Biochimica et Biophysica Acta 810 (1985) 370–376
Elsevier

BBA 41884

Fusion of bacteriorhodopsin with submitochondrial particles yields a new system with retention of energy coupling and acquisition of photophosphorylation activity

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(Received September 3rd, 1985)

Key words: Bacteriorhodopsin; Purple membrane; Mitochondrial membrane; Membrane fusion; Photophosphorylation; (Bovine heart, Submitochondrial particle)

Submitochondrial particles were fused with purple membranes of *Halobacterium halobium* cells by means of a freeze-thawe sonication procedure. It is reported that fusion of inner mitochondrial membranes with a bacterial membrane yields a new particle which shows not only retention of redox- and photon-linked energy-coupling activities, but also creation of an additional energy-coupling process, light-driven ATP synthesis.

Introduction

By reconstitution of vectorial proteins one generally means the extraction and purification of integral membrane proteins and their subsequent insertion into artificial phospholipid membranes. Such an approach has proved important for the understanding of the molecular basis of the structure-function relationship of many enzymes, as, for example, the H^+-ATP synthase [1] and the Ca^{2+}-Mg^{2+} ATPase from sarcoplasmic reticulum [2].

In particular, the establishment of a transmembrane translocation of protons driven by ATP hydrolysis, after insertion of the mitochondrial H^+-ATP synthase into liposomes [1], has been taken as evidence for an essential feature of the chemiosmotic theory of energy transduction, since in this hypothesis the H^+-ATP synthase is envisaged as a reversible proton pump [3]. In addition, reconstitution of ATP synthesis by insertion into liposomes of mitochondrial H^+-ATP synthase together with either cytchrome oxidase [4] or light-driven H^+-pump bacteriorhodopsin [5] has ruled out, in line with the chemiosmotic hypothesis [3], the necessity of any structural link between energy-yielding and energy-consuming enzyme complexes.

We have performed a novel type of insertion of a protein into a membrane through which bovine heart submitochondrial particles have been supplied with bacteriorhodopsin-containing liposomes.

This paper reports that, for the first time, an exogenous protein has been successfully inserted in an otherwise complete system. Moreover, this work shows that, in the new vesicle, bacteriorhodopsin proton pumps, respiratory chain proton

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Abbreviations: $\Delta\tilde{\mu}_H$, transmembrane electrochemical gradient for protons ($= F\Delta\psi + RT\ 2.303\ \Delta pH$, where F is the Faraday constant, R the gas constant, T the absolute temperature; $\Delta\psi$ the transmembrane electric-potential difference; ΔpH, the transmembrane pH difference); Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethil)ethyl]-amino}ethanesulphonic acid; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

pumps and ATP synthase proton pumps are all effectively operating so that synthesis of ATP can be elicited either by redox energy or photon energy.

Materials and Methods

Type II Mg-ATP bovine heart submitochondrial particles were prepared according to Ref. 6 and suspended in 20 mM Tes/5 mM magnesium acetate $Mg(CH_3CO_2)_2$ (pH 7.5). In the experiments where succinate was substrate, submitochondrial particles were preincubated with 2.5 mM sodium malonate for 30 min at 35°C to activate succinate dehydrogenase. Bacteriorhodopsin-containing purple membranes were isolated according to Ref. 7 from *Halobacterium halobium* cells (R1 strain) grown and harvested with a standard procedure [7].

Soy-bean phospholipid vesicles were prepared as described in Ref. 8, except that butylated hydroxytoluene (1% w/v alcoholic solution) was added during sonication at 1% v/v final concentration, in order to prevent peroxidation of phospholipid.

Reconstitution of purple membranes into presonicated liposomes was carried out by means of a freeze-thaw sonication technique as in Refs. 8 and 9, with a bacteriorhodopsin-to-phospholipid weight-to-weight ratio of 1 : 3. The freezing-thawing step was repeated twice. The mixture was then layered on top of a discontinuous sucrose density gradient (in 20 mM Tes (pH 7.5)/ 50 mM KCl) and run 14–18 h at $130000 \times g$. Thereafter a major band was collected, with a buoyant density of 1.1 ± 0.1 g/ml and containing bacteriorhodopsin and phospholipid at a molar ratio of 1 : 90–110. This band, in which bacteriorhodopsin concentration was in the range of 1–2 mg/ml, was used for subsequent fusions with submitochondrial particles.

Incorporation of bacteriorhodopsin-containing vesicles in submitochondrial particles membranes was obtained by means of the freeze-thaw sonication procedure [8,9], performed twice. More specifically, 0.3 ml Mg-ATP submitochondrial particles (corresponding to approx. 15–25 mg protein) were mixed with 0.2–0.3 ml of bacteriorhodopsin-containing liposomes (corresponding to approx. 0.3–0.6 mg protein of bacteriorhodopsin) and were frozen and thawed twice. The sample was then diluted to 3 ml with 20 mM Tes (pH 7.5)/50 mM KCl/5 mM $MgCl_2$ before sonication. The bacteriorhodopsin concentration of the product of the freeze-thaw sonication step was in the range of 5–15% of total protein, while the phospholipid content ranged from 1 to 1.5 μmol P_i/mg of total protein. Submitochondrial particles and bacteriorhodopsin-containing liposomes, which underwent the freeze-thaw sonication procedure, will be identified in the text as submitochondrial particles/bacteriorhodopsin-containing liposomes.

The protein concentration of submitochondrial particles was determined according to Ref. 10, while that of submitochondrial particles/bacteriorhodopsin-containing liposomes according to Ref. 11. Bacteriorhodopsin-protein concentration was determined according to Ref. 11 or from the absorption maximum in the visible region of the light-adapted form, assuming a molar extinction coefficient of 63000 [12]. Phospholipids were determined by the method of Petitou et al. [13] or according to Rouser and Fleischer [14].

L-α-phosphatidylcholine from soy-bean was purchased from Sigma (St. Louis, U.S.A.) and used without further purification. Oligomycin and P^1,P^5-di(adenosine-5′-)pentaphosphate (as lithium salt) were obtained from Sigma (St. Louis, U.S.A.), FCCP and ADP from Boehringer (Mannheim, F.R.G.). All other chemicals were of the highest quality commercially available.

Results and Discussion

After submitochondrial particles and bacteriorhodopsin-containing liposomes had undergone the fusion procedure, the sample was run on a discontinuous sucrose density gradient (25–50% w/v) in order to separate any fused submitochondrial particles/bacteriorhodopsin-containing liposomes from unfused brown submitochondrial particles and purple bacteriorhodopsin-containing liposomes. Unfortunately, this procedure proved inadequate because control experiments with submitochondrial particles and bacteriorhodopsin-containing liposomes simply mixed together showed that the two systems aggregate, as was apparent from the single band recovered. How-

ever, it was found that presence of 400 mM NaCl in the sucrose gradient prevented such phenomenon. Under these conditions, submitochondrial particles and bacteriorhodopsin-containing liposomes that had undergone the process of fusion produced a single major band, with only a faint purple band at a density corresponding to bacteriorhodopsin-containing liposomes alone (data not shown).

These data were interpreted as evidence that indeed most of the bacteriorhodopsin was physically part of the submitochondrial particle membranes after the fusion procedure. However, as control experiments showed that after exposure of submitochondrial particles alone to 400 mM NaCl ATP synthesis was completely inhibited, the product of the fusion procedure was not routinely purified after the freeze-thawe sonication step.

At this point it was necessary to demonstrate that the fusion procedure had not altered dramatically the integrity of the enzyme complexes of the particles nor that of the membrane in general, and, most importantly, that bacteriorhodopsin was still functionally active and that both light and succinate oxidation were promoting the pumping of protons into the same internal space. To this end, the experimental strategy adopted stemmed from the well-known observation that in mitochondria and bacteria there exists a pronounced non-linear relationship between $\Delta\tilde{\mu}_{H^+}$ and the rate of proton pumping such that, once a certain value has been attained, $\Delta\tilde{\mu}_H$ magnitude does not increase on raising further the proton-pumping activity [15]. This finding implies that, if after the freeze-thawe sonication procedure there remained substantial amounts of unfused submitochondrial particles and bacteriorhodopsin-containing liposomes, the extent of energisation due to light should have been independent of that due to succinate. On the contrary, if the same system contained both bacteriorhodopsin and respiratory chain proton pumps, energisation, or incremental increase in $\Delta\tilde{\mu}_H$, due to light should have varied depending on the preexisting energisation due to succinate. Consequently, in the experiments shown in Fig. 1A, the submitochondrial particles/bacteriorhodopsin-containing liposomes population was energised first with succinate and subsequently with light.

Unfortunately, it was not possible to determine the magnitude of $\Delta\tilde{\mu}_H$ by means of ion distribution followed by the flow-dialysis technique [15], as the available submitochondrial particles/bacteriorhodopsin-containing liposomes preparations were never enough to be used in the flow-dialysis method. Therefore, energisation was followed by means of 9-aminoacridine fluorescence quenching in presence of K^+ and valinomycin. These conditions are believed to favour the total conversion of $\Delta\psi$ into ΔpH, so that ΔpH, calculated from the percentage of the fluorescence quenching (see legend of Fig. 1), should be equal to the effective value of the electrochemical gradient of protons. As the estimation of the magnitude of ΔpH by 9-aminoacridine fluorescence quenching is generally considered to be less accurate than ion distribution, the absolute values of ΔpH given in Fig. 1 should be taken only as a guide of the state of energization of submitochondrial particles/bacteriorhodopsin-containing liposomes population.

In Fig. 1A the submitochondrial particles/bacteriorhodopsin-containing liposomes population was first supplemented with succinate which generated a ΔpH of 65 mV. When light was subsequently switched on, the increase of ΔpH due to bacteriorhodopsin activity was approx. 10 mV. In contrast, in the absence of respiration-driven ΔpH (i.e., when anaerobiosis was reached) bacteriorhodopsin proton-pumping activity could maintain a ΔpH of a much larger value (50 mV). These results clearly indicate that the two modes of energisation have a synergic behaviour as expected if light and succinate were promoting the translocation of protons into a common pool.

To prove further that such conclusion was correct, ΔpH generated by light-driven bacteriorhodopsin activity was followed in the submitochondrial particles/bacteriorhodopsin-containing liposomes particle under conditions where succinate induced energisation was selectively diminished by addition of increasing concentrations of malonate, a specific inhibitor of succinate dehydrogenase activity. Trace B of Fig. 1 was obtained in presence of malonate at a concentration such that succinate dehydrogenase activity was inhibited by approx. 70–80%. In such an instance succinate oxidation induced a ΔpH of lower value than that found when no malonate was added

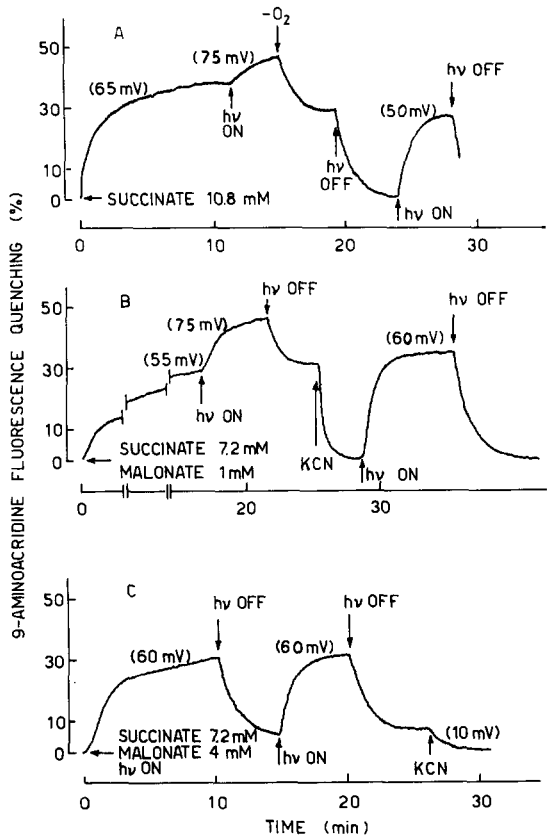

Fig. 1. Energisation by succinate and light in the submitochondrial particles/bacteriorhodopsin-containing liposomes particle in absence and presence of increasing concentrations of malonate. To a final volume of 2.5 ml were present 20 mM Tes (pH 7.5)/50 mM KCl/5 mM $MgCl_2$/valinomycin 1.3 μg per mg protein/5 μM 9-aminoacridine/1.5 mg protein of submitochondrial particles/bacteriorhodopsin-containing liposomes. When present, KCN was 0.5 mM. ΔpH generation was followed by means of 9-aminoacridine fluorescence quenching (Q) (excitation wavelength, 390 nm; emission wavelength, 460 nm) followed at a fluorimeter equipped with a 250 W quarz-iodine lamp, screened by a heat filter and a Corning glass long-path filter no. 368. The light intensity used was saturating for bacteriorhodopsin as shown in Ref. 8. ΔpH values were calculated according to the equation: $\Delta\lambda\ pH_{o-i} = \log Q/(100 - Q) + \log V_o/V_i$, where V_0 and V_i are the external and internal aqueous spaces accessible to the dye [23], respectively. The apparent internal volume (V_i) of the system was calculated from the intercept (at $\Delta pH = 0$) of the slope relating the extent of 9-aminoacridine fluorescence quenching and the magnitude of artificially imposed ΔpH of known values. Submitochondrial particles/bacteriorhodopsin-containing liposomes apparent internal volume was found to be 80 μl/mg total protein. In parentheses steady state values of ΔpH, expressed in mV, are given. In all calculations of ΔpH values the percentage of fluorescence quenching referred to the total initial 9-aminoacridine fluorescence. Temperature was room temperature. $-O_2$ indicates the conditions at which the oxygen dissolved in the reaction medium had been exhausted.

(compare trace A and B). Therefore bacteriorhodopsin increased its contribution to total energisation, as predictable in this condition where the thermodynamic pressure on light-driven proton pump had been partially alleviated. This prediction was once again confirmed by the greater extent of energisation that light induced after respiratory-chain proton pumps were blocked by KCN (trace B). In the experiment shown in trace C (Fig. 1), malonate concentration was so high that the system had to be illuminated from the beginning in order to decrease the time needed to reach the steady state. With a severely restricted succinate dehydrogenase activity, it is expected that contribution to total energisation by the respiratory chain would be minimal. This is proved by the large increase in fluorescence that occurred upon switching off the light and by the small ΔpH calculated when KCN was added in the dark.

In trace C it is also important to note that the magnitude of the total electrochemical gradient of protons was less than that found when malonate was absent or only partially limiting succinate oxidation. This finding can be taken to mean that bacteriorhodopsin activity by itself was insufficient to energise fully the particles. On the other hand also succinate-driven $\Delta\tilde{\mu}_H$ is less than that found, for example, in Mg-ATP submitochondrial particles [15]. Explanations for such a lower-energised state can be sought in the possibility that the membranes of the particles were more leaky to ions due either to the increased content of phospholipids after bacteriorhodopsin-containing liposomes incorporation or to some uncontrolled damage consequent to the freeze-thawe sonication procedure. That dilution of respiratory chain components in the submitochondrial particles/bacteriorhodopsin-containing liposomes population be responsible for a decreased electron-transport rate (which would then cause a concomitant drop in $\Delta\tilde{\mu}_H$) is a possibility though not definite. This is because, as also discussed previously, substantial inhibition of the electron-transport rate can be associated with small decreases in the magnitude of $\Delta\tilde{\mu}_H$ [15]. Moreover, it is also appropriate to

consider the possibility of a deleterious effect of valinomycin on the membrane of submitochondrial particles, as already has been found [16], that could have prevented full attainment of the thermodynamically possible $\Delta\tilde{\mu}_H$.

The rate of succinate oxidation by submitochondrial particles/bacteriorhodopsin-containing liposomes was found not to vary when a 250 W tungsten lamp, screened by a yellow filter, was shone on a glass-cell fitted with a Clark-type oxygen electrode and containing the submitochondrial particles/bacteriorhodopsin-containing liposomes population. This result was not entirely surprising because even in intact submitochondrial particles the rate of electron transport is rather insensitive to variations of the magnitude of $\Delta\tilde{\mu}_H$ (low-respiratory control). Thus, as shown in Fig. 1A, an increase in $\Delta\tilde{\mu}_H$ magnitude by approx. 10 mV upon illumination was insufficient to alter the rate of oxygen consumption by submitochondrial particles/bacteriorhodopsin-containing liposomes.

In concluding the discussion of this set of experiments, we believe that data reported in Fig. 1 constitute good evidence that after the fusion procedure bacteriorhodopsin was inserted into submitochondrial particle membranes in such a way that, when stimulated by light, pumped protons into the internal space of the submitochondrial particles/bacteriorhodopsin-containing liposomes particle.

Finally, if such a conclusion is correct, light should have been able to induce synthesis of ATP catalysed by the ATP-synthase complexes of the mitochondrial membrane. Table I reports that indeed ATP was synthesised also when energisation was due to bacteriorhodopsin proton-pump activity, though at 6% of the rate associated with succinate oxidation.

For exclusion of the possibility that ATP formed in presence of light was due to an adenylate kinase catalysed reaction, P^1,P^5-di(adenosine-5′-)pentaphosphate (Ap_5A) was present in all determinations in order to specifically inhibit adenylate kinase. Nevertheless, other conditions were sought to control against such possibility. As reported in Table I, no ATP was synthesised when the system was illuminated in presence of FCCP nor in the dark, thus confirming that ATP synthesis in presence of light was indeed a consequence of energisation due to bacteriorhodopsin activity and mediated by the oligomycin-sensitive mitochondrial ATP synthase (see also the control experiment in presence of oligomycin).

TABLE I

ATP SYNTHESIS RATE OF SUBMITOCHONDRIAL PARTICLES/BACTERIORHODOPSIN-CONTAINING LIPOSOMES PARTICLES INDUCED BY SUCCINATE AND LIGHT

The experiments were performed in a cell fitted with a magnetic stirrer, and thermostatically controlled. To a final volume of 1.4 ml were added 20 mM Tes (pH 7.5)/50 mM KCl/5 mM $MgCl_2$/35 mM glucose/10 mM potassium phosphate/50 mM succinate (or an equal volume of H_2O if light was the energy source)/50 units of salt-free yeast hexokinase/90 μM P^1,P^5-di(adenosine-5′-)pentaphosphate/1.1 μg per mg total protein of valinomycin/1.2 mg protein of submitochondrial particles/bacteriorhodopsin-containing liposomes/^{32}Pi (1 μCi, total). The reaction was initiated by addition of 100 μM ADP and then between four and six aliquots of 0.1–0.2 ml were withdrawn from the reaction mixture at intervals of between 1 and 2 min for the assay of esterified ^{32}P by the method as in Ref. 24. In order to obtain linear rates of ATP synthesis, the mixture was preincubated either with succinate or illuminated for 5 min before ADP addition. ATP synthesis rate was found to be linear up to 10 min with either energy source. When succinate was substrate, an H_2O-saturated stream of oxygen was blown over the incubation mixture throughout the experiment. As light source a 250 W tungsten lamp was used, screened by a yellow filter. No difference in the rate of photophosphorylation was found when an additional 250 W tungsten lamp was also shone on the cell. When added, FCCP was 16 μM and oligomycin 6 μg/mg protein. Temperature was 25°C.

Energy source	ATP synthesis rate ($nmol \cdot min^{-1} \cdot mg^{-1}$)	Specific activity (%)
Succinate (20 mM)	61.5	100
Light	3.7	6
Light + FCCP	0.0	0
Light + oligomycin	0.0	0
Dark, no succinate	0.0	0

Several factors might be responsible for the relative low rate of ATP synthesis elicited by light compared to that due to succinate oxidation. One possibility can be sought in the particular relationship between the rate of ATP synthesis and $\Delta\tilde{\mu}_H$, that has been found both in bacterial and mitochondrial membranes under restricted proton fluxes [15]. The relation is such that only small decreases in $\Delta\tilde{\mu}_H$ cause large variations in the rate of ATP synthesis. Though the magnitude of ΔpH

due to light and succinate was not measured in parallel with the rate of ATP synthesis (Table I) (thus their absolute value is not known), from the data presented in Fig. 1 it is rather likely that also in this case ΔpH induced by light was lower than that due to succinate, so accounting for the lower rate of ATP synthesis. The question now arises as to why energisation generated by light was smaller than that due to the activity of the respiratory chain. The most likely reason is that insufficiently correctly orientated bacteriorhodopsin molecules were present in each fused particle.

Nevertheless, for a reconstituted activity to be 6% of that observed using the native generator of an electrochemical gradient of protons compares favourably with specific rates of ATP synthesis catalysed by other classically reconstituted systems [5,17], the most likely reason being that, in the present work, H^+-ATP synthases were left in their natural environment. Recently, a reconstitution of bacteriorhodopsin and yeast mitochondria ATP synthase has been reported in which the rate of ATP synthesis is rather higher than has appeared hitherto [18]. In that work a light-driven rate of ATP synthesis of 73 nmol/min per mg protein for liposomes that contained ATP-synthase and bacteriorhodopsin as the only protein was measured, using as co-reconstitution procedure the freeze-thaw sonication technique. The rate of ATP synthesis expressed per mg protein (Table I) can be compared with the data in Ref. 18 only if an estimate of the ATP synthase content of submitochondrial particles is made. A common estimate is that approx. 10% of the inner mitochondrial membrane is ATP synthase, in which case, and without allowing for the incorporated bacteriorhodopsin, the rate of photophosphorylation shown in Table I must be multiplied 10-fold to be comparable with the data in ref. 18.

Moreover, it is also appropriate to point out that for the first time an exogenous protein as well as exogenous phospholipids have been incorporated in the intact inner mitochondrial membrane with retainment of energy-coupling properties. In this context it is worth noting that incorporation of mixed phospholipid liposomes in the inner mitochondrial membrane has already been carried out [19–21]. However, whereas incorporation of ammoniumsulfate-cholate fractionated membrane fragments of mitochondria into liposomes yielded a system capable of ATP-dependent enhancement of ANS fluorescence [19], fusion of liposomes with untreated inner mitochondrial membranes has always hitherto occurred concurrently with loss of both of ATP formation by oxidative phosphorylation and of respiration-dependent $\Delta\tilde{\mu}_H$ [20,21].

Therefore, the successful new type of reconstitution presented in this paper can give an important contribution to the methodology of incorporation in whole systems of exogenous proteins and phospholipids with retention of vectorial membrane functions, in the light of future prospects of more complex membrane reconstitution studies, such as, for example, fusion of cells with reconstituted proteoliposomes [22].

Acknowledgements

The authors are grateful to Prof. W. Stoeckenius and Dr. S.J. Ferguson for helpful discussion and for providing *Halobacterium halobium* strains, to Dr. M.P. Rigobello for the kind help in growing *H. Halobium* cells and to Ms. Monica Vettore for excellent secretariat help.

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