Properties of Membrane Fractions Prepared by Chromatophore-Liposome Fusion

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Intracytoplasmic membrane vesicles (chromatophores) isolated from *Rhodopseudomonas capsulata* cells were fused with liposomes by a pH transition procedure. Vesicles of lower density and higher lipid contents and larger diameter than chromatophores were obtained. Similar results were observed by Ca^{2+} induced fusion and by the freeze-thawing method. Respiratory and light-induced electron transport were measured in chromatophores and fused vesicles. Light-induced reaction center bleaching was observed in all types of vesicles, whereas repiratory electron transport was substantially diminished by lipid incorporation. Ubiquinone 10 restored to some extent respiratory electron transport and oxidative phosphorylation and it modified the photophosphorylation kinetics under continuous light. Electrochromic carotenoid band-shift and the 9-aminoacridine fluorescence quenching indicate that the capacity of the fused vesicles to maintain an electrochemical proton gradient has not been substantially diminished. From the kinetics of 9-aminoacridine quenching an increased K^+-permeability seems to be apparent.

Introduction

The photosynthetic and the respiratory apparatuses of facultative phototrophic bacteria contain several supra-molecular membrane bound complexes, namely the reaction center and light-harvesting complexes, the ubiquinol-cytochrome *b/c* oxidoreductase, the cytochrome oxidase and the coupling factor [1, 2]. The functional interaction of these complexes may proceed through direct close contact between the redox partners or through mobile carriers which diffuse laterally in the lipid double layer [3–6]. The observed oxidation half-times are quite different: 5 ms for cytochrome *c* oxidase, 80 ms for the cytochromes *b* and 500 ms for the dehydrogenases [7]. Because of a high protein/lipid ratio in the electron transport membranes and strong protein-protein as well as protein/lipid interactions the mobility of single membrane components is lower in electron transport membranes than in other membranes [8]. Although the function of the ubiquinol pool as a mobile electron carrier between the dehydrogenases and the cytochrome *b/c* oxidoreductase has been established [3–6], a direct contact between the integral membrane complexes by random collision and transitory or dominating protein/protein interactions within integral membrane complexes for electron transport seems to be of importance [3–6, 8–11].

In the experiments, described in this article, the amount of membrane phospholipids was increased by fusion of liposomes with intracytoplasmic membrane vesicles (chromatophores). The activities in electron transport and phosphorylation were measured in fused vesicles obtained by different methods.

Materials and Methods

Isolation of chromatophores

Rhodopseudomonas capsulata, strain 37b4 (German collection of microorganism, Göttingen, strain DSM 938) was grown phototrophically as described [12]. The chromatophores were isolated and purified as described [13]. The cells (300 mg of packed cells per ml buffer) were suspended in 5 mM Hepes buffer, pH 7.8 with 50 mM KCl (HK). Magnesium ions were avoided during separation because of its interference with the fusion process.

Abbreviations: Bchl, bacteriochlorophyll *a*; DTE, dithioerythritol; HK, Hepes-KCl; *R.*, *Rhodopseudomonas;* TMPD, N,N,N′,N′-tetramethyl-*p*-phenylendiamine dihydrochloride; UQ_{10}, ubiquinone 10.

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Preparation of liposomes: low pH induced fusion

Liposomes were prepared by suspending 1 g of L-phosphatidyl choline (type II, Sigma Chemical Corp.) in 10 ml HK buffer and sonication in an ice bath with the microtip of a Branson sonifier at position 4 (maximum output, 2 amp) for 20 min with several intervals of 2 min to avoid heating. The liposomes were size selected by differential centrifugation [14] and finally by Sepharose 4B column chromatography.

One ml of chromatophores (0.3 mg Bchl) was mixed with liposomes (about 300 mg lipid/ml). The fusion was induced at 30 °C by lowering the pH to 6.0 by addition of 10 mM HCl. After 15 min and 30 min two additional aliquots of liposomes were added and the pH maintained at 6.0. After 45 min the pH was adjusted to 7.8 by addition of KOH. The control membranes were treated in the same way except that the liposomes were omitted.

In order to incorporate ubiquinone the dissolved phospholipids were mixed with a chloroform solution of ubiquinone 10 (Sigma Chemie, Taufkirchen) to a final concentration of 1.75 mg ubiquinone 10/g lipid. The solution was evaporated under a stream of nitrogen at room temperature and the dried material was used for liposome preparation as described above.

The fused membrane-liposome fractions were selected according to their density in a discontinuous sucrose gradient consisting of 0.9 ml 60% and 2.4 ml of each 45%, 30%, 15% and 7.5% sucrose in a SW 41 rotor of a Beckman ultracentrifuge at 36000 rpm for 15 h. After centrifugation three Bchl containing main bands were collected, centrifuged and resuspended in HK buffer to a final Bchl concentration of 0.5 mg ml^{-1}.

$CaCl_2$ induced fusion

The chromatophores were resuspended in 10 mM K-phosphate buffer, pH 6.6. To 1 ml of chromatophores containing 0.3 mg Bchl ml^{-1}, 1 ml of the small unilamellar liposomes were added (300 mg of phospholipid ml^{-1}) and 50 µl or 100 µl of a 100 mM $CaCl_2$ stock solution were added. The samples were kept in the dark at 30 °C for 10 min and then the process was stopped by addition of 1 ml of Na-EDTA, 100 mM. The samples were immediately fractionated in a density gradient similar to that described above for the low pH transition procedure.

Freezing and thawing induced fusion

It was performed as described in [15] except that the buffer (pH 7.8) contained 10 mM K-phosphate and 5 mM Mg-acetate. Two freeze (liquid nitrogen)-thaw cycles were performed.

Aerobic electron transport

NADH dehydrogenase (EC 1.6.99.3) and succinate dehydrogenase (EC 1.3.99.1) were measured as described [16]. Succinate-cytochrome *c* reductase was measured in 10 mM KP buffer, pH 7.8 containing 5 mM KCN, 1 mg cytochrome *c* and either 20 mM Na-succinate or 2 mM NADH. The final volume was 1 ml. The reaction was started by addition of the substrate and recorded by measuring absorbance at 550 nm ($\varepsilon = 19\ mM^{-1}\ cm^{-1}$). When ascorbate-TMPD supported oxidase was measured the reaction was started by addition of the membrane preparation to 10 mM K-phosphate buffer, pH 7.8 containing 5 mM Na-ascorbate and 3 µM TMPD. The oxygen uptake was followed polarographically in air saturated buffer (O_2 concentration at 20 °C 233 µM).

Oxidative and light-induced phosphorylation

ATP formation was measured by the luciferin-luciferase procedure [17, 18]. ATP production linked to substrate oxydation was inhibited by addition of 5 mM KCN. Photophosphorylation was started by irradiation with saturating light (100 W/m^2). Oxidative phosphorylation linked to cytochrome *c* reduction was performed in presence of 180 µM KCN in order to inhibit the terminal oxidase. In this experiment 25 µM cytochrome *c* (horse heart, Sigma, type III) was added as the alternative electron acceptor.

Reaction center bleaching

The reversible, light-induced bleaching was determined at 870 nm using an extinction coefficient of 113 $mM^{-1}\ cm^{-1}$ [19]. To avoid interference with energy-linked absorption band shifts of Bchl [20], 5 µg · ml^{-1} valinomycin and 100 mM KCl were added. Antimycin (4 µg · ml) was added to block cyclic electron flow. The Bchl concentration was about 2 to 4 µg Bchl · ml^{-1}. The bleaching was recorded at a potential of +400 mV established by addition of K-ferricyanide. The membrane prepara-

tion was excited at 590 nm (NAL 590 interference filter, Schott & Gen., Mainz). The fluence rate was adjusted to 100 $W \cdot m^{-2}$. The portion of preexisting oxidized reaction center at the given potential was taken in the calculation.

Analytical procedures

The lipids were extracted according to Bligh and Dyer, modified according to Ames [21]. The lipid phosphorus was determined according to Parker and Peterson [22].

Bchl was determined in aceton-methanol extracts using an extinction coefficient of 75 $mM^{-1} \cdot cm^{-1}$ at 772 nm [23]. Protein was determined by the method described in [24]. Ubiquinone was determined in the lipid extract using an oxidized minus reduced extinction coefficient at 275 nm of 12.6 $mM \cdot cm^{-1}$. Reduction was carried out by addition small amounts of Na-borohydride to the ethanolic solution of the quinone.

9-Amino acridine fluorescence

The reaction mixture contained in 1 ml: 10 µmol Tricine, pH 8.0, 2 µmol Mg-acetate, 12 nmol 9 amino acridine, 100 µmol KCl, 0.5 µmol DTE, 5 µmol KCN and chromatophores in variable amounts of Bchl. The procedure was described in [25–27].

Carotenoid band-shift

It was measured with a Perkin-Elmer dual wavelength spectrophotometer type using the wavelength pair 522–508 nm. The calibration of the signal was performed by measuring the extent of the absorption change as a response to an imposed K^+ diffusion potential in the presence of 0.4 $\mu g \cdot ml^{-1}$ valinomycin and 100 mM KCl. The membranes, containing 5–10 µM Bchl, were suspended in Tris-acetate buffer, 50 mM, pH 7.8 and 2 mM Mg-acetate and 0.6 mM DTE.

The preparation was irradiated with a saturating beam of a halogen bulb.

Results

Chemical and spectroscopical properties of liposome-chromatophore fusion products

When unilamellar liposomes and chromatophores were fused by the low pH method three main bands were obtained after density gradient centrifugation. The control chromatophores sedimented at the same position as fraction 3 (Fig. 1). The phosphate content of the fractions has increased with respect to the chromatophores (Table I). Bchl was partly oxidized during low-pH-fusion procedure demonstrated by a new absorption band at 687 nm. This Bchl oxidation was not prevented when the membranes were treated in presence of 100 mM DTE or Na-ascorbate. In comparison with chromatophores of the absorption ratio 860/800 nm was increased to 2.13 in fraction 3, to 2.38 in fraction 2 and to 2.28 in fraction 1. The long wavelength absorption band became asymmetrical with increasing lipid content: This observation points to a preferential destruction of B800 of the B800–850 complex. In fraction 1 an additional absorption band at 687 nm appeared (Fig. 2). The diameter of the fused vesicles has no influence on the near infrared absorption spectrum.

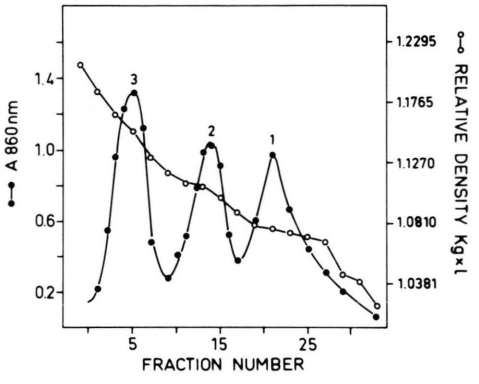

Fig. 1. Sucrose density centrifugation after low pH chromatophore-liposome fusion. The control membrane bands exactly at the same position as band 3. The equilibrium positions are: $\varrho = 1.1513$ for band 3 and control chromatophores; $\varrho = 1.1059$ for band 21; $\varrho = 1.0766$ for band 1.

Table I. Lipid phosphorous content of the fusion products induced by low pH by Ca^{2+} ions.

Fraction of the sucrose gradient	µg of phosphate low pH method	mg protein^{-1} Ca^{2+}
1	160 ± 8	–
2	60 ± 5	50 ± 4
3	18 ± 4	16 ± 2
chromatophores	12 ± 4	

In all cases small unilamellar liposomes were used for fusion with chromatophores. The final concentration of Ca^{2+} was 2.5 mM. The fractions to be analyzed were always repurified by a second sucrose gradient centrifugation.

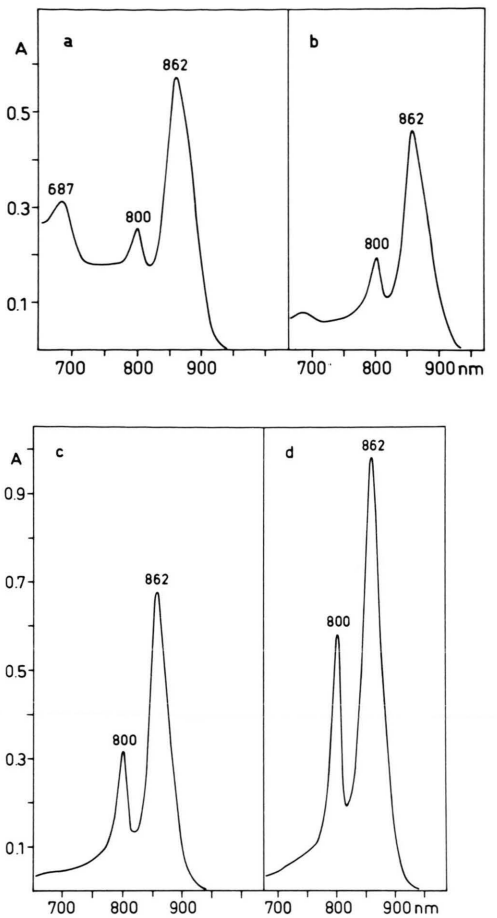

Fig. 2. Near infrared absorption spectra of the fused membranes obtained by the low pH procedure. 2a: fraction 1; 2b: fraction 2; 2c: fraction 3; 2d: chromatophores.

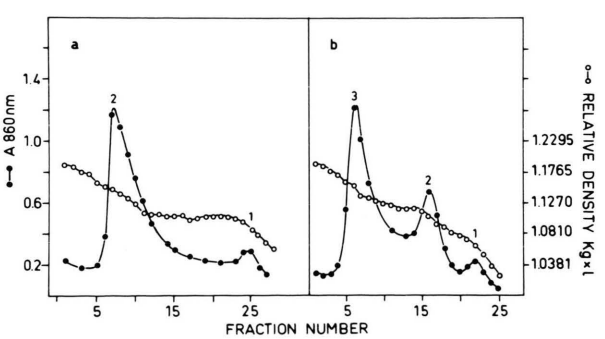

Fig. 3. Sucrose density centrifugation after chromatophore-liposome fusion induced by Ca^{2+} ions. 3a: Fusion obtained in the presence of 5 mM Ca^{2+}; 3b: Fusion obtained in the presence of 10 mM Ca^{2+}. The equilibrium positions are: $\varrho = 1.1513$ for fraction 1 (5 mM Ca^{2+}); $\varrho = 1.0899$ for fraction 2 (5 mM Ca^{2+}); $\varrho = 1.1415$ for fraction 3 (10 mM Ca^{2+}); $\varrho = 1.1013$ for fraction 2 (10 mM Ca^{2+}); $\varrho = 1.0592$ for fraction 1 (10 mM Ca^{2+}).

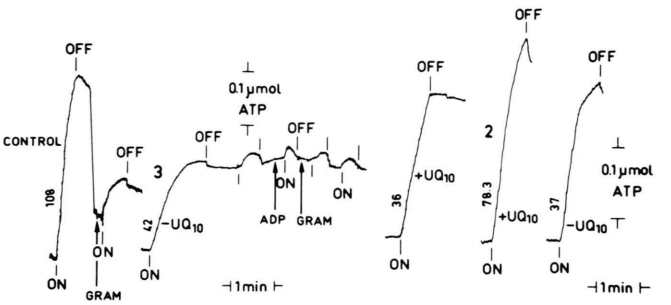

Fig. 4. Photophosphorylation kinetics for the control and for fraction 3 from the low pH procedure and fraction 2 from the freeze-thawing procedure. The numbers on the tracings indicate the corresponding rate of photophosphorylation in µmol ATP · h^{-1} · µmol $Bchl^{-1}$. The amounts of Bchl used are: 5.3 µM for the control and 0.73 µM for fraction 3 without UQ_{10} and 1.05 µM in the presence of UQ_{10} (low pH procedure) and 2.6 µM for fraction 2 (± UQ) (freeze-thawing). Where indicated GRAM (gramicidin) was added at a final concentration of 25 µg ml^{-1}. The photophosphorylation rate of untreated membranes was 280 µmol ATP · h^{-1} µmol $Bchl^{-1}$.

The Ca^{2+} induced fusion method gave similar products as the low pH method. However, the number of bands appearing in the sucrose gradient depended on the Ca^{2+} concentration (Fig. 3). Increase of Ca^{2+}-concentration resulted in aggregation of vesicles. Spectral changes, indicating Bchl destruction, were not observed. The absorbance ratio 860/800 nm was 1.8 in the fraction of higher density and 2.0 in the remaining fractions. Using large liposomes for fusion the absorption ratio was 1.75 for fraction 3, 1.9 for fraction 2 and 2.1 for fraction 1.

Three vesicle fractions were obtained at the same positions after freeze-thawing induced fusion and sucrose density centrifugation. The absorption spectra did not change during the procedure and no Bchl degradation was observed.

Electron transport and energy transducing capacities of fused membranes, photophosphorylation

The initial photophosphorylation rate of fraction 3 from low pH induced fusion was about 40% of the control. The reaction slowed down and came to a complete stop (Fig. 4). At 380 mV the photophosphorylation activity in the control membrane dropped down to 40% of their activity at 200 mV and to 1 to 2% in the fused vesicles.

Further addition of ADP did not restore the photophosphorylation. After a dark phase of 1 min

again a small photophosphorylation activity was observed. The results are in accordance with the hypothesis that in the fused vesicles the electron transport system became the limiting step.

It has been shown in chloroplasts and mitochondria that in fused vesicles the electron transport is inhibited by limited lateral diffusion of quinone [9, 10]. In order to test this idea chromatophores were fused with ubiquinone-loaded liposomes (see Materials and Methods). The liposomes contained 1.75 mg ubiquinone 10 per 10 g of phospholipids. The ratio of incorporated ubiquinone to total lipid phosphorous increased from fraction 3 to fraction 1. As the result of ubiquinone incorporation the photophosphorylation reaction was kept linear for a longer period, although the initial velocity was decreased in comparison with control membranes (Fig. 4).

The rates of photophosphorylation measured in the vesicle fraction obtained by the freeze-thawing method were higher than those obtained with the low pH method. The decrease of photophosphorylating activity in the vesicles prepared by freeze-thawing was found to be proportional to the amount of incorporated lipid. Addition of ubiquinone increased the activity (Fig. 4).

Electron transport activities

Phosphorylation activities linked to succinate and NADH oxidation were strongly reduced when fraction 3 of the fused vesicles, prepared by the low pH method, was used and compared with chromatophores. After complementation with ubiquinone a partial restoration of the succinate-linked oxidation was observed. NADH linked ATP formation was less activated (Table II). The activity of the succinate dehydrogenase was not impaired by liposome fusion, the activity of the NADH dehydrogenase was about 50% reduced (Table III). The activities of succinate and NADH-linked cytochrome *c* oxidoreductase were strongly reduced after liposome fusion and partially restored by addition of ubiquinone (Table III). The terminal oxidase activity was unaffected by the liposome fusion (Table III).

Table II. Oxidative phosphorylation in control membranes and fraction 3 obtained by the low pH induced fusion, with liposomes with or without ubiquinone 10.

			μmol ATP h^{-1} mg $prot^{-1}$		
Substrate	Acceptor	Inhibitor	Control	Fraction 3	Fraction 3 + UQ_{10}
Succinate	O_2	–	1.8	0.02	0.8
NADH	O_2	–	2.8	0.02	0.37
Succinate	O_2	Oligomycin	0.14	ND	0.02
Succinate	O_2	KCN	ND	ND	ND
NADH	O_2	Antimycin	0.34	ND	0.03
Succinate	Cyt *c*	–	0.60	0.02	0.2
NADH	Cyt *c*	–	1.2	0.02	0.02
NADH	Cyt *c*	Antimycin	0.03	ND	ND
NADH	Cyt *c*	CCCP	0.04	ND	ND

ND: Activity not detectable.

Table III. Respiratory activities of "low pH" fused vesicles with or without a supplement of ubiquinone 10 (UQ_{10}).

Fraction	Succinate dehydrogenase	NADH dehydrogenase	Succinate cyt *c* reductase	NADH cyt *c* reductase	Ascorbate DCPIP oxidase
Control	20	8	2.5	4.5	8
Fraction 1	15	5	0.22	0.6	7
Fraction 2	17	4.5	0.36	0.4	8.1
Fraction 3	17.8	3.5	0.39	0.5	8
Fraction 1 + UQ_{10}	15	3	0.19	0.45	6.3
Fraction 2 + UQ_{10}	18	5	0.54	1.8	6.8
Fraction 3 + UQ_{10}	17.4	5.5	0.69	1.7	8.2

The activities are expressed in μmol substrate oxidized per hour milligram of membrane protein.

Reaction center photobleaching

The size of the photosynthetic unit in low pH membrane fractions did not differ more than 5% for fractions 2 and 3 when compared to the control membranes. Fraction 1 showed, however, a lower value possibly due to the destruction of some of its B800–850 complexes during preparation (Table IV). It must be pointed out that the measurements of the RC photobleaching were performed in the presence of Antimycin, Valinomycin and 10 mM KCl to decrease the back pressure due to the electrical potential of the membrane. Likewise, maximum photobleaching was obtained by poising the external redox potential to 400 mV with ferricyanide. Similar results were obtained in Ca^{2+} or freeze-thawed samples.

Carotenoid band-shift and 9-aminoacridine fluorescence quenching

All fractions showed the characteristic light-induced electrochromic band-shift and also a K^+-diffusion induced band-shift in the dark. In low pH fused vesicles (Fig. 5) the relative values for $\Delta\Psi$ under irradiance were: 130 ± 7 mV in the control membranes, 150 ± 5 mV in fraction 3, 110 ± 6 mV in fraction 2, and 70 ± 8 mV in fraction 1. These values are consistently higher (except fraction 1) than those found in untreated chromatophore preparations, where a $\Delta\Psi$ of 100 mV ± 2 mV in three different chromatophore preparations was measured. This could be due to an effect of the fusion procedure changing membrane permeability. However, the interpretation of these results must be done with some reservations [28].

Under continuous light the extent of the signal has been decreased for fractions 1 and 2 and is identical in fraction 3 and control chromatophores. In the last case the decay of the signal in the dark is bi-phasic. When Antimycin was added to a concentration of 2.5 μg ml^{-1} the slower decay phase disappeared and the kinetics is monotonic. It is assumed that under the conditions of this experiment and in the absence of Antimycin the signal is due to the electrogenic operation of the electron transport sequence Cyt c_2-RC-UQ-cyt b and the decay is the consequence of a general H^+-permeability as well as to an electronic back reaction [29]. When the sequence of cyclic electron transport was interrupted by addition of Antimycin, then, probably, the decay signal is due to the electronic back reaction between UQ_{II} and the RC. It is interesting to point out that in the absence of Antimycin the slow phase of the decay became less conspicuous with increasing lipid content. In fraction 1 addition of Antimycin influences the size of the signal but it did not change the kinetics of the dark decay. This could be interpreted in agreement with the photophosphorylation results that the operativ-

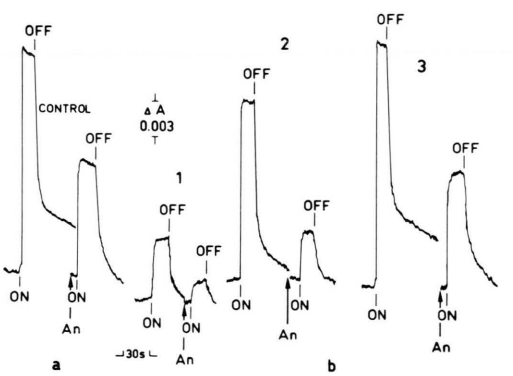

Fig. 5. Carotenoid band shift in low pH fused fractions. Where indicated (An) Antimycin A was added to a final concentration of 2.5 μg ml^{-1}. The concentrations used in this experiment were: Control 7.5 μM, fraction 3 6.7 μM, fraction 2 7.6 μM, fraction 1 3.3 μM. "ON", "OFF" light switched on and off, Time scale (30 s) and absorbance changes (0.003).

Table IV. Size of the photosynthetic unit, reaction center (RC) and bacteriochlorophyll content.

	Control	Fused membranes		
		Fraction 1	Fraction 2	Fraction 3
Photosynthetic unit	65 (± 2.5%)	50 (± 1%)	68 (± 1.5%)	62 (± 2%)
RC content pmol RC · mg $prot^{-1}$	240	236	238	245
Bchl content μmol Bchl · mg $prot^{-1}$	15	12	16.2	15.2

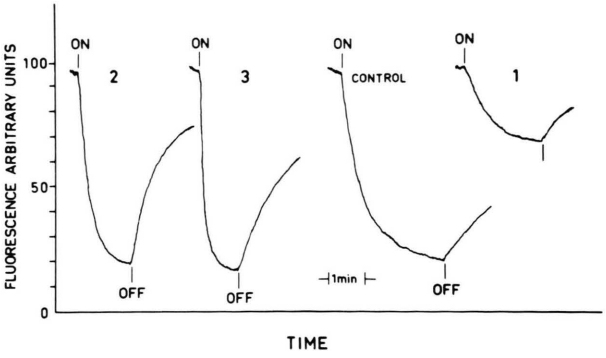

Fig. 6. 9-Aminoacridine fluorescence quenching in low pH fused fractions induced in the light. The Bchl concentration was: Control 7.7 μM, fraction 3 6.9 μM, fraction 2 8.0 μM, fraction 1 1.65 μM.

ity of the *b/c*-oxidoreductase complex had been highly diminished by the lipid incorporation and that, therefore, the decay observed is mainly due to the back reaction between the UQ secondary pool and the RC. Moreover, the decrease in the size of the signal in fraction 1 cannot be obviously explained unless it is assumed that Antimycin also increases membrane permeability to H^+.

All fractions showed a light induced fluorescence quenching of the 9-aminoacridine (Fig. 6). When fractions 2 and 3 were compared with the control a faster "on-off" kinetics indicating a higher permeability of the membrane to K^+ was observed. When Valinomycin was added to the control a similar "on-off" kinetics was induced. Since the specific volume of the fused samples is not known it is not possible to estimate the amount of ΔpH linked to the fluorescence quenching. In the control chromatophores, taking a value of 1.2 μl · mg protein^{-1} for volume [30], it was calculated that in the absence of Valinomycin the chromatophores can maintain a ΔpH of 3.5 units in the light. Assuming that in the fused samples the specific volume is a function of the vesicle size, it has been tentatively calculated (as in 31) that fractions 2 and 3 maintain a light induced ΔpH of 3.2 units and fraction 1 of 2.1 units.

Discussion

Intracytoplasmic membranes of photosynthetic bacteria contain, as other electron transport membranes, integral membrane complexes functional in respiratory and light driven energy conservation. These complexes must interact either by direct protein-protein contact or mediated by mobile carriers.

In the work described in this article liposomes prepared from phosphatidyl choline were fused with chromatophores. Although phosphatidyl-choline is not the major phospholipid component in *Rps. capsulata* and the fatty acid patterns in liposomes and chromatophores are different, we expected that the liposomes behave similar to chromatophores with regard to proton and ion permeability and particularly lateral diffusion of low molecular and high molecular components. The fusion was possible by three methods. The Bchl-protein complexes, especially B800 were found to be sensitive to lowering of pH. On the other hand, the Ca^{2+}-induced fusion and the freezing and thawing method resulted in spectroscopically unchanged vesicle fractions also having different lipid to protein ratios. Although all activities, which are dependent from cooperation of various membrane complexes, such as photophosphorylation of the respiratory chain activity, were found to be diminished in fused vesicles compared with chromatophores, the method seems to be useful to study interaction between the electron transport complexes in the membrane. The results showed that electron transport in the respiratory chain is impaired when the whole chain or the dehydrogenase-ubiquinone-cytochrome-*b/c*-oxidoreductase electron transport was measured (Table III), while the activities of the dehydrogenases and of the cytochrome oxidase are not changed or slightly reduced in vesicles fused with liposomes compared to chromatophores.

The tentative conclusion is that the increase in surface area, *i.e.* dilution of the mobile carriers, hindered electron transport between dehydrogenases and UQ-cytochrome *b/c* oxidoreductase.

Oxidative phosphorylation was completely inhibited in fused vesicles. Both processes, electron transport and oxidative phosphorylation were increased when ubiquinone was incorporated into liposomes (see also [10, 11]). The results presented here support the idea that the electron transport is greatly diminished in liposome-chromatophore-fused vesicles by dilution of the ubiquinone pool and a relative low lateral diffusional mobility of ubiquinol [19] because of the isoprenoid chain length (about 48 Å) (see [33]) and the dependence of the lateral mobility of lipid molecules on their length [32]. Addition of ubiquinone 10 restores also

some of the photophosphorylating capacity of fraction 2 (freeze-thawing method). In vesicles prepared by the low pH method the activity is partially lost due to the general effect of low pH on Bchl and may be other components of the system.

The carotenoid band-shift was observed in all fused vesicle preparations even when the electron transport activities were diminished.

It is concluded that proton permeability has not been diminished by liposome chromatophore fusion. This is further confirmed by the 9 aminoacridine fluorescence quenching activities.

Acknowledgements

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