared to reconstitute more efficiently when the incubation was performed at 26°C when membrane fluidity and phospholipid transitions could play a part in allowing reaction centres to move within the 01 membrane vesicle; increasing the cholate concentration at 4°C had the same effect. Over-disruption of membrane structure at high cholate concentrations and temperature may occur, leading to a loss of activity.

Reconstituted light-induced steady-state absorbance changes

Fig. 6 shows results obtained by illuminating cholate-treated reconstituted preparations; under the same conditions, chromatophores prepared from photosynthetically-grown R-26 mutant behaved in a similar manner. At the onset of steady-state illumination, reconstituted reaction centres become photo-oxidised with electron transfer to a cytochrome *b*, which becomes reduced. An electron from reduced cytochrome c_2 passes to the reaction centre, the resulting oxidised cytochrome c_2 in turn is reduced via an antimycin-sensitive pathway. The steady-state photoreduction of cytochrome *b* is

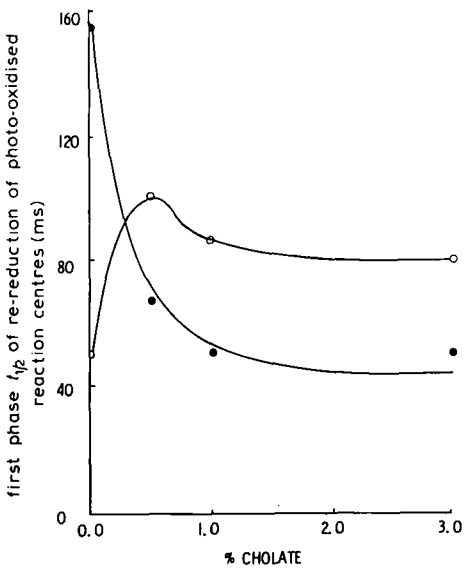

Fig. 5. The effect of varying cholate concentration and temperature on the reconstitution process. 7 nmol carotenoid-less reaction centres were mixed in the bulb of an anaerobic Thunberg cuvette with 10 mg 01 membrane protein, 20 mg sonicated soybean phospholipid and sodium cholate at the indicated concentration; the temperature of the 60 min incubation was either 26°C (○) or 4°C (●). By inverting the cuvette, each mixture was diluted at least 25-fold with anaerobic buffer containing 20 mM MOPS/100 mM KCl (pH 7.0), and 2 mM sodium succinate so that the final concentration was less than 0.1%. Following a single saturating flash of 40 μs duration the reaction centres become photo-oxidised and the rate of re-reduction was recorded at 598 minus 575 nm on the unchopped dual wavelength spectrophotometer. Points from the trace were plotted semi-logarithmically; all plots revealed biphasic kinetics, assuming a pseudo first order process. Points on the graph were obtained from replots of the first, fast phase. All kinetic measurements were made at 22°C.

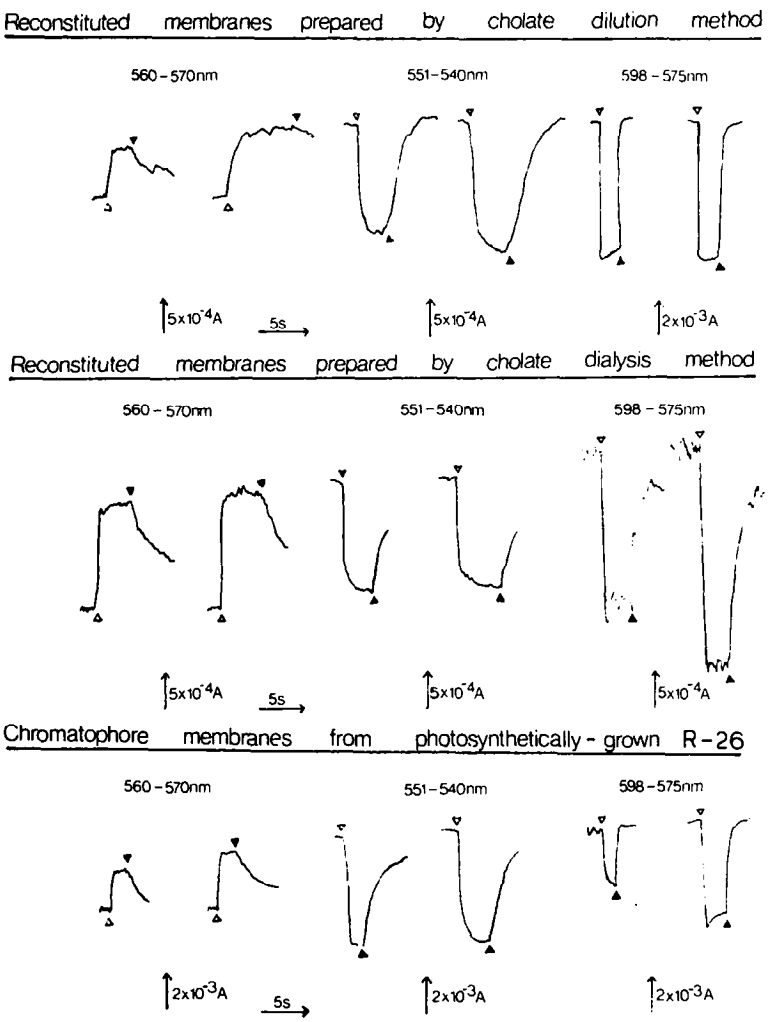

Fig. 6. A comparison of light-induced steady-state absorbance changes in reconstituted and chromatophore membrane preparations. The reconstituted membranes were prepared by mixing 10 mg 01 membrane protein, 7 nmol carotenoid-less reaction centres, 20 mg sonicated soybean phospholipid and sodium cholate to a final concentration of 1%. After incubation for 30 min the suspensions were diluted or dialysed as described earlier in Figs. 2 and 3. The cuvette contents were aerobic and the buffer was 20 mM MOPS/100 mM KCl/1 mM sodium ascorbate (pH 7.0). The time and absorbance scales are shown on the figure. Note the variation in absorbance scales. The cuvette contents were illuminated with a tungsten filament light passed through a far-red filter (Kodak Wratten No. 88A). Light on is denoted by △, light off by ▼. The second trace of each pair was recorded after the addition of 10 μg antimycin A.

enhanced by the addition of antimycin A, suggesting that a cyclic light-driven system has been reconstituted. Control measurements with membranes from photosynthetically-grown cells were consistent with this cyclic system.

Discussion

Examination of 01 membrane polypeptides after solubilization and electrophoresis in SDS (Fig. 1) does not conclusively demonstrate the three reaction

centre bands (H, M, L) observed in the purified centre and R-26 membrane samples [17]. The synthesis of some reaction centre components can occur in cells lacking photosynthetic pigments. Previous work by Takemoto and Lascelles [2] has shown that the synthesis of the H subunit is not tightly coupled to bacteriochlorophyll synthesis. However, since examination of membranes from a *R. sphaeroides* mutant (PM8bg II-15) lacking carotenoids and reaction centre peptides [17] demonstrates faint bands in the H and M region, there is some uncertainty in establishing the absence of reaction centre subunits in gels.

The bacteriochlorophyll-less mutant 01 lacks polypeptides in the molecular weight 9000—12 000 region which are normally ascribed to the subunits of the light-harvesting pigment-protein complex in several species of photosynthetic bacteria [27—30].

The SDS gels of light-harvesting pigment-protein complex purifed from a carotenoid-containing photosynthetically-grown GVP mutant has three low molecular weight Coomassie staining bands (Fig. 1). The 9 kilodalton protein described by Clayton and Clayton [19] ran as a very broad band and, we suggest, was incompletely resolved into its components. Membranes from carotenoid-less mutants R-26 and PM8-bg II-15 each possess two clearly distinguishable bands in this region. Spectral differences in bacteriochlorophyll absorption bands in the far red region between the isolated pigment-protein from GVP (peaks at 800 and 850 nm) and the carotenoid-less membranes (single peak at 870 nm) may therefore be reflected in the low molecular weight polypeptides resolved by our gradient gel.

The B-875 component present in chromatophores from *R. sphaeroides* GVP does not survive lauryldimethylamine oxide treatment used in the purification of the B-800 + 850 complex [19] but may still be present in some degraded form as a protein contaminant in the B-800 + 850 preparation. Coomassie-stained lipids must also be considered as a contributor to the gel pattern in this region [31].

Neither method of reconstituting reaction centres gave evidence for a specific binding of reaction centres to membranes. The results of this binding were an increase in cytochrome c_2 photo-oxidation and cytochrome *b* photoreduction per mg protein (Figs. 2 and 3). The division of these absorbance changes into apparent fast and slow phases produced an interesting correlation of increasing proportion of fast phase and increasing reaction centre content. For the cholate dilution method, the lower reaction centre content of the membrane resulted in more 'fast' cytochrome c_2 photo-oxidation than 'fast' cytochrome *b* photoreduction (Fig. 2b). This is to be expected if reaction centres bind to the surface of the membrane and react with cytochrome c_2 readily; this loosely bound reaction centre is not sufficiently submerged in the membrane to rapidly photoreduce a cytochrome *b* in the membrane. It appears to be necessary to bind many reaction centres to the membrane to obtain the few with the correct orientation to rapidly photoreduce cytochrome *b*. This is also consistent with the observation that reaction centres interact with cytochrome c_2 following mixing of reaction centres and 01 membranes in dilute suspension in the absence of added cholate. Dialysis produced similar results to the dilution method although it is noticeable that the maximum 'fast' cytochrome *b* photoreduction (Fig. 3b) was achieved at lower reaction centre content when the

dialysis method was used suggesting that the slower dialysis procedure was more efficient in promoting the interaction of reaction centres with membrane bound cytochrome *b*. The unexpected decrease in specific cytochrome c_2 photo-oxidation at high ratios of reaction centre to membrane (Fig. 3a) is possibly a consequence of the number of c_2-cytochromes photo-oxidised remaining constant while increasing the reaction centre continues to elevate the total protein present. It appears that the dialysis method binds more of the added reaction centres to 01 membranes compared with the dilution method, although the measurements are complicated by the possibility that membrane binding of reaction centres changes the extinction coefficient of reaction centre photo-oxidation. Such a phenomenon was noticed after reaction centre solubilization by lauryldimethylamine oxide addition to Ga chromatophore membranes [32]. An absolute requirement for the inclusion of soybean phospholipid in the reconstitution procedure outlined above has not been demonstrated, although the presence of some added phospholipid in the reaction centre/01 membrane mixture results in some enhancement of reaction centre binding to the membranes. At present the role of added phospholipid in the reconstitution process is unclear.

Interpretation of the pattern of bands produced by centrifugation of reconstituted membranes in sucrose gradients is complicated by the apparent separation of 01 membranes into light and heavy fractions, both of which bind reaction centres and show some cytochrome c_2 photo-oxidation. Addition of light-harvesting bacteriochlorophyll pigment-protein complex to 01 membranes together with reaction centres produces a fraction more dense than that observed following the addition of reaction centres alone. Added light-harvesting complex enhances both reaction centre binding and cytochrome c_2 photo-oxidation (Fig. 4). Such preparations also exhibit energy transfer between the light-harvesting complex and reaction centres (Hunter, C.N., van Grondelle, R., Holmes, N.G. and Jones, O.T.G., unpublished results). When purified reaction centres and light-harvesting complexes are mixed and dialysed, energy transfer between these pigment-proteins has been demonstrated [33]. We have preliminary evidence for energy transfer between antenna and reaction centre pigment-proteins in our reconstituted preparations [22].

We have used the detergent sodium cholate to solubilise the hydrophobic reaction centres, and to cause limited disruption of membrane structure to allow interaction of reaction centres with the cytochrome chain. This can be accomplished using the detergent lauryldimethylamine oxide or sonication (Hunter, C.N. and Jones, O.T.G., unpublished results). We interpret the influence of temperature on reaction centre re-reduction in the absence of cholate (Fig. 7) as a consequence of increasing the mobility of reconstituted membrane bound reaction centres. This allows cytochrome c_2 access to re-reduce photo-oxidised reaction centres more readily at 26 than at 4°C.

Photo-oxidation of solubilised cytochrome c_2 liberated from membranes by detergent disruption of 01 membranes can occur, but the qualitative and quantitative changes in reaction centre re-reduction following detergent removal, the separation of a reconstituted complex in a sucrose gradient and the reduction of cytochrome *b* suggest that we have been successful in promoting the interaction of added reaction centres with a membrane bound respiratory chain.

The results in Table I show that in dilute solution, reaction centres have access to 01 membrane cytochrome c_2 almost immediately upon mixing. The re-reduction of photo-oxidised reaction centres was monophasic and slow. The results in Table II suggest that a preincubation with 1% sodium cholate for as long as 60 min at room temperature is necessary to achieve the correct alignment with the cytochrome c_2. The criteria used to study the reconstitution of reaction centres do not preclude the possibility of liberation of cytochrome c_2 from 01 vesicles as mentioned above. However, we have observed that after a wide variety of treatments, such as prolonged washing with 20 mM MOPS/100 mM KCl buffer (pH 7.0), 1% ethanol, or concentrations of up to 3% cholate the ratio of total cytochrome c_2 : total cytochrome b estimated from reduced minus oxidised difference spectra remains almost constant. This suggests that despite the ready removal of some cytochrome c_2 from the membranes during disruption in the French press much of the cytochrome appears to be bound to the membranous fraction. Cytochrome c_2 reaction centre kinetics may reflect the partial integration of reaction centre protein into the membrane. We assume that the membrane provides the necessary hydrophobic environment round the reaction centre after removal of the cholate, which in turn replaced chromatophore phospholipid during reaction centre purification.

Fig. 6 compares absorbance changes observed upon illuminating reconstituted and chromatophore membrane with far-red light. The extents of these changes are comparable for the three preparations, when expressed per mg protein or as a percentage of the total cytochrome.

These results show that this system has potential in studies of photosynthetic membrane development in *R. sphaeroides*. The assembly of intracytoplasmic membranes bearing photoreactive pigments and a cyclic light-driven electron transport system requires the incorporation of newly synthesised pigment proteins into a membrane containing the appropriate cytochromes. We have demonstrated that a membranous fraction isolated from the aerobically-grown mutant 01 will bind reaction centre and antenna proteins, forming a system which will behave similarly in these experiments to chromatophore membranes. Further studies will concentrate on the ability of such preparations to show energy transfer from the antenna to the reaction centre, membrane potential induced carotenoid band shifts and flash induced kinetics of electron flow.

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