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ELECTRON TRANSPORT IN CHROMATOPHORES FROM *RHODOPSEUDOMONAS SPHAEROIDES* GA FUSED WITH LIPOSOMES

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Chromatophores from *Rhodopseudomonas sphaeroides* GA were fused with liposomes in order to dilute the components of the cyclic photosynthetic electron-transport chain within the membrane. This dilution led to a decrease in the rate of cytochrome *b*-561 reduction. The original rates could be restored at potentials around 100 mV (where a large part of the quinone pool is chemically reduced), if ubiquinone was incorporated into the liposomes prior to fusion. Similar dilution effects could be observed in synchronized cultures. The membrane obtained after division contained about twice the amount of phospholipids per reaction center when compared to chromatophores prepared from cells harvested just before division. Chromatophores from synchronized cultures are more uniform with respect to the concentration of the different electron-transport components in the membrane than the membranes from normally grown cells. The kinetic behaviour both of fused chromatophores and of membranes from synchronized cultures are in agreement with a modified Q-cycle model for photosynthetic electron transport in *Rps. sphaeroides*. The results presented in this paper cannot be explained by postulating the presence of a firmly bound quinone, Q_Z, in the ubiquinol: cytochrome c_2 oxidoreductase, as previously proposed.

Introduction

Chromatophores from photosynthetic bacteria contain ubiquinone in a large excess over the other electron transport components [1]. It has been shown that most of these molecules behave as a uniform group of thermodynamically equivalent species [2], the so-called quinone pool. In *Rps. sphaeroides* GA the size of the quinone pool has been determined to be about 25–30 ubiquinones per reaction center [2]. Until recently, the role of these quinones was very poorly understood. We have proposed a model of the cyclic electron transport [3], in which quinone from the pool acts as a diffusable H-carrier between reaction centers and ubiquinol : cytochrome c_2 oxidoreductase (the so-called b–c_1 complex). The model proposed is a modified Q-cycle [4–6], but differs from previous Q-cycles suggested to explain the behavior of the chromatophore system [7–10] in several significant features, as discussed in detail elsewhere [3,11]. Among these is the postulate that oxidation of ubiquinol by the ubiquinol : cyt c_2 oxidoreductase complex does not proceed through a firmly bound quinone of fixed stoichiometry and modified redox potential. We suggested that at the quinol oxidase site of this complex (the Q_Z-site [3]), quinol from

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Abbreviations: Q, QH_2, oxidized and reduced forms of ubiquinone-10; Q_Z, hypothetical bound ubiquinone of the quinol oxidase site; $b-c_1$ complex, ubiquinol : cytochrome c_2 oxidoreductase; cyt, cytochrome; Mops, 4-morpholinepropanesulfonic acid; $(BChl)_2$, primary donor of the photochemical reaction center; RC, reaction center.

the pool is oxidized in a second-order concerted reaction, reducing the Rieske-type iron sulfur protein and one of the *b* cytochromes. At ambient potentials between 250 and 130 mV, where 0–3 quinols are present in the pool in the dark, depending on ambient potential, each saturating single turnover flash introduces one quinol per two reaction centers into the pool; in this E_h range the second-order reaction between QH_2 and the b–c_1 complex has been shown to be rate-determining [3]. At E_h values lower than 130 mV, the quinol oxidation was no longer the only rate determining reaction. Therefore, quantitative studies of the kinetics of the turnover of the quinol oxidase site, as assayed by the reduction kinetics of cytochrome *b*-561, have been limited to the range between 200 and 130 mV in native chromatophores.

Fusion of liposomes with membranes from different sources has been previously used to dilute membrane proteins within the lipid bilayer in a number of vesicle systems. Several techniques for fusion have been developed for submitochondrial particles [12], chloroplasts [13], chromatophores [14,15]. In this paper, we have used fusion to study the diffusion-controlled reactions of the quinone pool. Differences in the protein-to-lipid ratio can also be obtained with synchronized cultures. It has been shown by Kaplan and co-workers [16–20], that membranes from cells harvested just before cell division have a high protein-to-lipid ratio. Shortly after division, as a result of incorporation during the division process, the amount of lipid in the membranes has dramatically increased to give a higher proportion of lipid. A difference in membrane fluidity was also detected using a fluorescent probe [21]. Samples from those two extremes have been used in experiments to assay the kinetics of turnover of the quinol oxidase site, and the results are compared with those from fusion experiments.

Materials and Methods

Asynchronous cell cultures were grown in 1 l bottles at high-light intensity over night, using the medium of Sistrom [22]. At the time of harvest, the cells were in the exponential or late-exponential phase of growth. Synchronized cell cultures were grown using the stationary phase cycling technique of Cutler and Evans [23], as modified by Lueking et al. [16]. Two 1 l pre-cultures were grown in sequence to about 0.8 mass doublings after cessation of exponential growth, and the cell suspension was used to inoculate a 5 l bottle to a cell density of about 60 units on the Klett-Summerson colorimeter equipped with a No. 66 filter. Growth and degree of synchrony were measured as in Ref. 16. Two samples of cells were collected, one just before or at the beginning of division, the second about 40 min later, after division. The samples (about 2 l each) were immediately cooled by passing the suspension through a copper coil placed in ice-water, and were kept thereafter on ice, to prevent any changes in the cells after the start of collection. The cells were pelleted and subjected to the normal chromatophore preparation procedure.

Chromatophores were prepared as described in Ref. 24. After preparation they were additionally purified by centrifugation on a continuous sucrose gradient (0.6–1.4 M sucrose in Mops/KCl buffer). Chromatophores from the sucrose gradient were mixed with Mops buffer (50 mM Mops (adjusted to pH 7.0)/100 mM KCl), sedimented and resuspended in the same buffer.

Liposomes were prepared starting from a multilammellar suspension (40 mg/ml) of soybean phospholipids (Sigma phosphatidylcholine from soybean type II-S, commercial grade, purified by two acetone precipitations from chloroform) in Mops buffer. In some experiments, phospholipids isolated from chromatophores (see below) were used to ensure that phospholipid specificity was not a controlling factor. The phospholipid suspension was either passed four times through a French press [25], or sonicated [26], and then centrifuged at 16000 rpm for 45 min to yield a homogeneous preparation of vesicles. In cases where ubiquinone was required in the liposomes, solid ubiquinone-10 (a gift from the Roche Co., Switzerland) was added to the dry phospholipids before suspension.

Chromatophores were fused with liposomes by a modification of the method by Schneider et al. [12]. Chromatophores were diluted in Mops buffer to about 0.3 mM bacteriochlorophyll. To 12 ml of membranes, 1 ml of liposomes containing about 40 mg phospholipid was added. The mixture was kept at 30 °C in the dark, the pH was lowered to 6.1 by addition of 0.1 M HCl. After 10 min, another 1 ml of liposomes was added and the pH corrected to

6.1. This was repeated from one to three times. After 60 min, the pH was brought to 7.1 with 0.1 M KOH and the membranes were cooled to 4°C to stop the fusion. The mixture was layered on top a continuous sucrose gradient (0.6–1.4 M) and centrifuged at 25000 rpm for 16 h.

Phospholipids were extracted from chromatophores using the method of Folch et al. [27] and purified by acetone precipitation from chloroform/methanol (2:1) followed by column chromatography on silica gel.

Protein concentrations were determined by Peterson's modification [28] of the Lowry et al. [29] procedure. Bovine serum albumin was used as a standard. Determination of the phospholipids was performed on lipid extracts [27] of chromatophores, using the method of Petitou et al. [30].

Redox titrations were carried out as in Ref. 24. Cytochrome *b*-561 was measured at 561–569 nm, using an extinction coefficient of 19.5 $mM^{-1} \cdot cm^{-1}$, and cytochrome ($c_1 + c_2$) was measured at 551–542 nm, using the same extinction coefficient [3,24,31]. The concentration of reaction centers was measured from the change of $(BChl)_2^+$ induced at 542 nm by a train of eight flashes spaced at 32 or 64 ms, delivered to chromatophores poised at $E_h = 200$ mV in the presence of antimycin, using an extinction coefficient of 10.3 $mM^{-1} \cdot cm^{-1}$ [24,31]. The concentrations of cyt *b*-561 and cyt ($c_1 + c_2$) were routinely measured by a similar procedure using a train of four or eight flashes. The cytochrome concentrations were also checked by full-spectrum dark redox titrations [33,34] in some preparations. Also in some preparations cyt c_1 and cyt c_2 were measured independently, using the kinetic method above, and the wavelength pairs previously described [34]. Values for extinction coefficients for cyt *b*-561 and cyt c_1 in situ are not well characterized. The extinction coefficients in the isolated b–c_1 complex have recently been measured by Gabellini et al. [35], who suggested values of 17.5 $mM^{-1} \cdot cm^{-1}$ at 553 nm for cyt c_1, and 25 $mM^{-1} \cdot cm^{-1}$ at 561 nm for cyt *b*-561, both values being taken from the reduced-oxidized difference spectra. In view of the wide range of values for the cytochromes in situ quoted in the literature, the choice of a particular set of values must be empirically based. The values chosen here may be in error, and the kinetic constants derived using them may need to be revised in the light of more precisely determined values. However, we believe the values chosen are correct within ±20%.

Kinetic traces of cyt b_{561} reduction were tested for second-order reaction kinetics using a computer program which calculated k_2 from the equation:

$$\ln \frac{[QH_2]_t}{[b^{ox}\text{-}561]_t} = k_2 t([QH]_0 - [b^{ox}\text{-}561]_0) + \ln \frac{[QH_2]_0}{[b^{ox}\text{-}561]_0}$$

where the subscripts 0 and t indicate concentrations at zero time and at time t after the start of the reaction, respectively. The use of this equation has been discussed in our previous paper [3]. The assumed values for the concentration of cyt *b*-561 and QH_2 are outlined in the result section of this paper. The concentration of cyt *b*-561 in the oxidized form [b^{ox}-561] was calculated from its concentration before the flash (as determined by E_h, pH), minus the amount reduced at any given time, which was derived from the kinetic trace. A similar calculation was used for $[QH_2]_t$, by subtracting any QH_2 consumed from the concentration immediately after the flash. The value of k_2 was calculated from the slope of the line $\ln[QH_2]_t/[\text{cyt } b^{ox}\text{-}561]_t$ vs. time, the slope itself was derived by a linear regression with least square fit. For the calculation of the second order reaction constant, zero time was usually displaced by the lag time from the point of the flash to the point of onset of cyt *b*-561 reduction, determined by extrapolation from the maximal slope of the kinetic trace. This displacement did not have a significant effect on the value of k_2, but changed the correlation factor of the linear regression from about 0.67 to values between 0.80 and 0.98 for the 40 experimental points usually used.

Results

Separation of liposome-fused chromatophores

The fusion mixture was usually separated on the sucrose gradient into two major green bands, one or two minor green bands and a yellow band at the top of the gradient. This upper band contained only small amounts of bacteriochlorophyll, with a red absorption maximum at about 770 nm. We therefore concluded that this must be excess

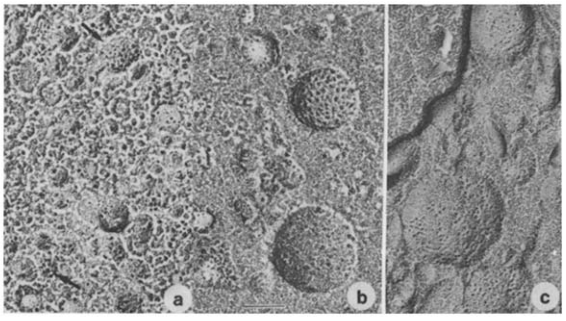

Fig. 1. Electron micrographs of chromatophores. For freeze fracturing the membranes were suspended in 20% glycerol in 50 mM Mops buffer. After 1 h, they were frozen in liquid freon and later fractured at −110 °C in a Balzers 301 freeze etch unit. The samples were rotary shadowed with platinum and carbon (except sample c, which is shaded by an angle). The replicas were examinated in a Joel 100C electron microscope. (a) Normal chromatophore preparation; (b) chromatophores fused with plain phospholipids; (c) chromatophores after fusion with liposomes containing ubiquinone. Magnification, 55000, bar represents 100 nm.

liposomes with some extracted bacteriochlorophyll. The lower of the two major bands comigrated with untreated chromatophores; preliminary results from electron transport studies, however, suggested that it did contain some enlarged particles. We think that it is a mixture of unchanged chromatophores and products of chromatophore-chromatophore fusion. The second major band, containing up to 60% of the total bacteriochlorophyll, was the product of liposome-chromatophore fusion. Since the other two green bands (probably also the products of chromatophore-liposome fusions) did not contain much material, all the studies reported here have been carried out with this second band.

Freeze-fracture electron micrographs of the liposome-fused chromatophores showed an increase of the average size of the vesicles as well as a decrease of particle density on the outer fracture faces of the membranes (Fig. 1). For those membranes fused with liposomes containing ubiquinone-10, the increase in size was even more pronounced. This agrees with a slightly shifted band of these membranes on the sucrose gradient compared to chromatophores fused with liposomes containing phospholipid alone. These findings are confirmed by the measurements of phospholipids per reaction center (Table I). The increase of this ratio after fusion was between 2.2 and 2.5 for plain liposomes and 2.3 and 2.9 for liposomes containing ubiquinone. When fusion experiments using the same chromatophores and two different types of liposomes were performed in parallel, the amount of phospholipid incorporated into the chromatophore membranes was always higher for those liposomes which contained ubiquinone in addition to the phospholipids.

Integrity of fused chromatophores

Integrity of the fused membranes could be demonstrated in two ways. First, the amount of cytochrome c_2 per reaction center as measured by redox titration in the dark was usually only 2–5% less in fused preparations than in the starting chromatophores. Nevertheless, the amount of flash-oxidized total cytochrome *c* per reaction center after four flashes, with 32 ms between the flashes, in the presence of antimycin A was reduced by 8–20%. The amount of total cytochrome *c* measured in this way was dependent on the time between flashes, giving higher values for longer times between flashes. Secondly, the electrochromic carotenoid band shift in the presence of antimycin was measured. This bandshift measured at 503 nm was decreased by about 40% in liposome-fused as compared with control chromatophores. The decay of the shift in freshly prepared fused membranes was increased. The decay of the carotenoid band shift in the fused membranes was clearly biphasic, with a slow component which was comparable to the decay in the control chromatophores, and a fast phase accounting for about 25% of the total extent. Since the decay after 4 flashes was about the same as after one flash, the fast component of the decay is probably due to a fraction of membrane vesicles which are partly uncoupled. Upon storage for two or three days, the decay of the carotenoid band shift in fused membranes became enhanced, which was not the case with the control chromatophores.

The amount of flash reducible cyt *b*-561 was checked in the membrane preparations by measuring the extent of reduction induced by using four flashes, usually with 32 ms in between. For untreated chromatophores, when measurements were made at an E_h of 200 mV, the amount of cyt *b*-561

reduced was found to be independent of the flash frequency between 60 and 7.5 Hz. In fused membranes the ratio of flash reducible cyt *b*-561 to reaction center, measured with four flashes at 32 ms intervals, was decreased by 5–25% compared to unfused chromatophores. The decrease was dependent on the concentration of cyt c_2 in the fused chromatophores. Since in most cases, the loss of cyt c_2 during fusion was negligible with this fusion method, the initial concentration of cyt c_2 in the unfused chromatophore preparation determined the amount of flash reducible cyt *b*-561 in the fused product. Low cyt c_2 concentrations led to a lower apparent ratio of cyt *b*-561 to reaction centers as assayed by this method. In addition, there was a strong dependence on the time between flashes, giving more reduced cyt *b*-561 for a lower flash

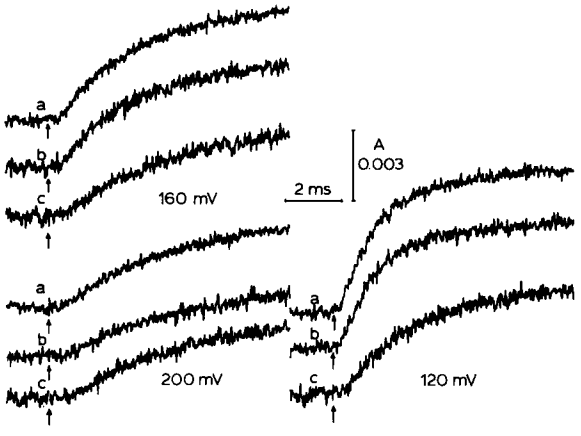

Fig. 2. Examples of kinetic traces of cyt *b*-561 reduction. Kinetic traces of cyt *b*-561 reduction at three different potentials (200, 160 and 120 mV) for the following preparations are shown: (a) control chromatophores, after purification by a sucrose gradient; (b) chromatophores fused with liposomes, containing ubiquinone; (c) chromatophores fused with liposomes prepared from phospholipids only. The traces are normalized to the same reaction center content. They are the average of 16 traces (60 s dark time between single traces), with a sweep of 10 ms and an instrument response time of 10 μs. Chromatophores were suspended in MOPS buffer (100 mM KCl/50 mM Mops, pH 7.0); mediators present were 1 μM each of phenazine methosulfate, phenazine ethosulfate and pyocyanin; 10 μM each of 1,2-naphthoquinone, 1,4-naphthoquinone, *p*-benzoquinone and duroquinone; 2 μM DAD. Valinomycin and nigericin at 2 μM, and antimycin A at 10 μM were also added. Photo-oxidized reaction center concentration produced by the flash was 0.31 μM for control, and 0.28 μM in the fused chromatophores (corresponding to a 90–92% saturation produced by the flash as outlined in Ref. 3).

frequency. At potentials around 300 mV, where a major fraction of the Rieske-type iron sulfur center and cyt c_1 were oxidized before the flash, a series of four flashes 64 ms apart gave approximately the same ratio of cyt *b*-561 reduced per reaction center in all preparations, and this protocol was used when appropriate. For all chromatophore preparations, the concentration of cyt *b*-561, cyt $(c_1 + c_2)$ and $(BChl)_2$ of the reaction center were routinely measured, as described above and in Materials and Methods. In some preparations, cyt c_1 and cyt c_2 were also assayed independently [34]. The concentrations of cyt c_1 and cyt *b*-561 were found to be similar when both were assumed to have the same extinction coefficient. However, a trend in the ratio of cyt *b*-561/cyt c_1 to values greater than 1 suggests that the in situ extinction coefficient for cyt *b*-561 is higher than that for cyt c_1 [cf. 35], if both are at the same concentration in the membrane. The relative concentration of cyt c_2 was somewhat more variable, but was generally in the range 0.6–1.3 cyt c_2/cyt c_1 (assuming the same extinction coefficient), with a mean value close to 1 for preparations showing rapid re-reduction of $(BChl)_2^+$ in over 85% of centers. The concentration of cyt $(c_1 + c_2)$ was generally about equal to that of the reaction center, but this value varied between preparations, due to variation of the cyt *b*-561/RC ratio over the range 0.4–0.7. The concentration of the b–c_1 complex was taken to be the same as the concentration of cyt *b*-561.

Effect of fusion on electron transport rates through the b–c$_1$ complex

In the presence of antimycin A the rate of cyt *b*-561 reduction reflects the turnover of the quinol oxidase site of the b–c_1 complex [3]. Fig. 2 gives some examples of the cyt *b*-561 reduction kinetics following flash illumination of control and fused chromatophores at three different potentials. At high redox potentials (200 mV and above), were the quinone pool is almost completely oxidized in all samples before illumination, fusion of chromatophores with liposomes led to two differences of the kinetic traces of cyt *b*-561 reduction. First, the rate of reduction was obviously slowed in fused chromatophore samples with or without added ubiquinone. In the case of chromatophores fused with ubiquinone-containing liposomes, the

slower rate of reduction was somewhat more pronounced. Second, the delay (lag) between the flash and onset of cyt *b*-561 reduction was increased in both types of fused chromatophores. This lag decreased at lower redox potentials, but remained somewhat longer even at 100 mV, in the fused preparations. The rate of cytochrome *b*-561 reduction also became faster at lower redox potentials as previously observed [36–38], but the titration curves for the increase of the reduction rate as a function of the ambient redox potential were consistently displaced in the fused chromatophore preparations when compared to controls (see Discussion below). The increase in rate observed using chromatophores fused with ubiquinone containing liposomes titrated in at higher potentials than when using chromatophores which were fused with plain liposomes. The ubiquinone-enriched preparations reached almost the same maximal reaction rate as the controls at an E_h around 120 mV. The chromatophores fused with plain phospholipid liposomes on the other hand reached maximal reduction rates only when the potential was below 100 mV, and showed a lower maximal rate.

Fig. 3 shows full titration curves for the effects described above. The two fused chromatophore preparations showed at all potentials slower cyt *b*-561 reduction rates than the control. The lower reduction rate observed at 200 mV in the chromatophores fused with ubiquinone-containing liposomes is attributable to the fact that in those liposomes, incorporation of additional phospholipids, and hence dilution of the membrane components, was greatest. In other preparations, fused with either plain or ubiquinone-enriched liposomes, a more marked effect of dilution on the rates of cyt *b*-561 reduction at $E_h > 200$ mV compared with control chromatophores was observed. At lower redox potentials the ubiquinone-enriched membranes showed much faster rates than those fused with plain phospholipids; this can be explained by the concomittent increase in ubiquinol per reaction center, which was able to reverse the effect of dilution as soon as a few quinones from the pool became chemically reduced.

Results similar to those obtained with liposomes prepared from soybean phospholipids were obtained when chromatophores were fused with liposomes prepared from phospholipids extracted

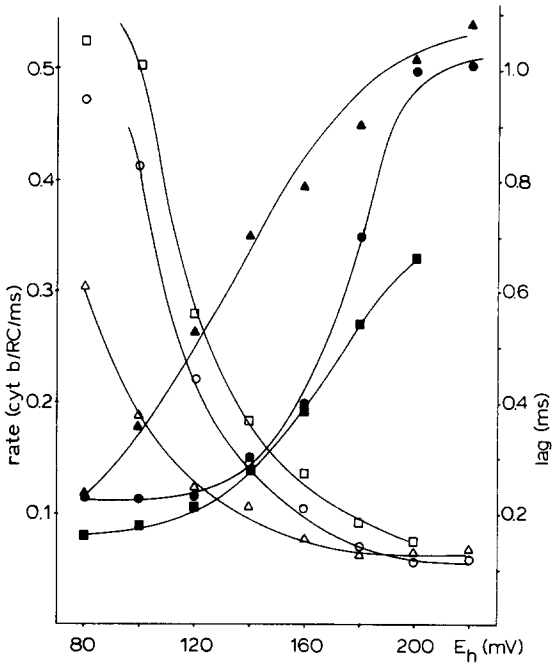

Fig. 3. Titration curves of rate of cyt *b*-561 reduction vs. E_h. The values of initial reduction rate and of the lag between flash and onset of reduction were taken from traces similar to those in Fig. 2. Filled symbols were used for the lag, and open symbols for the initial reduction rate. ■, □, control (900 phospholipids per RC (M/M), about 30 UQ per RC (M/M); △, ▲, chromatophores fused with plain liposomes (1980 phospholipids per RC (M/M)); ○, ●, chromatophores fused with ubiquinone-containing liposomes (2350 phospholipids per RC (M/M), about 50 UQ per RC (M/M)).

from chromatophores. In this case, however, the lipid enrichment was not as great as with the soybean phospholipids, and there was no marked difference in phospholipid incorporation between plain liposomes and liposomes containing ubiquinone. Fig. 4 shows the titration curves for the three chromatophore preparations. Again the fused membranes showed slower cyt *b*-561 reduction, and the maximal rate of cyt *b*-561 reduction showed that in the chromatophores fused with ubiquinone-containing liposomes the kinetic effects associated with the increase in volume of the membrane could be compensated for at lower redox potentials, by the increased size of the quinone pool. The titration curves for the lag between the flash and the onset of cyt *b*-561 reduction are similar to those in Fig. 3. It has to be mentioned, however, that the determination of the

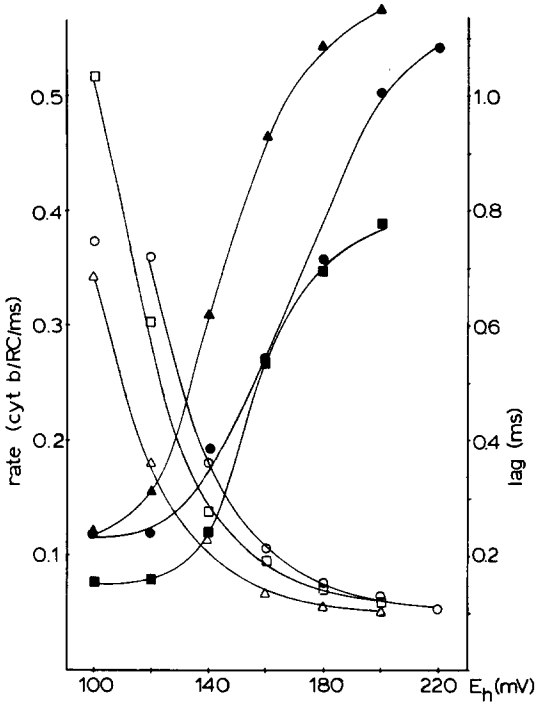

Fig. 4. Titration curves for the chromatophores fused with liposomes prepared from phospholipids isolated from chromatophores. The data points were obtained in the same way as in Fig. 3. □, ■, control; △, ▲, chromatophores fused with liposomes; ○, ●, chromatophores fused with liposomes, containing ubiquinone.

lag in traces with slow reduction rates is less precise than in cases with fast cyt *b*-561 reduction rates.

Kinetics observed with chromatophores from synchronously grown cells

In synchronized cell cultures of *Rps. sphaeroides* GA, the protein-to-lipid ratio was found to vary in synchrony with the cell division cycle, as has been described by other authors before [16–20]. Chromatophores from cells harvested after the cell division had about twice as much phospholipid per protein (w/w) as the membranes prepared from cells which were harvested just before division (Table I). Because of the large scatter between determinations, quinone measurements using the method of Takamiya and Dutton [2] gave no unambiguous evidence as to whether the quinones were incorporated together with the phospholipids (discontinuously) or with the proteins (continuously).

Fig. 5 shows the titration curves for chromatophores from two phases of a synchronized culture. The chromatophores from the cells harvested before division gave a titration curve for the rate of cyt *b*-561 reduction which coincided over a substantial range with the theoretical curve expected from a second-order reaction determined by $[QH_2]$. The chromatophores from cells after division, having a membrane which contained about twice as much phospholipids as the other preparation, showed the lower cyt *b*-561 reduction rate typical for the dilution of the quinone pool. Also with respect to the lag between flash and onset of cyt *b*-561 reduction, there was a remarkable difference, with the chromatophores having the greater lipid volume showing a more pronounced lag phase.

It has been shown previously [39,40] that chro-

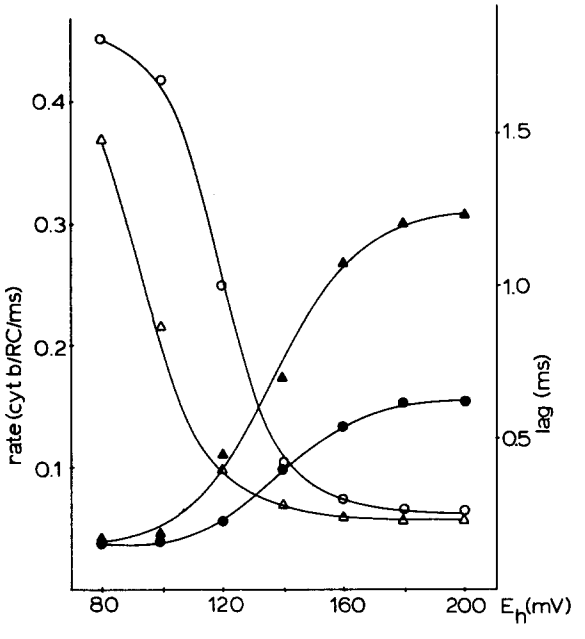

Fig. 5. Titration curves of the initial rate of cyt *b*-561 reduction, and the lag, for two different samples of chromatophores from a synchronously grown culture. The values for the initial rate and the lag were measured from traces similar to those in Fig. 2. Filled symbols were used for the lag, and open symbols for the rate of cyt *b*-561 reduction. ○, ●, chromatophores from cells harvested just before division (protein-to-lipid ratio, 3.0 (w/w)); △, ▲, chromatophores from cells harvested after the division (protein-to-lipid ratio, 1.6 (w/w)).

TABLE I

COMPOSITION OF THE DIFFERENT MEMBRANES USED

The values given are for a representative preparation of each of the listed membrane samples. The lipid and protein values were the mean of four determinations with an error of about 5% for the lipids and 8% for the protein. For measurement of the ubiquinone, four parallel determinations were made on the same sample. These showed up to 15% deviation between values.

Preparation	Lipid [a]	UQ [a]	Protein [b]	RC [c] (mM)	$b - c_1$ complex [c] (mM)	UQ [c] (mM)
Normal chromatophores	10.0	0.33	16.7	2.1	1.1	63
Liposome-fused chromatophores	21.1	0.31	16.2	0.98	0.48	27
Chromatophores, fused with liposomes that contain ubiquinone	24.1	0.61	15.9	0.87	0.40	48
Pre-division chromatophores	7.6	0.28	14.3	2.5	1.25	69
Post-division chromatophores	13.6	0.35	15.1	1.56	0.72	49

[a] Units in mol/per mol bacteriochlorophyll.

[b] Units in mg per mol bacteriochlorophyll.

[c] Concentration in lipid phase of membrane, calculated as outlined in the text using the experimental values obtained from the sample.

matophores from synchronized cultures represent a much more homogeneous membrane preparation with respect to particle density (particles per unit area) on freeze-fracture faces, than the chromatophores from normally grown cells; the latter show quite a range of particle density within the same batch of cells. Since the lipid-to-protein ratio undergoes changes by a factor of two during the cell cycles, asynchronous cultures are expected to give quite heterogeneous populations of chromatophores. Membranes from synchronized cultures are therefore more useful for experimental determinations of the physico-chemical properties of the electron transport components.

Fig. 6 shows double-reciprocal plots for the rate of cytochrome *b*-561 reduction in chromatophores from synchronized cells at different ambient redox potentials, in which the concentrations of quinol (substrate) were calculated from the midpoint of the pool, the ambient redox potential, and the measured concentration of ubiquinone (quinone plus quinol forms) per unit phospholipid volume. It is worth noting that the points measured for both chromatophore samples (before and after division) are on the same line. The apparent K_m value for QH_2 at the quinol oxidase site derived from these plots is 9.5 mM. The saturation effects

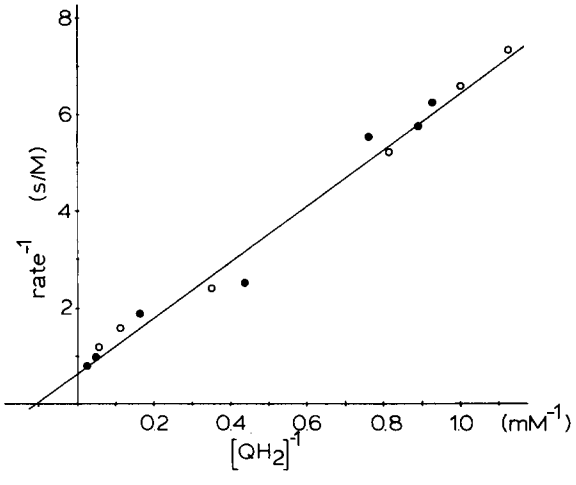

Fig. 6. Double reciprocal plot of rate of cyt *b*-561 reduction as a function of ubiquinol concentration. The reciprocal value of the initial rate of cyt *b*-561 reduction calculated from traces similar to the ones in Fig. 2 is plotted against the reciprocal value of the quinol concentration in the membrane calculated from the total quinone and ambient redox potential as outlined in methods and the text, plus the flash generated quinol. The open symbols are values from post-division membranes, the closed ones are from pre-division chromatophores. The intercept on the *y*-axis is 0.599 $M^{-1} \cdot s$ which corresponds to a V_{max} of 1.67 $M \cdot s^{-1}$, on the *x*-axis the value is 0.106 mM^{-1}, corresponding to an apparent K_m of 9.5 mM.

indicated by the straight lines show that these reactions can also be treated by the classical methods of enzyme kinetics with respect to the saturation of the reduction rate of cyt *b*-561 with increasing quinol concentrations. The values for V_{max} derived from these plots are 1.67 $M \cdot s^{-1}$, rather higher than the maximal reduction rate of 1.37 $M \cdot s^{-1}$ measured experimentally at around 100 mV in these chromatophores. It should be noted that for the same stoichiometric ratio of quinone to reaction center, the increased lipid/protein ratio observed after cell division represents a considerable dilution of the quinone pool in terms of local concentration.

Kinetic traces of the cyt *b*-561 reduction in chromatophores from synchronized samples have also been used to calculate the second order reaction constant, k_2, as described in Materials and Methods. The values for k_2 calculated are of course dependent on concentration as determined by the volume of lipid in which the quinones are dissolved. Measurements of the size of chromatophores by electron microscopy show an average diameter of 60 nm. Biochemical and biophysical measurements [39–43] show that there are about 40 reaction centers per vesicle, and 25–30 quinones per reaction center. The membrane thickness was estimated to be around 10 nm and the lipid part of the membrane was assumed to amount to about 45% of the membrane volume. Using these values, the concentration of reaction centers in the lipids turns out to be about 1.9 mM. Values between 1.8 and 2.6 mM were calculated from the measured ratio of phospholipid to reaction center, using 0.95 g/ml as the specific weight for the lipids (Table I). Calculations of k_2 were made using the experimentally determined phospholipid to reaction-center ratio, and the specific weight of 0.95 g/ml for the lipids. For the initial calculation of the second order reaction constant, traces at 200 mV were used, since at this potential the only reductant for cyt *b*-561 is the quinol produced by the reaction centers. If a pair of reaction centers cooperate to reduce Q to QH_2 [3,24,38], then 0.92 QH_2 per b–c_1 complex would be produced photochemically (in the case of a flash saturation of 92% of the reaction centers), and at this potential the size of the quinone pool would not have much effect on the calculated values of k_2. Using this assumption, traces at 200 mV were used to calculate a value for k_2, and theoretical fits to the kinetic data obtained at lower potentials were generated, assuming different values for the stoichiometry of the quinone pool. In this way the pool size giving the best fit with the experimental results could be tested against the measured pool size in different preparations. In chromatophores from synchronized cells, k_2 was measured to be between 1.2 and $2.0 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ (for potentials between 220 and 150 mV) for a number of preparations, and the kinetically estimated size of the quinone pool before division was around 25 quinones per reaction center, with slightly higher values after division. These values are the same within experimental error as the values estimated directly from chemical measurements (Table I), and provide strong support for our assumption that about 1 QH_2 is produced in the pool per b–c_1 complex on excitation with a saturating flash.

Using a value for k_2 of $1.4 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ determined for a specific preparation of chromatophores from synchronized cells, and the maximal cyt *b*-561 reduction rate derived from the double-reciprocal plot, the QH_2 concentration which would be required to achieve this rate in a simple second order process can be calculated from the equation, $v = k_2$ [QH_2][b^{ox}-561]. The value of 10 mM QH_2, which would be present at an ambient potential of approx. 120 mV, shows that at lower potentials the redox state of the quinone pool would be such that the quinol concentration would saturate the second order collisional process between the b–c_1 complex and QH_2, as suggested earlier [3]. A concentration of 10 mM QH_2 represents 3–5 QH_2 per reaction center.

When k_2 was calculated from kinetic traces of cyt *b*-561 reduction at potentials below 150 mV as described in Materials and Methods, the value started to decrease due to the saturation phenomenon discussed above. The apparent value for k_2 calculated from the trace at 120 mV is $0.78 \cdot 10^5$ $M^{-1} \cdot s^{-1}$. This is similar to the value of $0.74 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ calculated using the half maximal rate and the corresponding quinol concentration taken from the double-reciprocal plot. Since these values were derived independently (in the first case from an analysis of a kinetic trace of cyt *b*-561 reduction, and in the second from a titration of the

initial reduction rate at different ambient redox potentials), but using the same assumptions with respect to the concentration of the components in the membrane, their agreement shows that the assumptions about the size of the chromatophore, reaction center content, etc. are reasonable.

Discussion

The method of Schneider et al. [12] used for the fusion of chromatophores with liposomes increased the amount of phospholipids in the membrane by a factor of 2–3. Similar results have been obtained by Costa et al. [14], who also showed that the change in protein-to-lipid ratio after fusion is dependent on the nature of the lipids used for preparation of the liposomes. The incorporation of lipids into the membrane can be demonstrated by the change in the specific weight, the increase of the average size as well as by the decrease of particle density in freeze-fracture electron micrographs. The traces of the carotenoid band shift indicate that, after fusion, the membranes still form closed vesicles which are not drastically more permeable to ions, and the lower amplitude of the carotenoid change is in line with the increased capacitance expected from the increased surface area, and classical physics. Nevertheless, the fused membranes do not seem to be as stable as chromatophores, since the decay of the band shift is more rapid after storage at 4 °C. Most experiments on electron transport were done in the presence of nigericin and valinomycin as uncoupler, and therefore no marked effect due to additional uncoupling associated with this aging process are to be expected.

The fusion procedure was able to dilute the chromatophore membranes without a significant loss of cyt c_2. This is very important in the light of electron-transport measurements through the b–c_1 complex. Oxidation of the Rieske-type iron sulfur center is a prerequisite for the turnover of the quinol oxidase site of the complex, so that a sufficient amount of cyt c_2 is required before the measurement of cyt b-561 reduction kinetics can be made without loss of rate due to a slower oxidation rate of the Rieske FeS center.

In terms of the modified Q-cycle previously proposed [3], dilution of the membrane components by fusion would be expected to have a number of kinetically measurable effects [44].

(a) Since the reduction of cyt b-561 requires a second-order process (first-order in QH_2), dilution of QH_2 would be expected to lead to a slowing of the reaction rate. If the E_m value for the Q/QH_2 couple is the same in normal and fused membranes, the ratio of Q/QH_2 at any given value of E_h will be the same, but for a fixed amount of quinone per reaction center, the local concentration of QH_2 will be lowered in the fused chromatophores. This would be expected to give rise to titration curves of rate of cyt b-561 reduction against E_h which are systematically displaced in fused compared to normal chromatophores. The rates at all E_h values where $[QH_2]$ is rate determining should be lower in the fused preparations, but the inflection point of the titration curve showing the appearance of QH_2 in the pool due to dark redox equilibrium should be at the same value of E_h.

(b) If quinol from the pool acts as a diffusional H-carrier, the diffusion distances will be greater in the fused preparations. We have suggested that the lag observed between the flash and onset of cyt b-561 reduction may in part be due to the diffusion from reaction center to b–c_1 complex. If this is the case, the lag would be expected to be more pronounced in the fused preparations.

(c) When ubiquinone is included in the liposomes used for fusion, altered effects should be expected. At values of E_h where the pool is oxidized, the plain-liposome and ubiquinone-liposome fused systems should behave the same (for equivalent dilutions). However, the titration curves (rate vs. E_h) for the preparations enriched in ubiquinone should show an inflection at higher E_h values, since, at any particular value of E_h below 200 mV, a higher local concentration of QH_2 will be produced by chemical reduction.

The results presented in this paper show good agreement with these predicted changes. The chromatophores fused with pure phospholipids show lower rates of reduction than the control at any given potential above 100 mV, though over the high E_h range, the reduction in rate due to dilution shown in the figures is rather less than expected from the increase in lipid volume. However, this factor was somewhat variable between prepara-

tions, and in other experiments a greater effect was observed. Although in all cases, dilution led to slower rates, and the range of values was consistent within experimental error with the model discussed above, we cannot exclude the possibility that other factors, ignored in our simplifying assumptions, may be important. The most obvious potential source of error is our assumption that the protein does not contribute to the lipid phase. If the protein contributes a significant fraction of the phase in which ubiquinone is mobile, then the dilution factor due to addition of lipids on fusion would be less than that assumed in these experiments. The rate of cyt *b*-561 reduction starts to increase around 160 mV as in the control, but the quinone pool in the diluted sample has to be reduced to a much higher extent to get the same reduction rate as in the control. These results are incompatible with models proposing the presence of a tightly bound quinone Q_Z with a fixed E_m of 150 mV [36,38,45–48]. Calculations of the second-order reaction constant in the control and in the diluted sample fitted best with the assumption of a 2–2.5-fold dilution due to the fusion. These values are in good agreement with the increase of phospholipid as measured by the lipid phosphorous assay.

The chromatophores fused with liposomes containing ubiquinone showed an even slower cyt *b*-561 reduction rate at 200 mV and above, in agreement with lipid phosphorous assays which showed a larger incorporation of phospholipids in this case than in the case of plain phospholipid liposomes. On titration at potentials lower than 200 mV, the reduction rate began to increase at higher values of E_h than in the other sample, reaching values close to the control. This behavior is also that predicted by the diffusional Q-cycle model [3], and cannot be explained in terms of a tightly bound quinone. The calculations of second-order reaction constants gave best fits with the assumption of a 2.3–2.8 fold dilution of the membrane, and an increase of quinone per reaction center from between 25 and 30 to about 50, in good agreement with chemically determined values (Table I).

It has been reported earlier [2,46,49], that a large portion of the quinone pool can be extracted from chromatophores without changing the kinetics of reduction of cyt ($c_1 + c_2$), the slow phase of the carotenoid change, or the reduction kinetics of cyt *b*-561, all measured at E_h around 100 mV. Although this may seem in contradiction with our present results, the rates in extracted chromatophores were measured at E_h of 100 mV and lower, and the small ubiquinone pool remaining after extracting would have been about half reduced. As our calculation with chromatophores of synchronized cells has shown, the quinol concentration needed to saturate the second order reaction is in the range of 10 mM. In other words, only about 3–4 quinols per reaction center would be needed in the pool to obtain maximal cyt *b*-561 reduction rates; this range coincides with the stoichiometry of quinone required to observe the phenomena previously ascribed to the bound quinone, Q_Z. It would appear, therefore, that the discrepancy between the extraction experiments, and those reported here using fusion, is more apparent than real, and that the extraction experiments cannot be regarded as showing the presence of a firmly bound quinone at the quinol oxidase site of the b–c_1 complex.

The lag between the flash and the onset of cyt *b*-561 reduction at potentials of 200 mV and above has previously been attributed in part to the leaving time of the quinol from its site of formation (the Q_B site of the reaction center), and in part to the diffusion time from the reaction center to the b–c_1 complex [3]. Since the usual lag of about 1 ms is decreased in membranes very rich in protein (for instance, in chromatophores of synchronous cells harvested before division, the lag can be decreased to 0.6 ms), one would expect that a substantial fraction of the lag was due to this diffusion time. If the lag were due solely to the time needed for diffusion from the reaction center to the b–c_1 complex, a somewhat ordered structure would have to be postulated, since a random distribution of the two protein complexes would ensure that, for a fraction of the centers, the diffusion distance would be minimal and no real lag would be expected. In fact, several reports on freeze-fracture electron microscopic studies indicate that the distribution of particles on the protoplasmic fracture face (PF) of chromatophores is more ordered than random [39,40,50–52]. In addition, EPR measurements of lipid mobility using spin labels revealed that quite

a large portion of the lipids seems to be immobilized by the proteins. A fraction of the lag between the flash and the onset of cyt *b*-561 reduction, therefore, might quite well be the result of the diffusion time.

Fusion of chromatophores with liposomes led to an increase of this lag by about a factor of 1.5–2. This may be an indication that the partial order discussed for the chromatophore membrane is not maintained completely after fusion. If the order were maintained, one would expect an increase in lag proportional to the square of the increased distance. In view of the difficulty of measuring the lag with any precision, and the uncertainty as to the relative contributions of leaving-time and diffusion, it seems premature to derive any precise kinetic parameters from our measurements. However, the results are certainly consistent with our previous suggestion [3,11] that ubiquinone must have a relatively slow diffusion coefficient in the membrane ($D < 10^{-9}$ cm$^2 \cdot$ s^{-1}).

We have previously shown that the lag between flash and onset of cyt *b*-561 reduction decreases as the E_h is lowered through the range in which the first QH_2 is produced chemically in the pool, presumably because at these lower values of E_h, QH_2 is already available before the flash [3,38]. As the E_h is lowered further, the lag reaches a minimal value of about 160 μs. We have suggested that this residual lag is due to the time needed to oxidize the Rieske FeS center, the other reactant required for the concerted reaction at the quinol oxidase site. Fused membranes never reached the same small value for the lag as observed in the control chromatophores at potentials around 100 mV. This is probably due to the fact that the oxidation of cyt c_1 is slower in fused membranes [44], and consequently, the rate of oxidation of the Rieske type iron sulfur center is also slower. This lower rate of oxidation of Rieske FeS center might also be responsible for the fact that in fused membranes the maximal rate of cyt *b*-561 reduction was always slower than in the controls, even in the case where a lot of quinones were added together with the lipids. However, in all cases where the rate of oxidation of cyt c_1 was measured directly, the rate was at least an order of magnitude more rapid than the rates of reduction of cyt *b*-561 from which values of k_2 were derived, and the slowing of the cyt c_1 oxidation would not have contributed significantly to the slowing of the rates or increase in the lags measured for cyt *b*-561 at higher values of E_h. We will discuss factors controlling the rate of oxidation of cyt c_1 at greater length elsewhere.

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