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Functional Reconstitution of Photosystem I Reaction Center from Cyanobacterium *Synechocystis* sp PCC6803 into Liposomes Using a New Reconstitution Procedure

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Photosystem I reaction center from the cyanobacterium *Synechocystis* sp PCC6803 was reconstituted into phosphatidylcholine/phosphatidic acid liposomes. Liposomes prepared by reverse-phase evaporation were treated with various amounts of different detergents and protein incorporation was analyzed at each step of the solubilization process. After detergent removal the activities of the resulting proteoliposomes were measured. The most efficient reconstitution was obtained by insertion of the protein complex into preformed liposomes destabilized by saturating amounts of octylglucoside. In the presence of N-methylphenazonium methosulfate and ascorbic acid, liposomes containing the reaction center catalyzed a light-dependent net H^+ uptake as measured by the 9-aminoacridine fluorescence quenching and the pH meter. An important benefit of the new reconstitution procedure is that it produces a homogeneous population of large-size proteoliposomes with a low ionic permeability and with a majority inwardly directed H^+ transport activity. In optimal conditions, a light-induced ΔpH of about 1.8 units could be sustained at 20°C in the presence of valinomycin. In the absence of valinomycin, a "back-pressure" effect of an electrical transmembrane potential decreased both the rate and the extent of the H^+ transport. The reaction center was also co-reconstituted with F_0F_1 H^+-ATPases from chloroplasts and from the thermophilic bacterium, PS3. The co-reconstituted system was shown to catalyze a light-dependent phosphorylation which could only be measured in the presence of a high concentration of PSI (low lipid/PSI ratios) while no ΔpH could be detected.

KEY WORDS: Photosystem I reaction center; reconstitution; H^+-ATPase; proton gradient; proteoliposomes.

INTRODUCTION

The most important feature common to photosynthetic and respiratory organisms is an energized state that acts as an intermediate between electron flow and ATP synthesis. According to Mitchell's chemiosmotic hypothesis (Mitchell, 1966), this energized state is an electrochemical gradient for H^+ that acts as a driving force not only for ATP synthesis but also for many other reactions such as the translocation of ions other than H^+. Unambiguous approaches to mechanisms of energy coupling in intact cells or organelles use models in which the energy-transducing systems are incorporated into closed lipid bilayer membranes. A number of important results have been obtained with the use of reconstituted proteoliposomes in the area of oxidative and photosynthetic phosphorylation (Racker, 1972; Hinkel 1973; Gromet-Elhanan and Leiser, 1975; Orlich and Hauska, 1980; Hauska *et al.* 1980; Rigaud *et al.*, 1995; Casey, 1984; Van Walraven, 1990). In particular, reconstituted liposomes have been an

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advantageous system for the study of the generation and/or consumption of transmembrane electrochemical H^+ gradients, which are the obligatory energy-rich intermediate steps in energy transduction through mitochondria, bacteria, and chloroplast. However, only few combinations of functionally coupled proteins have been successfully reconstituted so far, and studies on ATP synthesis in co-reconstituted vesicles have mostly employed the light-driven proton pump bacteriorhodopsin (BR)[4] to generate a proton gradient (Rigaud *et al.*, 1995; Casey, 1984; Van Walraven, 1990).

In this framework we have recently set up optimal conditions for the reconstitution into liposomes of BR and F_0F_1 H^+-ATPases to perform studies on the mechanism of energy coupling between a $\Delta\mu_{H+}$ generator and a $\Delta\mu_{H+}$ consumer (Pitard *et al.*, 1996a). A stable $\Delta\mu_{H+}$ in the right orientation for ATP synthesis could be generated by the light-driven H^+-pump, and constant rates of ATP synthesis could be maintained for many hours. Such proteoliposomes have already proved to be useful for the study of the mechanism of ATP synthesis by ATP synthase from a thermophilic bacillus (TF_0F_1)[4] and offered new opportunities for exploring the coupling mechanisms between ATP synthesis and the protonmotive force (Pitard *et al.*, 1996b; Richard *et al.*, 1995). However, a general drawback of BR-F_0F_1 H^+-ATPase proteoliposomes was the low turnover of ATP synthesis [300–800 nmol ATP/(mg CF_0F_1 min)] as compared to those obtained with natural membranes [about 5000 nmol ATP/(mg CF_0F_1 min) obtained with thylakoids] or with F_0F_1 H^+-ATPase proteoliposomes under artificially imposed $\Delta\mu_{H+}$ [about 2500 nmol ATP/(mg CF_0F_1 min)] (Pitard *et al.*, 1996b; Van der Bend *et al.*; 1984; Kuprinsky and Hammes, 1986; Richard *et al.*, 1990). It was demonstrated (Pitard *et al.*, 1996b) that this limitation was more related to a property of the light-driven H^+-pump bacteriorhodopsin, which is retroinhibited by the $\Delta\mu_{H+}$ it generates (see also Westerhoff and Dawcshazy, 1984). The consequence of this back-pressure effect of ΔpH was to level off the magnitude of the ΔpH attainable in proteoliposomes to about 2 pH units at 20°C and 1.3 pH units at 40°C. Correspondingly the turnovers of photophosphorylation were found very low.

Surprisingly, very few studies have dealt with other light-dependent H^+-generators incorporated into liposomes (Krenn *et al.*, 1993; Graf *et al.*, 1985; Gabellini *et al.*, 1989). Reaction centers of photosystem I (PSI) from spinach chloroplasts reconstituted into lipid vesicles by sonication have previously been reported by Orlich and Hauska (1980) and Hauska *et al.* (1980). These authors presented a reconstituted system able to translocate protons upon illumination of these small proteoliposomes. Only a minor fraction of the vesicles contained the photosystem I reaction center in the appropriate polarity for proton uptake. Nevertheless, photophosphorylation occurred when the photosystem was co-reconstituted with the F_0F_1 H^+-ATPase from chloroplasts. However, the small size of the liposomes and the heterogeneity of the preparations have hampered further thermodynamic and kinetic studies. In the present work we have optimized the reconstitution into liposomes of the photoactivable redox proton gradient generator PSI from the cyanobacterium *Synechocystis* sp PCC6803, as an alternative to bacteriorhodopsin (BR). PSI proteoliposomes with a low permeability and good asymmetric protein orientation were prepared, able to develop a large light-induced pH gradient in the presence of reduced N-methylphenazonium methosulfate (PMS). It was further demonstrated that co-reconstitution of the ATP synthase from chloroplast (CF_0F_1) or thermophilic bacterium PS3 (TF_0F_1) with the reaction center could catalyze under specific conditions a light-induced ATP synthesis.

MATERIALS AND METHODS

Materials

Phosphatidylcholine (EPC) was purified from egg yolk as described by Singleton *et al.* (1965). The sources of the chemicals used in this study were as follows: egg phosphatidic acid (EPA) (Avanti); cholesterol (Sigma); Triton X-100 (Sigma); *n*-octyl β-D-glucopyranoside (OG) (Sigma); 9-aminoacridine (Eastman Kodak). All reagents were of the highest commercially available grade. SM-2 Bio-beads (Bio-Rad) were extensively washed before use as described

[4] Abbreviations: PSI, photosystem I reaction center; TF_0F_1, H^+-ATPase from thermophilic bacterium PS3; CF_0F_1, H^+-ATPase from chloroplast; BR, bacteriorhodopsin; EPC, egg phosphatidylcholine; EPA, egg phosphatidic acid; ΔpH, transmembrane pH gradient; $\Delta\Psi$, transmembrane electrical potential gradient; $\Delta\mu_H+$, transmembrane electrochemical potential gradient for protons; OG, *n*-octyl β-D-glucopyranoside; 9AA, 9-aminoacridine; PMS, N-methylphenazonium methosulfate; FCCP, carbonylcyanide-*p*-trifluoromethoxy phenylhydrazone.

by Holloway (1973). Polycarbonate filters were purchased from Nucleopore Corporation.

Preparation of Enzymes

Monomeric photosystem I reaction centers from *Synechocystis* sp PCC6803 were prepared according to Rögner *et al.* (1990a). Membranes were solubilized with 1% (w/v) *n*-dodecyl β-D-maltoside and the purification was carried out up to the sucrose density gradient purification. Reaction centers were then equilibrated in 10 mM sodium Tricine-NaOH, pH 7.8, containing 0.03% (w/v) β-D-maltoside, and concentrated by ultrafiltration (Centriprep 100, Amicon). Chlorophyll concentration was determined according to Arnon (1949). The chlorophyll-to-P700 ratio was found to be 90 in accordance with previously reported data (Sétif and Bottin, 1995).

The ATP synthase (TF_0F_1) from the membranes of thermophilic bacillus PS3 was extracted and purified according to Yoshida *et al.* (1975). Purified TF_0F_1 was lyophilized from 5 mM Tris/H_2SO_4 buffer, pH 8.0, and 10% sucrose and stored at −80°C. Before use the enzyme was resuspended at a concentration of 3 mg/ml in the medium used for reconstitution supplemented with 1 mg/ml Triton X-100.

The ATP synthase (CF_0F_1) from chloroplasts was isolated and purified as described earlier (Fromme *et al.*, 1987). After purification, 3 mg CF_0F_1 protein/ml were obtained in 2 mg/ml Triton X-100.

Preparation of Liposomes

Large unillamelar liposomes were prepared by reverse-phase evaporation as described previously (Rigaud *et al.*, 1983) with a 9:1 molar mixture of EPC and EPA as lipids. The final phospholipid concentration was 16 mg/ml (20 mM) in a buffer containing 25 mM KH_2PO_4·KOH, pH 7.3 (KPi buffer), or 25 mM sodium Tricine, pH 8.0 (Tricine buffer), both supplemented with 50 mM Na_2SO_4 and 50 mM K_2SO_4. These liposomes were then extruded through 0.4 and 0.2 μm polycarbonate membranes in order to obtain unilamellar vesicles within a narrow size distribution around 200 nm in diameter (Szoka *et al.* 1980; Rigaud *et al.*, 1983).

Reconstitution of Photosystem I into Liposomes

Liposomes were diluted to a phospholipid concentration of 4 mg/ml (5 mM) in the buffer used for their preparation and treated with different amounts of Triton X-100 or OG. The final detergent concentrations were 2, 4, and 8 mg/ml Triton X-100 or 7.5, 10, and 12 mg/ml OG to reach onset, half, and complete liposome solubilization respectively (Rigaud *et al.*, 1995; Paternostre *et al.*, 1988). These three solubilization stages can be followed by turbidity changes and correspond to the following: lipid bilayers saturated with detergent (onset); coexistence of lipid-detergent mixed micelles and saturated bilayers (half solubilization); and liposomes totally converted into mixed micelles (complete solubilization). After equilibration of the lipid detergent mixtures, the PSI preparation was added under vortexing, at a chlorophyll concentration ranging from 0.04 to 0.4 mg chlorophyll/ml (chlorophyll/PSI molar ratio from 100 to 10 w/w). The mixture was incubated for 5 min and then the detergent was removed by direct addition of SM-2 Bio-beads to the lipid/detergent/protein mixtures (Lévy *et al.*, 1990). This was generally performed by three successive additions: 80 mg wet beads/ml for one hour, followed by a second portion of 80 mg wet beads/ml for one hour. Then 160 mg wet beads/ml were added for an additional hour. The suspensions containing the reconstituted proteoliposomes were pipetted off and stored at 4°C for subsequent assays.

Co-reconstitution of PSI and F_0F_1-ATPases

Reconstitution of PSI/TF_0F_1 proteoliposomes was performed from liposomes totally solubilized in octylglucoside (OG/lipid = 3, w/w) supplemented with solubilized PSI and TF_0F_1 at the desired lipid-to-protein ratios. Due to the inhibitory effect of OG on CF_0F_1 and to the presence of ATPase aggregates in the purified CF_0F_1 preparations (Pitard *et al.*, 1996a), reconstitution of PSI/CF_0F_1 proteoliposomes was started from solubilized samples, by adding to liposomes a Triton X-100/PSI/CF_0F_1 mixture to give a final Triton X-100/lipid ratio of 2 (w/w).

Light-Induced Proton Movements in Reconstituted PSI Proteoliposomes

Two different methods were used to detect the pH gradient formation:

Light-induced ΔpH formation was monitored as changes in the fluorescence intensity of 9-aminoacridine. Fluorescence was monitored with an SLM Instruments spectrofluorimeter using 400 and 460 nm for excitation and emission, respectively. Illumination was performed with a 250 W xenon lamp through a flexible glass fiber guide equipped with a red filter with a low-wavelength cutoff at 600 nm and a heat filter. The cuvette holder was connected to a water bath in order to work at a constant temperature of 20 or 38°C. The reaction mixture always contained 10 mM ascorbic acid and 40 μM phenazine methosulfate (PMS) to support electron transport by PSI upon illumination (Orlich and Hauska, 1980; Hauska *et al.*, 1980).

Light-induced proton uptake into reconstituted proteoliposomes was also determined with a pH meter essentially as described earlier (Seigneuret and Rigaud, 1986a). pH measurements were carried out in 2-ml samples containing 0.5 ml of the proteoliposome preparation diluted with 1.5 ml of a nonbuffered solution (50 mM Na_2SO_4 and 75 mM K_2SO_4). During the pH measurements the samples were continuously stirred and kept at the desired temperature. O_2 was removed by gassing with N_2 since anaerobiosis is required to avoid proton uptake during chlorophyll-sensitized photooxidation processes.

Light-Induced ATP Synthesis in PSI-F_0F_1 ATP Synthase Proteoliposomes

Proteoliposomes were diluted 5- to 10-fold in the buffer used for their preparation and supplemented with 0.1 μM valinomycin, 10 mM ascorbate, 40 μM PMS, 1 mM ADP (for TF_0F_1), or 0.4 mM ADP (for CF_0F_1), and 25 mM KH_2PO_4 when Tricine buffer was used. The reaction mixtures were illuminated in stirred tubes immersed in a transparent water bath at the desired temperature. After illumination for 5–10 min to allow the generation of a stable transmembrane gradient, ATP synthesis was initiated by addition of 1.5 mM $MgSO_4$. Aliquots were taken at different reaction times and quenched with an equal volume of 4% trichloroacetic acid. ATP was determined by means of the luciferin-luciferase enzymatic assay using an LKB luminometer (Pitard *et al.*, 1996a, b; Richard *et al.*, 1995).

Determination of the Proteoliposome Size

The diameter of the proteoliposomes was determined using a Microtrac ultrafine particle analyzer (Pennsylvania, USA), designed to measure the distribution of particle sizes by a dynamic light scattering technique in the range between 3 nm to 6.54 μm. Samples were diluted with the corresponding buffer to a final phospholipid concentration of 2 mg/ml.

RESULTS AND DISCUSSION

Reconstitution of PSI into Liposomes

Optimal Reconstitution Conditions

The standard procedure for reconstitution of PSI was developed from a strategy for investigating the mechanisms by which proteins associated with the lipids in detergent-mediated reconstitutions (for a review see Rigaud *et al.*, 1995). The protocol was based on the idea that such reconstitution procedures are a reverse of membrane-detergent solubilization. Accordingly, liposomes prepared by reverse-phase evaporation were first treated with different amounts of detergents in order to get onset, half, or total liposome solubilization. Then, a solution of solubilized PSI was added. The detergent–protein–phospholipid mixtures at each adjusted step in the lamellar-to-micellar transition were kept at room temperature for 5 min. After detergent removal by successive addition of SM-2 Biobeads, the resulting proteoliposomes were analyzed for their biological activities.

The efficiency of the reconstitution was analyzed by measuring the formation of a light-induced transmembrane pH gradient in the presence of an artificial redox system (Orlich and Hauska, 1980; Hauska *et al.*, 1980). In the presence of N-methylphenazonium methosulfate which translocates protons from outside to inside the liposomes during its redox cycle, the light-induced charge separation of PSI incorporated into the liposomal membrane led to the formation of a pH gradient (see Fig. 1). The fluorescence quenching of 9-aminoacridine was taken as a qualitative reporter for this transmembrane proton gradient. Figure 2 shows some representative fluorescence records for different PSI-proteoliposomes. In all cases actinic illumination of PSI proteoliposomes in the presence of PMS induces a time-dependent decrease of fluorescence intensity related to the accumulation of 9-aminoacridine upon a ΔpH acidic inside. This effect slowly reverted in the dark due to the passive H^+ re-equilibration along its gradient.

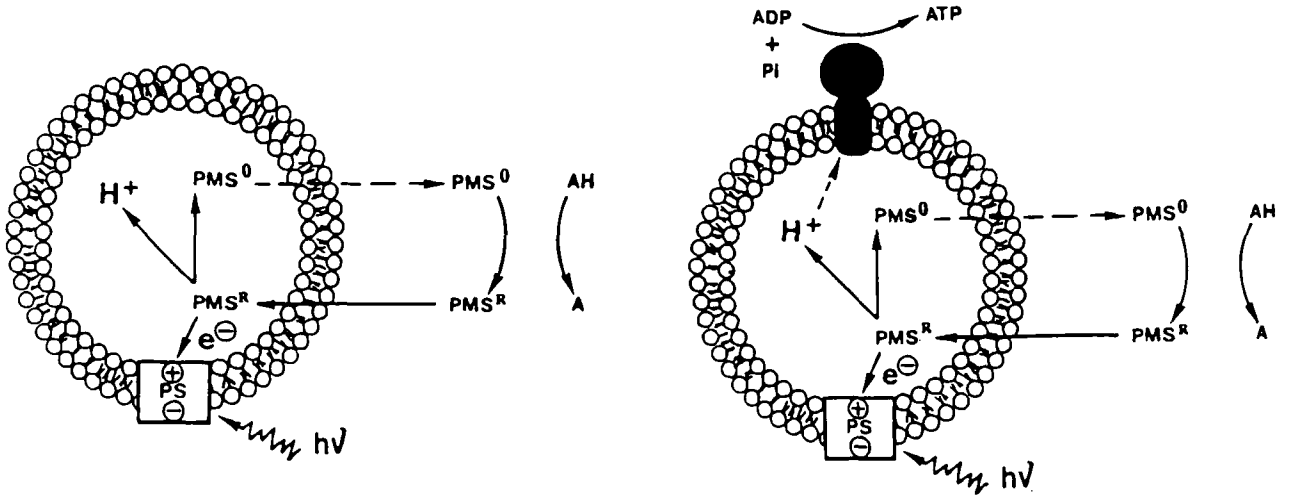

Fig. 1. Scheme of the Photosystem I–methylphenazonium methosulfate–ascorbate transmembrane redox cycle. PMS^O and PMS^R are the N-methylphenazonium methosulfate oxidized and reduced forms, respectively. Detailed redox reactions are described in Evans and Bolton (1979).

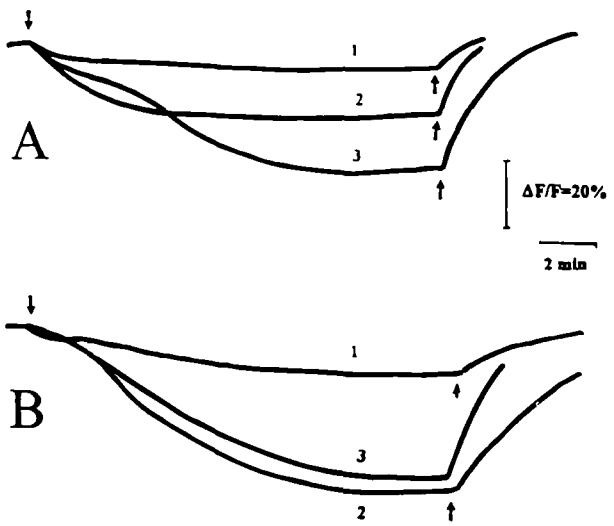

Fig. 2. Detergent-mediated reconstitution of PSI reaction center. Liposomes (4 mg/ml) prepared by reverse-phase evaporation were treated with variable amounts of octylglucoside or Triton X-100 (1, 2, 3 correspond to the onset of solubilization, half solubilization, and total solubilization respectively). Solubilized PSI (40 μg chlorophyll) was then added to each sample under vortex mixing (incubation medium:50 mM Na_2SO_4, 50 mM K_2SO_4, 25 mM KH_2PO_4, pH 7.3, 20°C). After incubation for 5 min detergent was removed by treatment with SM-2 Bio-beads. The resulting proteoliposomes were resuspended at 0.2 mg lipid/ml in the same buffer used for their preparation supplemented with 0.1 μM valinomycin, 40 μM PMS, 10 mM ascorbate, and 0.1 μM 9AA. Light-induced fluorescence changes of 9AA were measured at 20°C (λ_{exc} = 400 nm, λ_{em} = 460 nm). Panel A: Triton X-100 mediated reconstitutions. Curves 1, 2, 3 correspond to Triton X-100/lipid ratios of 0.5, 1, and 2 (w/w) respectively. Panel B: octylglucoside mediated reconstitutions. Curves 1, 2, and 3 correspond to OG/lipid ratios of 1.9, 2.5, and 3 (w/w) respectively. (↓) Light on, (↑) light off.

Figure 2A shows the effect of the initial Triton X-100 concentration on the light-dependent H^+ transport activity of reconstituted PSI proteoliposomes. Maximal activities, and thus optimal reconstitutions, were obtained when starting from totally solubilized samples. Interestingly, the results of the reconstitution studies with octylglucoside (Fig. 2B) indicated that maximal activities were obtained using OG concentrations much lower than those necessary for complete solubilization of the initial phospholipid suspensions. Systematic studies indicated that optimal H^+ transport activities occurred starting from lipid/detergent suspensions where 30–50% of the initial liposomes were solubilized. It was further observed that optimal incorporation of PSI into vesicles occurred rapidly, since after 5 min of incubation of solubilized photosystem with OG-treated liposomes, the resulting initial rates and total proton pumpings were already maximal. Furthermore, when two reconstitutions were performed, one such that solubilized PSI was added to OG-treated liposomes (experiments reported in Fig. 2B) and the other such that the appropriate amount of OG was first added to the protein and next added to preformed liposomes, identical activities were obtained after only 5 min of incubation. Therefore, a rapid equilibration occurred, i.e., movement of PSI and/or phospholipids between micelles and detergent-saturated liposomes.

Under optimal conditions light-induced internal acidification led to a time-dependent decrease of 9-aminoacridine fluorescence intensity, reaching a steady level of 45% decrease when OG was used for reconstitution, or 22% when Triton X-100 was employed. Even

taking into account the differences in the mean diameter of OG and Triton X-100 reconstituted proteoliposomes (120 and 175 nm, respectively), the 9-aminoacridine fluorescence quenching observed for OG-mediated reconstitution corresponds to a much larger ΔpH than when using Triton X-100-mediated reconstitution.[5]

Other detergents like Chaps, Chapso, and *n*-dodecyl β-D maltoside were also tested but in all cases the light-dependent proton pumping activities were found much lower than with Triton X-100 and OG.

Thus, the most striking feature of our systematic studies of detergent-mediated reconstitution experiments is that the most efficient PSI-proteoliposomes were obtained using OG-mediated reconstitutions in which rapid transfer of the protein from micelles into preformed liposomes saturated with OG was evidenced. It is interesting to compare the results of PSI reconstitution with previous findings obtained under similar experimental conditions with other membrane proteins (Rigaud *et al.*, 1988; Lévy *et al.*, 1992; Richard *et al.*, 1990). In particular, it was demonstrated that bacteriorhodopsin, Ca^{2+}-ATPase, and F_0F_1 H^+-ATPases could be directly incorporated at the onset of liposome solubilization by octylglucoside. Results from PSI reconstitution indicated that this protein can also be incorporated into OG-saturated liposomes but by a mechanism which required the presence of micellar structure since optimal reconstitution was found at 30–50% of liposome solubilization. This observation can be analyzed taking into account the mechanisms proposed by Rigaud *et al.* (1995) (see also Helenius *et al.*, 1981, and Christiansen and Carlsen, 1985) to explain the incorporation of membrane proteins into preformed liposomes destabilized by saturating amounts of detergents.

It was proposed that the insertion of protein oligomers into preformed liposomes occurred via the monomeric form and would depend upon the stability of protein oligomers. In the case of PSI complex, probably due to the high propensity of this protein for self-association (Boeckema *et al.*, 1989; Rögner *et al.*, 1990; Ford and Holzenberg, 1988) the size and/or stability of protein oligomers might be so high that they would need an excess of OG to allow dissociation of protein oligomers and insertion of monomers into the remaining detergent-saturated liposomes. Another important aspect when dealing with membrane protein reconstitution mechanisms concerns the final orientation of the protein in the bilayer. In many cases in which protein reconstitution occurred through a mechanism of direct incorporation into preformed liposomes, proteins were found almost unidirectionally oriented whereas proteoliposomes with a more random orientation were obtained by detergent removal from phospholipid–detergent–protein micelles (Rigaud *et al.*, 1995; Eytan, 1982). Although we have not performed experiments directed at determining the precise orientation of PSI molecules in the vesicles, it should be stressed that the most efficient PSI proteoliposomes were obtained using OG-mediated reconstitutions in which PSI incorporation occurred well before total solubilization, i.e., in the presence of detergent-saturated liposomes.

Light-Induced Proton Transport

Energization of the reconstituted proteoliposomes by light was further analyzed in detail. In the first place, we have shown that the fluorescence quenching of 9AA reflected, at least qualitatively, the formation of a H^+ gradient across the liposomal membrane. Three ways were used to abolish the acidic pH gradient inside the vesicles (Fig. 3): NH_4Cl, which generates a dissipative NH_3-uptake/NH_4^+-release cycle (Orlich and Hauska, 1980); FCCP as a known protonophore; and nigericin as an ionophore that catalyzes an electroneutral exchange of K^+ for H^+. It was observed that when FCCP (0.1 μM), nigericin (7 μM), or NH_4Cl (150 μM) were added to the reaction medium, no 9AA decrease was detected. These results confirm the generation of a proton gradient, acidic inside, upon illumination of PSI proteoliposomes.

Secondly, to provide evidence for the formation of a membrane potential upon illumination of PSI liposomes, we have measured the influence of valinomycin on the extent and kinetics of the 9AA fluorescence quenching. As shown in Fig. 3, the presence of valino-

[5] Fluorescence quenching of 9-aminoacridine has been related to the transmembrane ΔpH according to Deamer *et al.* (1972): $\Delta pH = \log (Q/(1 \cdot Q))\ (V_{out}/V_{int})$, where Q is the fraction of the total fluorescence that is quenched, V_{int} and V_{out} are the internal and external volumes respectively, and ΔpH is the internal pH minus the external pH. Using the Q values given in the text and the internal volumes calculated from the average diameters, we have estimated the ΔpH values for OG and Triton X-100 reconstitutions. Since the ΔpH calculation from 9-aminoacridine measurements have been criticized, we have not used these values to give an absolute quantification of the ΔpH but just to compare two different reconstitution conditions which gave different internal volumes.

mycin allowed proton pumping to develop with a much higher initial rate and led to a steady-state ΔpH about 2-fold higher than in the absence of the antibiotic. This stimulating effect of valinomycin on the initial H^+ uptake and the final H^+ gradient obtained in PSI proteoliposomes might be related to the retroinhibitory effect of the membrane potential upon the activity of PSI. While this effect has already been reported by Orlich and Hauska (1980), it appears to be much stronger in our liposomes since the total extent of H^+ translocation is shown to drastically increase in the presence of valinomycin. In the presence of valinomycin, the $\Delta\Psi$ is overcome by compensatory K^+ movements and a large ΔpH (acidic inside) can develop due to the high rate of proton pumping. In addition, Fig. 3 indicates that the proton passive efflux rates, measured through V_{OFF}, increase with valinomycin, confirming the good degree of impermeability of the proteoliposomes able to catalyze the formation of a light-dependent transmembrane $\Delta\Psi$.

Thirdly, since the use of 9AA fluorescence quenching as a quantitative ΔpH reporter has been questioned (Fiolet *et al.*, 1974) we have measured the proton movements of the PSI proteoliposomes through the use of a glass electrode. As shown in Fig. 4 the illumination of PSI proteoliposomes in a low KPi buffered solution (pH 7.3) produces an alkalinization of the external medium. This result was expected taking into account the results obtained with 9AA and was in contrast with the results reported by Orlich and Hauska (1980). These authors, using sonicated PSI proteoliposomes, measured the external medium acidification upon illumination besides of measuring 9AA fluorescence quenching for the same proteoliposomes. They proposed that this fact originated from the exis-

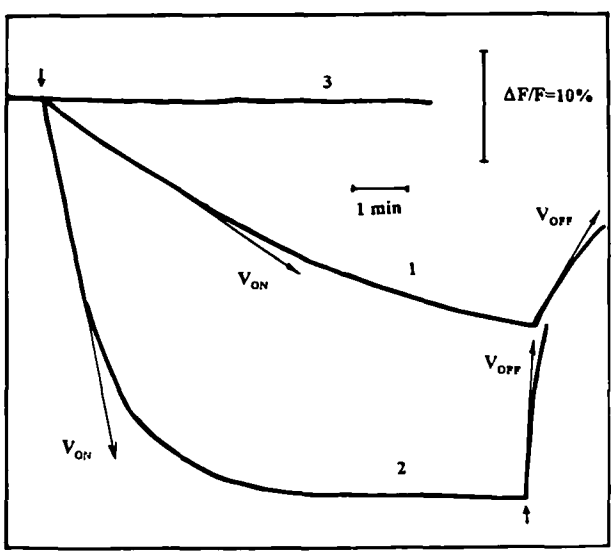

Fig. 3. Effect of valinomycin, NH_4Cl, nigericin, and FCCP on the light-induced 9AA fluorescence quenching. PSI proteoliposomes were reconstituted from liposomes half-solubilized with OG in a medium containing 50 mM Na_2SO_4, 50 mM K_2SO_4, and 25 mM Tricine, pH 8.0 (lipid/chlorophyll = 100 w/w). After reconstitution proteoliposomes were assayed for light-dependent H^+ transport following 9AA fluorescence quenching as described in Fig. 2. Light-induced fluorescence changes were measured at 20°C: (1) control; (2) in the presence of 0.1 μM valinomycin; (3) in the presence of 150 μM NH_4Cl or 0.1 μM FCCP or 7 μM nigericin. (↓) Light on, (↑) light off.

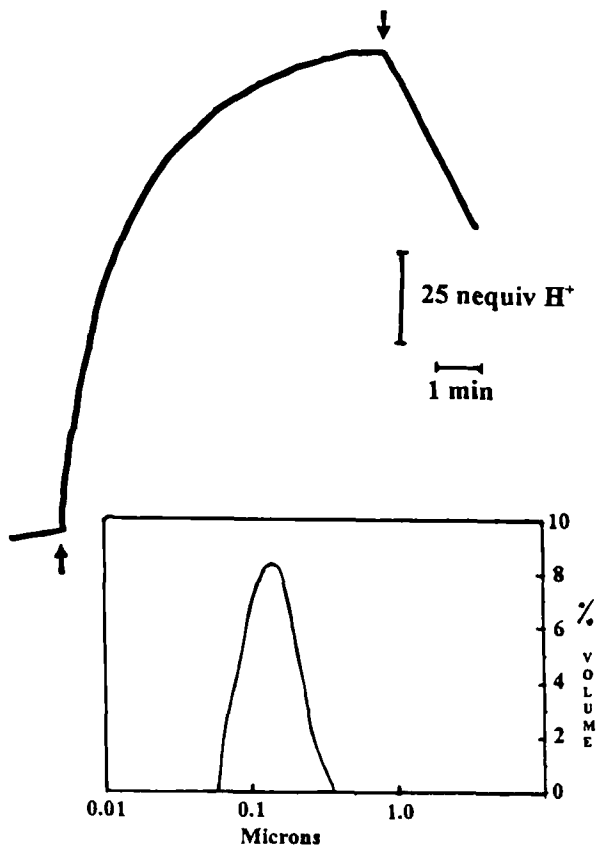

Fig. 4. External medium alkalinization due to light-induced H^+ pumping into PSI proteoliposomes. PSI proteoliposomes were reconstituted from liposomes half solubilized with OG in a medium containing 50 mM Na_2SO_4, 50 mM K_2SO_4, and 25 mM KH_2PO_4, pH 7.3 (lipid/chlorophyll = 100 w/w). After reconstitution pH measurements were carried out in 2 ml cuvettes containing 0.5 ml of the proteoliposomes diluted with 1.5 ml of a nonbuffered saline solution (50 mM Na_2SO_4 and 75 mM K_2SO_4), supplemented with 10 mM ascorbate, 40 μM PMS, and 0.1 μM valinomycin and gassed with N_2 (temperature 20°C). Inset: percentage of the total internal volume of the proteoliposomes corresponding to each diameter. (↓) Light on, (↑) light off.

tence of two vesicle populations in their reconstituted preparations: one right-side oriented (responsible for the 9AA quenching) and one oriented inside-out (responsible for the external medium acidification) with respect to the orientation of the incorporated reaction center complex.

Therefore, one important benefit of our reconstitution procedure is to produce a homogeneous proteoliposome preparation with one predominant protein orientation. This is a clear advantage compared to a nonhomogeneous distribution of two PSI orientations among two populations of proteoliposomes which exhibit oppositely directed proton pumping. Indeed the occurrence of inwardly and outwardly pumping proteoliposomes can introduce serious errors in measurements of the components of the electrochemical proton gradient. In this context, the amplitude of the light-induced H^+ gradient can be estimated in our proteoliposomes from the pH meter measurements. From the number of protons which have been pumped into liposomes, the elicited ΔpH can be calculated knowing the internal buffer capacity (deduced from titration of the buffer used for reconstitution) and the internal volume of the liposomes (deduced from the mean size of the liposomes). The inset in Fig. 4 shows the size distribution of our proteoliposome preparation. As can be seen, it is a very homogeneous population with a mean diameter of 120 nm corresponding to a proteoliposome internal volume of 4.29 μl/mg lipid, assuming that the double lipidic layer is 40 Å thick and an area of 70 $Å^2$ for the polar phospholipid headgroups. From all the experiments performed with the pH meter during the course of our studies, a mean maximal ΔpH of 1.8 ± 0.3 was calculated. For comparison, recall that the highest light-induced ΔpH values reported for bacteriorhodopsin proteoliposomes prepared with the same methodology were about 2–2.5 pH units (Rigaud *et al.*, 1988).

Influence of Different Physical and Chemical Parameters on the H^+-Pumping Activity

The extent and the kinetics of the 9AA fluorescence quenching have been further analyzed as a function of various reaction conditions. One critical factor is the respective concentrations of methylphenazonium methosulfate and ascorbate. As shown in Fig. 5A, 80 μM PMS inhibits pH gradient formation, probably because of improper functioning of the redox cycle (Orlich and Hauska, 1980). Concerning ascorbate, a minimal concentration of 5–10 mM was found to be necessary, in agreement with previous results (Orlich and Hauska, 1980).

We have also tested the influence of the internal buffer upon light-induced proton pumping. For these experiments, PSI proteoliposomes were prepared with different concentrations of phosphate buffer. Figure 5B shows the 9AA fluorescence quenching responses for preparations internally buffered with 10–50 mM phosphate. Apparent initial rates of H^+ pumping decrease with increasing buffering power. In this instance this only means that to elicit a given ΔpH, more protons are required at high buffer capacity than at low buffer capacity. Steady-state ΔpHs, on the other hand, appear independent of the internal buffer capacity, indicating that the increase of buffering power is compensated by a proportional increase in the extent of proton translocation, leading to a constant steady-state pH gradient. Such buffer-independent ΔpH values are expected for a simple pump and leak system.

The fluorescence responses of 9AA at different light intensities are shown in Fig. 5C for PSI proteoliposomes. The corresponding values of initial apparent pumping rate (V_{ON}) and $(\Delta F/F)_{max}$ are plotted as a function of light intensity. From this figure it can be seen that both parameters increase with light intensity and level off at the same saturating light intensity. These results are in contrast with those previously reported for bacteriorhodopsin proteoliposomes (Seigneuret and Rigaud, 1986b; Hellingwerf *et al.*, 1979) in which the total proton uptake was found to level off rapidly while the initial uptake rate was still increasing with light intensity. This observation furnished one of the early pieces of evidence for the retroinhibition of bacteriorhodopsin by the proton gradient it generated. The consequence of this retroinhibitory effect was to level off the maximum ΔpH attainable across BR proteoliposomes. From our results varying the light intensity, it can be seen that with PSI proteoliposomes there are no conditions in which the steady-state ΔpH became independent of the initial rate of H^+ pumping. So, a back pressure effect of ΔpH, similar to that observed for BR, can be discarded for PSI-proteoliposomes, which behave as a simple pump and leak system. Finally, Fig. 5D indicates the light-induced fluorescence quenching dependence on the pH of the medium. Maximal activities were found at pH 8.0 and almost no activity at pH 6.0

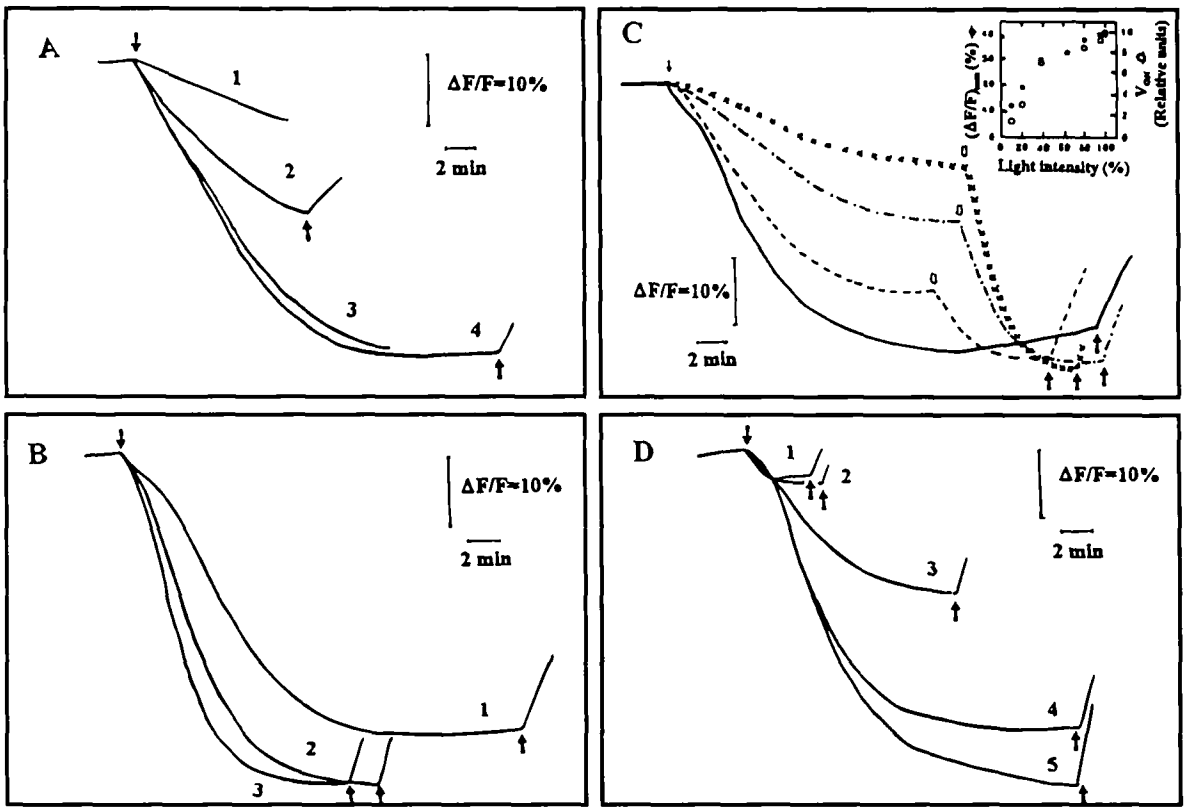

Fig. 5. Influence of different physical and chemical parameters on light-dependent H^+ pumping activity. PSI proteoliposomes were reconstituted as described in Fig. 4 in a medium containing 50 mM Na_2SO_4, 50 mM K_2SO_4, and variable amounts of KH_2PO_4 at different pH (lipid/chlorophyll = 100 w/w). After reconstitution, proteoliposomes were resuspended at 0.2 mg lipid/ml and analyzed for their light-dependent H^+ transport activity using 9AA. Panel A: effect of PMS and ascorbate concentration on 9AA fluorescence quenching. Proteoliposomes were reconstituted and resuspended in a medium containing 50 mM Na_2SO_4, 50 mM K_2SO_4, and 25 mM KH_2PO_4, pH 7.3. Samples were supplemented with 0.1 μM 9AA, 0.1 μM valinomycin, and different amounts of PMS and ascorbate: (1) 20 μM PMS, 10 mM ascorbate; (2) 80 μM PMS, 10 mM ascorbate; (3) 40 μM PMS, 10 mM ascorbate; (4) 40 μM PMS, 5 mM ascorbate. Panel B: effect of internal buffer capacity on 9AA fluorescence quenching. Proteoliposomes were prepared and resuspended in a medium containing 50 mM Na_2SO_4, 50 mM K_2SO_4, and (1) 50 mM, (2) 25 mM, and (3) 12.5 mM KH_2PO_4, pH 7.3. Panel C: effect of light intensity on 9AA fluorescence quenching. Proteoliposomes were reconstituted and resuspended in a medium containing 50 mM Na_2SO_4, 50 mM K_2SO_4, and 25 mM KH_2PO_4, pH 7.3. Samples supplemented with 0.1 μM 9AA, 0.1 μM valinomycin, 40 μM PMS, and 10 μM ascorbate were illuminated at 20°C through different actinic light intensities: (——) 100%, (---) 38%, (-·-·-) 20% and (xxxx) 10% light intensity. Inset: effect of actinic light intensity upon initial rates of light-induced 9AA fluorescence change (V_{ON}, -○-) and upon maximum steady-state fluorescence quenching (-●-). (↓) Filter removed to recover 100% light intensity. Panel D: effect of pH on the 9AA fluorescence quenching. Proteoliposomes were reconstituted in a medium containing 50 mM Na_2SO_4, 50 mM K_2SO_4, and 25 mM KH_2PO_4, pH 7.3. After reconstitution they were resuspended at 20°C in the same medium adjusted to the indicated pH and supplemented with 40 μM PMS, 10 mM ascorbate, 0.1 μM valinomycin, and 0.1 μM 9AA. (1) pH 6.2; (2) pH 6.6; (3) pH 7.1; (4) pH 7.3; (5) pH 8.0. All samples were supplemented with 40 μM PMS, 10 mM ascorbate, 0.1 μM valinomycin, and 0.1 μM 9AA and incubated at 20°C. (↓) Light on, (↑) light off.

Effect of Cholesterol, Temperature, and Lipid/PSI Ratio

Another approach to analyze a pump and leak system is to vary the proton passive permeability or the number of H^+-pumping units. In this context we have studied the influence of cholesterol, known to affect the membrane fluidity and consequently the passive permeability. An important finding was that cholesterol strongly stimulated the efficiency of reconstituted proteoliposomes: Fig. 6 shows that total H^+ uptake increases up to 2-fold in the presence of 10% (w/w) cholesterol. Smaller increases were found when the cholesterol concentration was increased up

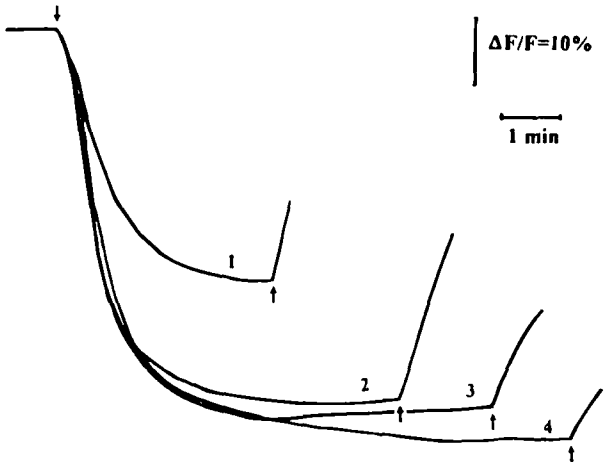

Fig. 6. Effect of cholesterol on the light-induced H^+ transport activity of PSI proteoliposomes. Liposomes containing different amounts of cholesterol were prepared by reverse-phase evaporation. After total solubilization with OG they were supplemented with solubilized PSI (lipid/chlorophyll = 100 w/w). After detergent removal, proteoliposomes were assayed for their light-dependent H^+ transport activity in a medium containing 50 mM Na_2SO_4, 50 mM K_2SO_4, 25 mM Tricine, pH 8.0, 40 μM PMS, 10 mM ascorbate, 0.1 μM valinomycin, and 0.1 μM 9AA and incubated at 20°C. (↓) Light on, (↑) light off.

to 30%. Interestingly it can be observed that the initial rates of proton pumping are not significantly changed by the cholesterol content, indicating similar PSI incorporation. On another hand, note that the efflux of H^+ when light was switched off decreased with increasing cholesterol although in the presence of a much higher ΔpH under steady-state conditions. These observations point to a decrease in passive permeability of PSI proteoliposomes upon increasing cholesterol content, the final consequence being to increase the amplitude of the light-induced pH gradient across the membrane of the liposomes.

Another critical factor determining the extent of H^+ uptake in proteoliposomes is the temperature of the reaction medium since 9AA quenching was not observed at 38°C for any given lipid/PSI ratio and whatever the buffer used for reconstitution. This may be related to a higher proton (and counterion) passive permeability, which has been shown to drastically increase with temperature (Seigneuret and Rigaud, 1986a). However, for comparison, BR proteoliposomes which develop a light-dependent ΔpH of 2 pH units at 20°C were still able to develop a pH gradient of about 1.3 pH units at 40°C (Pitard *et al.*, 1996ab). Thus, other parameters than a simple increase in passive permeability with temperature have to be taken into account to explain the absence of a measurable ΔpH in PSI liposomes at 38°C. This may be due to an inhibition of the activity of the reaction center at high temperature but also, more probably, to an increase in the permeability of both PMS and ascorbate which would affect drastically the efficiency of the artificial redox compounds to convert the charge separation in the PSI reaction center into a H^+ gradient.

We have also investigated the effect of varying the rate of proton pumping upon formation of the proton gradient by varying the number of pumping units. Figure 7 shows the light-induced 9AA fluorescence responses for PSI proteoliposomes reconstituted at lipid-to-chlorophyll weight ratios of 100, 50, 25, and 10, in the presence of valinomycin. Surprisingly, increasing the PSI content of the proteoliposomes diminishes drastically the extent of 9AA fluorescence quenching and at a lipid/chlorophyll ratio of 10 (w/w) the light-induced fluorescence decrease becomes almost undetectable (Fig. 7). From the initial slopes of fluorescence variations when the actinic illumination is switched off (back-leakage) it can be concluded that increasing the protein content in the liposomes increases significantly the passive proton permeability of the membrane (at a lipid/chlorophyll ratio of 100 about 12 molecules of PSI were incorporated per vesicle). However, more puzzling is the observation that

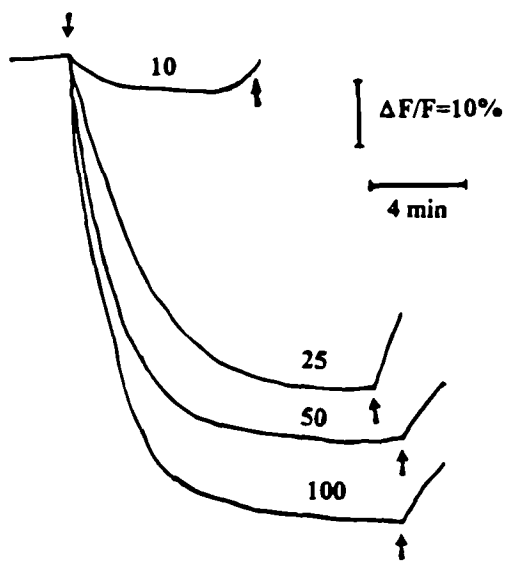

Fig. 7. Light-induced H^+ movements in PSI proteoliposomes reconstituted at different lipid/PSI ratios. Proteoliposomes containing 20% cholesterol were reconstituted at lipid/chlorophyll ratios of 100, 50, 25, and 10 (w/w). Light-induced changes in 9AA fluorescence were measured in a medium containing 50 mM Na_2SO_4, 50 mM K_2SO_4, 25 mM Tricine, pH 8.0, 40 μM PMS, 10 mM ascorbate, 0.1 μM valinomycin, and 0.1 μM 9AA and incubated at 20°C. (↓) Light on, (↑) light off.

the initial rates of H^+ pumping when the actinic illumination is switched on, decrease with protein content. This clearly indicates a less efficient PSI incorporation and/or orientation at low lipid-to-chlorophyll ratio. In this context, we have studied the homogeneity of PSI proteoliposomes at different lipid-to-protein ratios. Figure 8 shows the volume distribution vs. the diameter of proteoliposome reconstituted at lipid/chlorophyll ratios of 100:1 and 50:1. It can be foreseen that a large-size particle population (around 1400 nm diameter) appears upon increasing the protein content. This large-size particle population may correspond to large multilamellar liposomes, to vesicle aggregates, and/or to aggregates of nonincorporated proteins. Although more detailed studies are needed for a final interpretation of this observation, it should be recalled that PSI has a high tendency toward aggregation and/or oligomerization which could greatly influence the incorporation of this protein into liposomes at high protein content: it might be that the second particle population which appears with increasing PSI content resulted from a heterogeneous incorporation of PSI monomers and PSI trimers (Boeckema *et al.*, 1989; Rögner *et al.*, 1990b; Ford and Holzenberg, 1988).

Co-reconstitution of PSI and F_0F_1 ATP Synthases into Liposomes

The results reported above indicate that the detergent-mediated reconstitution method is useful for obtaining PSI proteoliposomes able to generate a light-dependent proton gradient acidic inside. Next, we tried to co-reconstitute the PSI with a F_0F_1-ATP synthase, in order to investigate the ability of the ATPsynthase to use this protonmotive force to synthesize ATP from ADP and P_i. With this goal in mind co-reconstitution experiments of PSI with the thermophilic H^+-ATP synthase (TF_0F_1) and with the chloroplast H^+-ATP synthase (CF_0F_1) were carried out. No ATP synthesis was observed when PSI-TF_0F_1 or PSI-CF_0F_1 proteoliposomes (lipid/chlorophyll = 100) were analyzed at 38°C. This result was expected since no 9AA quenching could be observed at 38°C. Surprisingly no ATP synthesis was observed with proteoliposomes containing CF_0F_1 and PSI (lipid/chlorophyll = 100) at 20°C, conditions in which, however, a clear 9AA fluorescence quenching was measured. The absence of ATP synthesis is in contrast with the photophosphorylation reported by Hauska *et al.*, (1980) with their reconstituted system, containing photosystem I and coupling factor complex from spinach chloroplasts. Noteworthy is the fact that when the lipid/chlorophyll ratio was lowered to 10:1, a significant ATP synthesis was observed with PSI-TF_0F_1 and PSI-CF_0F_1 proteoliposomes at 38°C (Fig. 9), although in these cases, no 9AA fluorescence quenching could be detected. The rates of light-induced ATP synthesis determined from different experiments with PSI-TF_0F_1 proteoliposomes are 150 ± 20 nmol ATP/(mg TF_0F_1 min) and 30 ± 3 nmol ATP/(mg CF_0F_1 min) with PSI-CF_0F_1 proteoliposomes. These values are comparable to those pre-

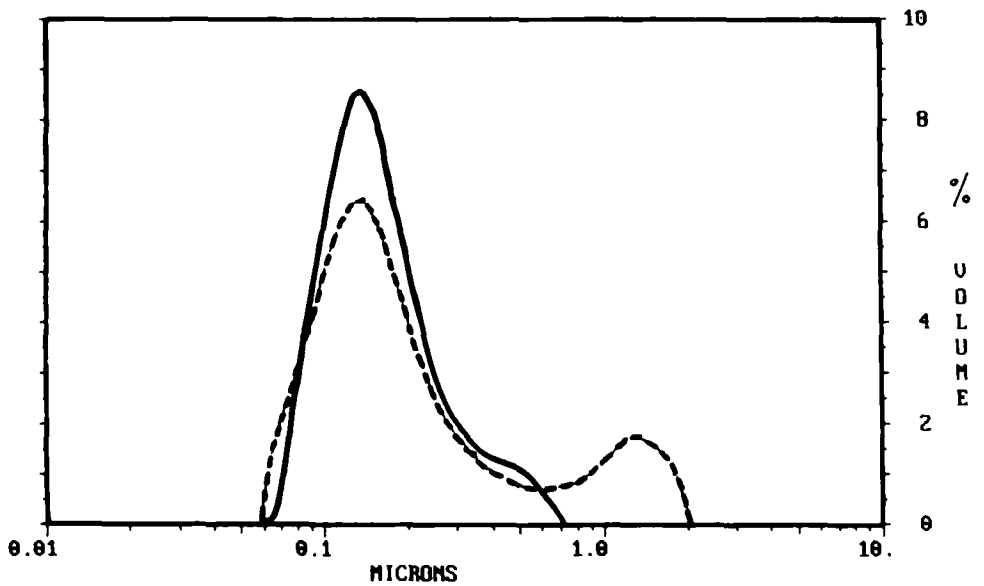

Fig. 8. Percentage of the total internal volume of PSI proteoliposomes corresponding to each diameter. (——) lipid/chlorophyll = 100 (w/w); (----) lipid/chlorophyll = 50 (w/w).

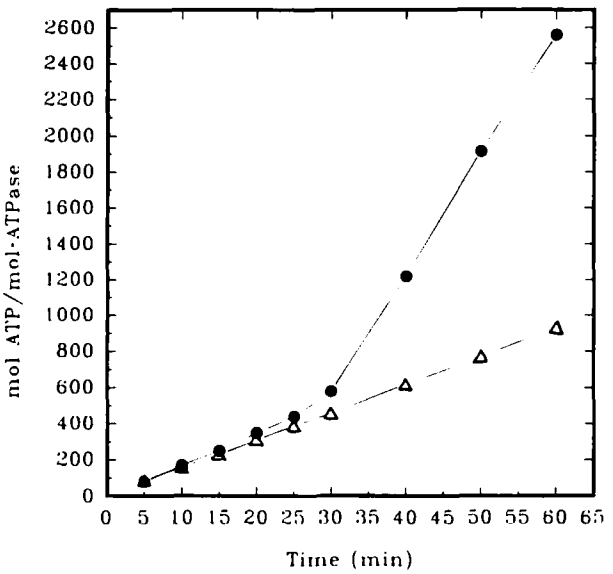

Fig. 9. Light-induced ATP synthesis by PSI-TF_0F_1 and PSI-CF_0F_1 co-reconstituted proteoliposomes. Proteoliposomes containing 20% cholesterol were co-reconstituted with PSI/TF_0F_1 or PSI/CF_0F_1 as described in Materials and Methods (lipid/chlorophyll = 10 w/w; lipid/F_0F_1 = 133 w/w) in a medium containing 50 mM Na_2SO_4, 50 mM K_2SO_4, and 25 mM Tricine, pH 8.0. The resulting proteoliposomes were resuspended at 0.4 mg/ml at 38°C in the same buffer used for reconstitution and supplemented with 40 μM PMS, 10 mM ascorbate, 0.1 μM valinomycin, and and 0.4 mM ADP. After 5 min illumination, ATP synthesis reaction was initiated by addition of 1.5 mM $MgSO_4$. Light-induced ATP synthesis was analyzed as a function of time by the luciferin-luciferase assay. (●) PSI-TF_0F_1 proteoliposomes; (Δ) PSI-CF_0F_1 proteoliposomes.

viously reported by Hauska *et al.* (1980) [10–50 nmol ATP/(mg CF_0F_1 min)]. For another comparison, rates of photophosphorylation driven by bacteriorhodopsin in BR-CF_0F_1 and BR-TF_0F_1 proteoliposomes displayed, in optimal cases, activities of about 500–800 nmol ATP/(mg F_0F_1 min) in the presence of cholesterol (Pitard *et al.*, 1996a,b). Thus, referring to the photophosphorylation activity of co-reconstituted proteoliposomes containing F_0F_1 ATPases and light-dependent H^+ generators, it appears that PSI does not allow one to improve the efficiency of cyclic phosphorylation as compared to that obtained with bacteriorhodopsin (Pitard *et al.*, 1996a,b). On the other hand, it should be stressed that ATP synthesis reported in the present work occurs only under specific experimental conditions, i.e., at lipid/chlorophyll ratio of 10 (w/w) where no detectable H^+ driving force could be measured. One interpretation could be that under those conditions, energy transduction between illuminated PSI molecules and the ATPase complex does not occur by translocation of H^+ via the internal phase but rather via pathways confined to the membrane proper or by the existence of some kind of direct interaction between PSI and the ATPase. In this framework, a direct transfer of H^+ from bacteriorhodopsin to F_0F_1 ATPases has been recently clearly evidenced in co-reconstituted proteoliposomes: in particular, it was observed that increasing the number of H^+ pumping units led to a tenfold increase in the rate of ATP synthesis while no changes, or even decreases, in the magnitude of the bulk-to-bulk $\Delta\mu_{H^+}$ could be detected (Pitard *et al.*, 1996a,b). In this framework Van Walraven (1990) reported a light-dependent ATP synthesis in BR-F_0F_1 ATPase from the thermophillic cyanobacterium *Synechococcus* in the absence of any measurable $\Delta\mu_{H+}$. However, another simple explanation, taking into account the experiments reported in Fig. 8, could be related to the heterogeneity of the vesicle population observed upon increasing PSI content of the proteoliposomes. The distribution and/or orientation of PSI molecules between the two vesicle populations and the structural characteristics of each population (multilamellarity, vesicle aggregation) could strongly influence the 9AA fluorescence quenching. Further structural analysis is needed to achieve a better understanding of the nature and the role of the observed heterogeneity in terms of protein distribution and orientation among and in the liposomes. Such studies could give new useful data to distinguish between the different hypotheses which at present can explain the ATP synthesis reported in the present work, i.e., the existence of a vesicle population generating a proton gradient that we are not able to detect or the existence of some kind of localized proton pumping between the PSI and the F_0F_1 ATPase.

CONCLUSIONS

To obtain detailed information about the partial reactions involved in the energy-transducing functions of enzymes involved in oxidative and photosynthetic phosphorylation, we describe a new method for the reconstitution into liposomes of the photosystem I reaction center from cyanobacteria. This method allows a rapid and easy determination of the optimal experimental conditions for detergent-mediated reconstitutions. We believe that an important benefit of our study is the finding that the reconstitution described in this paper is the method of choice for the reconstitution of PSI, more appropriate than the sonication method previously reported (Orlich and Hauska, 1980;

Hauska *et al.*, 1980). Proteoliposomes which satisfied most of the criteria for an efficient reconstitution could be produced, capable of sustaining, in the presence of PMS, a light-induced pH gradient of 1.8 pH units. The relatively large size of the proteoliposomes, the good asymmetric orientation of the protein among and in the liposomes, as well as the low ionic permeability are also definite advantages. From another point of view, our studies of the co-reconstitution of PSI and F_0F_1 ATPases have failed to produce efficient coupling between these two proteins. Light-driven ATP synthesis activities were very low, or the reconstituted proteoliposomes very heterogeneous, hampering further exploration of the coupling mechanisms between ATP synthesis and the amplitude of the protonmotive force. In conclusion, despite the shortcomings of our studies in terms of reconstituting cyclic photophosphorylation, we have demonstrated that the reconstituted system containing PSI reaction center can be a powerful tool to further analyze some functional and structural aspects of this membrane protein.

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