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FACTORS DEFINING THE FUNCTIONAL COUPLING OF BACTERIORHODOPSIN AND ATP SYNTHASE IN LIPOSOMES

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Optimal conditions for the co-reconstitution of bacteriorhodopsin and yeast mitochondria ATP synthase were determined. Reconstitution was achieved with a quick two-step procedure. Preparations obtained by this method displayed in optimal cases 2–3-times higher activities (up to 500 nmol ATP/min per mg protein) compared with maximal values reported in the literature, when light-driven ATP synthesis was measured under similar conditions. The final activities depended on the purification method used for the ATP synthase, and it is shown that the oligomycin-sensitive ATP hydrolysis activity was not a good measure for the ability of the ATP synthase preparations to perform ATP synthesis after co-reconstitution. Light-driven ATP synthesis activities depended also on the type of phospholipid used, soybean phospholipid giving the best results. A close relation to the bacteriorhodopsin proton pump activity was found. Using different phospholipids, different H^+/ATP ratios were found, calculated from ATP synthesis activities and initial and steady-state light-driven proton pump activities. From this, together with the findings that the ATP synthase displayed the same ATP hydrolysis and ATP-$^{32}P_i$ exchange activities with these different phospholipids used, it is concluded that the protein distribution for the two proteins among the liposomes is different relative to each other for the different phospholipids. The light-driven ATP synthesis activity did not correlate with the variation in leakiness of the membrane for protons when different phospholipids were used. An explanation is given by the finding that at high light intensities, the ATP synthesis became independent of the presence of protonophore.

Introduction

Quantitative studies on the mechanism of ATP synthesis have always been hampered by the complexity of the systems used (mitochondria, chloroplasts, etc.). In order to study the relationship between ATP synthesis and electrochemical proton gradients, which for instance is predicted by the chemiosmotic theory [1], co-reconstitution of purified ATP synthase with bacteriorhodopsin, the light-driven proton pump, is useful [2–4]. Bacteriorhodopsin used as an electrochemical proton-gradient generator has several favourable aspects for this purpose: (1) it can be purified very easily and reconstituted in liposomes with unidirectional orientation; (2) its substrate is clean, i.e., light; (3) electrochemical proton gradients can easily be varied by varying light intensity; and (4) one would not expect the formation of a complex between bacteriorhodopsin from *Halobacterium halobium* and ATP synthase from other organisms.

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

Functional coupling between bacteriorhodopsin and ATP synthase from various sources has already been reported [2–8]. However, in the reported cases, the light-driven ATP synthesis activity was always very low compared with ATP synthesis activities in vivo. Causes for this might be found in the purification method of ATP synthase and/or in the co-reconstitution method. The ATP synthase is, for example, rather sensitive to the conditions under which it is kept. For bacteriorhodopsin, this seems to be no problem, as it is stable under rather stringent conditions (cf. Refs. 9, 10). Apart from this, the protein distribution and the orientation of both proteins among and in the liposomes may be not optimal. In order to be able to do thermodynamic and kinetic studies on liposomes containing bacteriorhodopsin and ATP synthase, we tried to define conditions that give optimal functional coupling between bacteriorhodopsin and ATP synthase. In the present study, the different co-reconstitution parameters were systematically analysed. In order to obtain a better understanding of the factors which determine the functional coupling of the two proteins, preparations were tested for six parameters: ATP-$^{32}P_i$ exchange, light-driven ATP synthesis, ATP hydrolysis, stimulation of ATP hydrolysis by FCCP, the steady-state phosphate potential (ΔG_p) and the light-driven proton pump activity. Furthermore, the preparations were analysed by density gradient centrifugation and gel filtration in order to obtain information about the distribution of both proteins among the liposomes. Information about this is also important for further thermodynamic and kinetic studies. Studying the co-reconstitution technique, important information may be obtained which can be used in general for the incorporation of two or more membrane-bound proteins together in artificial bilayer systems.

Materials and Methods

Materials. Soybean phospholipid was obtained from Associated Concentrates (Woodside L.I., New York, U.S.A.), washed with acetone, extracted with ether according to the method of Kagawa and Racker [11], dissolved in chloroform/methanol (95 : 5, v/v), and kept at 200 K. Yeast mitochondrial phospholipid was obtained by extraction with chloroform/methanol (2 : 1, v/v) of isolated mitochondria (see below), according to the method of Folch [12]. After extraction, yeast mitochondrial phospholipid was washed with acetone and extracted with ether [11], after which it was kept at 200 K in chloroform/methanol (95 : 5, v/v). All other lipids were obtained from Sigma (St. Louis, MO, U.S.A.), except yeast phosphatidylinositol which was obtained from Koch Light (Colnbrook, Berkshire, U.K.). Octylglucoside, oligomycin, FCCP and hexokinase were also from Sigma. Glucose-6-phosphate dehydrogenase, lactate dehydrogenase and pyruvate kinase were from Boehringer (Mannheim, F.R.G.). Cholate and deoxycholate were from BDH (Poole, U.K.) and once recrystallised from ethanol and dissolved as potassium salt or sodium salt. Triton X-100 and DEAE-cellulose were from Serva (Heidelberg, F.R.G.). Coomassie brilliant blue R250 was from Bio-Rad Laboratory (Richmond, CA, U.S.A.). [2,4(n)-^{3}H]Cholic acid and [^{32}P]orthophosphoric acid was from New England Nuclear (Dreieich, F.R.G.). Sepharose 2B-CL, Sepharose 4B-CL and Sephadex G-50 (coarse) were from Pharmacia (Uppsala, Sweden). CHAPS was kindly donated by Dr. W.J. de Grip of the University of Nijmegen, The Netherlands. All other chemicals were of analytical grade.

Mitochondria were isolated from commercial baker's yeast by the procedure described by Stutterheim et al. [13] using a Dynomill and kept at 200 K. Specific ATP hydrolysis activity measured after freeze-thawing was about 2 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. Bacteriorhodopsin was purified from *Halobacterium halobium* strain S9 by the procedure described by Oesterhelt and Stoeckenius [14]. Bacteriorhodopsin concentrations were determined spectrophotometrically using $\epsilon_{570\ nm} = 63\,000\ M^{-1} \cdot cm^{-1}$ [15] at a Triton X-100 concentration of 0.5–1.0% (w/v).

Purification method of Ryrie [16]. ATP synthase was purified as described by Ryrie [16], except that Sepharose 4B-CL gel filtration was performed before density gradient centrifugation on 8–30% (w/v) sucrose gradients. Before sucrose density gradient centrifugation, yellow contaminants were removed by batch incubation with DEAE-cellulose in the presence of 100 mM K_2SO_4. At this salt concentration, ATP synthase did not bind to the DEAE-cellulose.

Purification method of Rott and Nelson [17]. This was done exactly as described by Rott and Nelson [17].

Co-reconstitution by the sonication-gel filtration procedure. The procedure was performed in 85 mM K_2SO_4/25 mM KCl/0.2 mM EDTA/10 mM Tricine (pH 7.5) (standard medium). Bacteriorhodopsin liposomes were prepared by sonication of purple membranes and phospholipid together for 900 s at 10–15 °C according to Hellingwerf et al. [18]. The concentrations of bacteriorhodopsin and phospholipid were 2 and 20 mg/ml, respectively, in this step. Bacteriorhodopsin liposomes were next incubated at a concentration of 1 mg bacteriorhodopsin/ml and 10 mg phospholipid/ml together with ATP synthase (0.2–0.25 mg/ml) for 30 min at 0 °C in the presence of 1.1% cholate (w/v) and 5 mM $MgCl_2$ in the dark in standard medium. After the incubation, detergent was removed by a quick centrifugation step on Sephadex G-50 columns for 1 min [19,20]. More than 95% of all cholate was removed by this step, as found by using [^{3}H]cholate in four experiments.

Measurement of light-driven ATP synthesis. The prepared liposome suspensions were incubated in tubes at a phospholipid concentration of 1–2 mg/ml (2 ml total volume), in a water-bath containing a 1% $CuSO_4$ solution (as infrared filter) at 32 °C. Light came from a 150 W slide projector (tungsten-halogen lamp) and had to pass 2 cm of the 1% $CuSO_4$ solution before reaching the tubes. ATP formed was trapped as glucose 6-phosphate by adding hexokinase and glucose to the incubation medium, composed of standard medium supplemented with 0.3 mM ADP, 33 mM K_2HPO_4, 27 mM glucose, 5 mM $MgCl_2$, 8.5 mM AMP and 100 μg/ml hexokinase. At 15 and 30 min time intervals, samples were taken and added to perchloric acid (final concentration 5%; w/v). After 15 min at 0 °C, precipitated protein was removed by centrifugation and the supernatant neutralised with 5 M K_2CO_3, before freezing at 200 K. After thawing, the precipitate $KClO_4$ was removed by centrifugation and then glucose 6-phosphate was determined by the glucose-6-phosphate dehydrogenase assay [21]. ATP synthesis was linear with time during the first 30 min of illumination. Varying the liposome concentration or adding extra yeast F_1 (purified according to the method of Stutterheim et al. [13]) during illumination did not affect glucose 6-phosphate formation per mg ATP synthase. This proves that ATP formed will not be hydrolysed before it is trapped as glucose 6-phosphate. Light intensity was varied with neutral density filters (Oriel Corp., Stanford, CT, U.S.A.).

Measurement of ΔG_p. Liposomes were illuminated as described for the measurement of ATP synthesis. The medium consisted of standard medium to which were added 30 μM ADP, 3.3 mM K_2HPO_4, 5 mM $MgCl_2$. Samples were treated in the same way as for the measurement of ATP synthesis. During incubation with perchloric acid, also 30 mM EDTA was present to minimise ATP hydrolysis. ATP was measured after neutralisation and removal of $KClO_4$ by centrifugation in an assay using hexokinase and glucose-6-phosphate dehydrogenase [21]. ADP was measured in an assay using pyruvate kinase and lactate dehydrogenase [21]. ΔG_p was calculated using a $\Delta G'_0$ of 30.12 kJ/mol [22].

Measurement of ATP-P_i exchange. ATP-P_i exchange was measured according to the method described by Berden and Henneke [20]. The medium consisted in this case of standard medium to which 10 mM ATP, 25 mM Na_2HPO_4, 5 mM $MgCl_2$, 1.5 mg/ml bovine serum albumin and [^{33}P]phosphate were added. ATP synthase was used at a concentration of 50 μg/ml.

Measurement of ATP hydrolysis activity. ATP hydrolysis activity was measured in an assay with pyruvate kinase and lactate dehydrogenase, monitoring the conversion of NADH to NAD^+ at 30 °C and pH 8.0. The assay medium consisted of 85 mM sucrose/30 mM Tris/5 mM $MgCl_2$/10 mM $KHCO_3$/0.25 mM NADH/0.5 mM phospho*enol*pyruvate/5 mM ATP. To the assay medium, 45 μg pyruvate kinase and 45 μg lactate dehydrogenase were added. The ATP hydrolysis activity during each assay was maximally 50 nmol $\cdot min^{-1} \cdot ml^{-1}$. The final volume in the cuvette was 2 ml. Oligomycin sensitivity was measured by adding 2 μl oligomycin (1 mg/ml in methanol) during each assay; the ATP hydrolysis activity of yeast F_1 was not inhibited at this concentration. The FCCP stimulation factor was calculated by dividing the ATP hydrolysis activity in the presence of FCCP (2 μl of a 1 mg/ml solution in

ethanol) by the ATP hydrolysis activity measured without FCCP.

Measurement of light-driven proton pump activity. This was done with the same equipment as described by Hellingwerf et al. [18], except that illumination was done with a 150 W slide projector, the same as used for ATP synthesis (tungsten-halogen lamp). Light had to pass first 1 cm of a 1% $CuSO_4$ solution before reaching the cuvette which was thermostatically controlled at 30 °C. After co-reconstitution liposomes were brought in standard medium without Tricine by means of the gel filtration procedure as described for the co-reconstitution procedure. Measurements were done in the same medium. Initial rates and H^+ efflux were determined by measuring the increase or decrease in pH of the extraliposomal medium during the first 10 s. Extent values were determined after steady-state conditions had been reached during illumination.

Calibration of the pH was done by titration with a 5 mM oxalic acid solution. Light intensities were varied as described in the section on measurement of ATP synthesis.

Fractionation of liposome suspensions. Sucrose gradient centrifugation of liposome suspensions was performed by layering 1.5 ml of the suspension on top of a 8–30% (w/w) linear sucrose gradient (14 ml) with a 1-ml 50% sucrose (w/w) cushion at the bottom. Centrifugation took place for 15 h at 70000 × *g*. Linear gradients were prepared before the run and fractionated after centrifugation from the top, using a Density Flow from Buchler Instruments, (Fort Lee, NJ, U.S.A.). Sepharose 2B-CL column chromatography of liposome suspensions was performed by chromatographing 1.5 ml of the liposome suspension through a 1.5 × 45 cm column at an elution rate of 9 ml/h. All fractions were 2.5 ml.

Miscellaneous assays. In all cases, phospholipid was determined by measuring the amount of inorganic phosphate after complete oxidation of the organic components in 70% perchloric acid at 80 °C [23]. In the case of analysing sucrose-gradient fractions, the phospholipids were first extracted with chloroform/methanol (2 : 1, v/v); this was done to remove sucrose because of the danger of explosion during oxidation in perchloric acid. After the extraction and before the oxidation, samples were evaporated to dryness.

Protein determination was performed according to the method of Peterson [24] or Bradford [25] using bovine serum albumin as a standard. SDS-polyacrylamide gel electrophoresis was performed on slab gels as described by Laemmli [26].

Results

Optimisation of the light-driven ATP synthesis activity

Comparison of two types of ATP synthase preparations obtained by different purification methods. In order to obtain an ATP synthase preparation with maximal light-driven ATP synthesis activity, two methods of purification were followed [16,17]. In Table I, the activities (ATP hydrolysis, ATP-P_i

TABLE I

COMPARISON OF THE ACTIVITIES OF TWO ATP SYNTHASE PREPARATIONS

	'Ryrie' preparation		'Rott-Nelson' preparation	
	present study [a]	literature [5,14]	present study [a]	literature [15]
ATP hydrolysis (μmol · min^{-1} · mg^{-1})	20–30 [b]	50	20– 30 [b]	
ATP-P_i exchange (nmol · min^{-1} · mg^{-1})	20 [c]	200	100–250 [c]	250
Light-driven ATP synthesis (nmol · min^{-1} · mg^{-1})	10–150 [d]	178	100–500 [d]	

[a] 'Range' values obtained for different preparations are given.

[b] Measured in the presence of soybean phospholipid. Oligomycin sensitivity was greater than 80%.

[c] After reconstitution in soybean phospholipid by incubation with cholate (1.1%), removing cholate afterwards by the gel filtration method.

[d] After co-reconstitution in bacteriorhodopsin liposomes according to the sonication-gel filtration method (soybean phospholipid as phospholipid). For both ATP synthase preparations, no ATP synthesis in the dark could be observed after co-reconstitution with bacteriorhodopsin.

exchange and light-driven ATP synthesis) measured after purification are compared with those reported by Ryrie [5,16] and by Rott and Nelson [17]. We obtained two types of preparation which had about the same oligomycin-sensitive ATP hydrolysis activity. Our preparation according to Rott and Nelson [17] had, however, much higher ATP-P_i exchange and light-driven ATP synthesis activities than our 'Ryrie' preparation (Table I). The latter also showed lower ATP-P_i exchange and light-driven ATP synthesis activities than reported by Ryrie himself [16]. With the Rott and Nelson preparation, in eight out of thirty experiments performed at the same optimal conditions, light-driven ATP synthesis activities of 300–500 nmol/min per mg were found. An average value of 237 nmol/min per mg with a standard deviation of 95 nmol/min per mg was found in all thirty experiments.

In Fig. 1, SDS-polyacrylamide gel electrophoresis patterns after staining with Coomassie brilliant blue are shown for our 'Ryrie' preparation (lane a) and our 'Rott-Nelson' preparation (lane b). The ATP synthase subunits were coded according to Rott and Nelson [17]. The bands in the lower parts of the gels were difficult to detect, so that a complete comparison of the two patterns is not possible.

The main differences between the preparations which could be found with Coomassie brilliant blue staining concern bands which probably do not belong to ATP synthase.

Comparison of the sonication-Sephadex gel filtration procedure with other co-reconstitution procedures. In Table II it is shown that the two-step procedure we used (first incorporation of bacteriorhodopsin in liposomes by sonication, and subsequently incorporation of ATP synthase in bacteriorhodopsin liposomes by incubation with cholate, followed by removal of cholate through gel filtration) gives the best results for the co-reconstitution of bacteriorhodopsin and ATP synthase. Comparison of different periods of sonication of bacteriorhodopsin together with phospholipid before the second step was performed shows that at least a 300-s sonication period should be used (Table II, Expt. 1). Sonication of ATP synthase together with bacteriorhodopsin and phospholipid resulted in less active preparations,

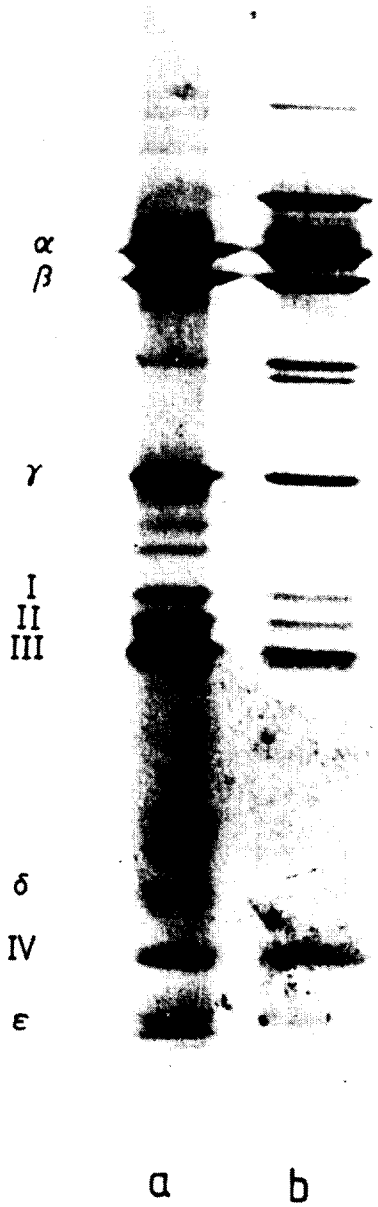

Fig. 1. Coomassie brilliant blue staining pattern of SDS-polyacrylamide gels of ATP synthase preparations. Lane a, 'Ryrie' preparation; lane b, 'Rott-Nelson' preparation. About 50 μg protein was loaded per slot. Denaturation was done by pouring the samples in boiling denaturation buffer. The coding of the subunits is according to Rott and Nelson [15].

as compared to the sonication-gel filtration method (Table II, Expt. 2). The concomitant decrease in ATP-hydrolysis activity indicates that the ATP synthase is sensitive to sonication (results not shown).

TABLE II

CO-RECONSTITUTION OF BACTERIORHODOPSIN AND ATP SYNTHASE USING DIFFERENT PROCEDURES

The sonication-gel filtration method was done as a control in expts. 2 and 4 and gave activities of 127 and 150 nmol/min per mg, respectively. In all cases the 'Rott-Nelson' ATP synthase preparation was used. bRh, bacteriorhodopsin.

Procedure [a]	Time (s)	Light-driven ATP synthesis
Expt. 1		
Sonication bRh and phospholipid	0	28
	15	181
	300	433
	900	425
Expt. 2		
Sonication of protein and phospholipid	0	7
	15	38
	45	39
	75	34
	150	13
Expt. 3		
Removal of cholate by dialysis [b]		17
dialysis [c]		12
gel filtration [b]		127
gel filtration [c]		127
Expt. 4		
Freeze-thaw and sonication [d]	0	73
	15	61
	45	31
	200	4

[a] Protein and phospholipid concentrations were the same as described in Materials and Methods in all experiments (soybean phospholipid was used).

[b] Dialysis gel filtration of a mixture of bacteriorhodopsin, ATP synthase, phospholipid and cholate.

[c] Dialysis gel filtration of a mixture of sonicated bacteriorhodopsin liposomes, ATP synthase, phospholipid and cholate.

[d] Freeze-thawing of bacteriorhodopsin liposomes and ATP synthase was done by freezing at −70 °C and thawing at room temperature. Afterwards, different times of sonication were done.

Instead of performing the second step by gel filtration, we also tested the classical dialysis procedure to remove cholate. In this case, we performed the dialysis procedure for sonicated bacteriorhodopsin liposomes and ATP synthase, and for a mixture of bacteriorhodopsin, phospholipid and ATP synthase without sonication. It has been reported [27] that bacteriorhodopsin cannot be reconstituted in liposomes by using gel filtration to remove cholate. This may explain the low activity found for the preparation which was obtained without sonication (Table II, Expt 1). Bacteriorhodopsin can be reconstituted properly by the cholate dialysis procedure [27,28], but in Table II, Expt. 3, it can be seen that if one starts with a mixture of bacteriorhodopsin, phospholipid and ATP synthase, a low activity is also found with the dialysis procedure, comparable to that found with the gel filtration method. Starting from sonicated bacteriorhodopsin liposomes and ATP synthase, the dialysis procedure gave the same results as the gel filtration procedure: the ATP synthesis activity was 8-times higher than that of the preparation obtained without sonication. The gel filtration method, which involves centrifugation of Sephadex columns, has several advantages over the dialysis method: it is quick, the losses are minimal, it can be performed in a very reproducible way, and it allows one to do many reconstitutions at the same time.

In Table II, Expt. 4, it is shown that by freeze-thawing of bacteriorhodopsin liposomes and ATP synthase together, a 2-times lower activity is obtained as compared with the cholate-gel filtration method. Sonication after freeze-thawing [27] leads to preparations with lower ATP synthesis activities.

Dependence of co-reconstitution on detergent concentration and type of detergent

In Fig. 2, the dependence of light-driven ATP synthesis activity on the concentration of different detergents is shown. It can be concluded from Fig. 2 that cholate gives better results than octyl-glucoside and CHAPS [29], a cholate derivative. The optimal cholate concentration was 1.1% for both ATP synthase preparations. After removal of the cholate by gel filtration, still approx. 5% of the total amount of cholate used during the co-reconstitution was present, as could be found with [^{3}H]cholate. In the ATP synthesis assay, the cholate concentration would be 0.005% when co-reconstitution was performed at 1.1% cholate. In order

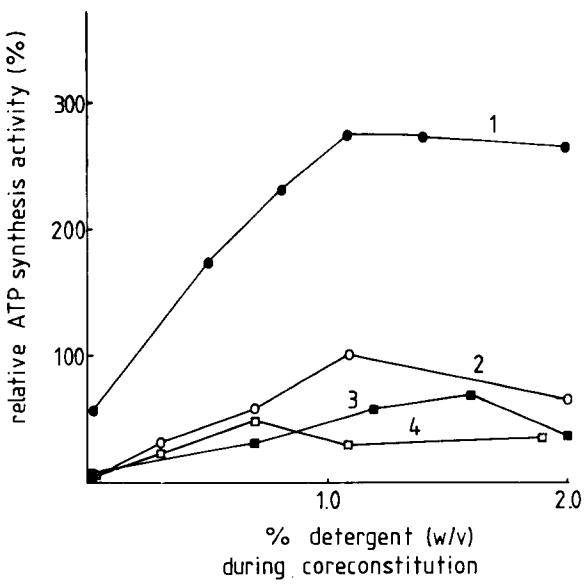

Fig. 2. Dependence of co-reconstitution on the type of detergent and concentration during the second step. Co-reconstitution conditions were as described for the sonication-gel filtration method (Materials and Methods). (1) 'Rott-Nelson' ATP synthase preparation used. (2), (3) and (4) 'Ryrie' ATP synthase preparation used. (1) and (2) cholate, (3) octylglucoside and (4) CHAPS used as detergents. 100% equals 50 nmol/min per mg in case of (1) and (2), 63 nmol/min per mg in case of (3) and 45 nmol/min per mg in case of (4). The 100% values were measured for sonication-gel filtration preparations obtained with 1.1% cholate using 'Ryrie'-ATP synthase preparation.

to test whether residual cholate remaining after co-reconstitution performed at higher concentrations than 1.1% would not have a negative effect on the activities measured, extra cholate was added to a final concentration of 0.01% during illumination. No change in rate of ATP synthesis was found. In order to test whether complete solubilisation of the liposomes takes place at 1.1% cholate, we measured light scattering of the mixture of liposomes and ATP synthase before and after the addition of cholate. Minimal light scattering was found at this concentration of cholate (results not shown).

Co-reconstitution using different types of phospholipid

In Table III, results are presented which were obtained after co-reconstitution using different phospholipids. It can be concluded that soybean phospholipid gives the highest light-driven ATP synthesis activity when compared with egg yolk phosphatidylcholine, dioleoylphosphatidylcholine, and also yeast mitochondrial phospholipid (Table III-1). In Table III-1 it is also shown that the light-driven ATP synthesis measured for the soybean phospholipid liposomes was fully sensitive to uncoupler and oligomycin. From Table III-2, it can be seen that when dilinoleoylphosphatidylcholine was used (soybean phospholipid contains a large amount of linoleic acid residues [30]), also a lower light-driven ATP synthesis activity was obtained than with soybean phospholipid. In the experiments presented in Table III-3, phospholipids with different headgroups were used. Co-reconstitution with phosphatidylserine of phosphatidylinositol resulted in preparations with the same light-driven ATP synthesis activity as was found with egg yolk phosphatidylcholine. ΔG_p showed the same pattern as the light-driven ATP synthesis. In Table III-4, different egg yolk phosphatidylcholine/egg yolk phosphatidylethanolamine ratios were used for co-reconstitution. In an experiment described by Alfonzo et al. [6], optimal coupling between bacteriorhodopsin and ATP synthase was found at a phosphatidylcholine/phosphatidylethanolamine ratio of 1 (mol/mol) or lower. Using phosphatidylcholine and phosphatidylethanolamine mixtures, we found a slightly higher light-driven ATP synthesis than with pure phosphatidylcholine. However, ΔG_p values became about the same as for soybean phospholipid at a ratio for phosphatidylcholine/phosphatidylethanolamine of 1 mol/mol. In Table IV it is shown that ATP-P_i exchange activities are comparable for the different phospholipids.

Another aspect found was that the FCCP stimulation factor of ATP hydrolysis was low for soybean phospholipid but high for egg yolk phosphatidylcholine, dioleoylphosphatidylcholine and dilinoleoylphosphatidylcholine liposomes (Table III-1,2). The low FCCP stimulation factor for soybean phospholipid was only found when ATP synthase was co-reconstituted together with bacteriorhodopsin. After reconstitution of ATP synthase without bacteriorhodopsin in soybean phospholipid (as in the case of the experiment in Table IV), an FCCP stimulation factor of about 3

TABLE III

DEPENDENCE OF CO-RECONSTITUTION ON THE TYPE OF PHOSPHOLIPID

In all cases, the 'Rott-Nelson' ATP synthase preparation was used. n.d., not determined.

Phospholipid	ATP hydrolysis [a] (μmol·min^{-1}·mg^{-1})	FCCP stimulation	Light-driven ATP synthesis (nmol·min^{-1}·mg^{-1})	ΔG_p (kJ·mol^{-1})
Expt. 1				
Egg yolk phosphatidylcholine	36	4.2	58 (1.4) [b]	n.d.
Dioleoylphosphatidylcholine	40	3.6	74 (3.8) [b]	n.d.
Yeast mitochondrial phospholipid	30	1.8	80 (5.5) [b]	n.d.
Soybean phospholipid	19	1.2	163 (16.5) [b]	n.d.
Soybean phospholipid + FCCP [c]			1	
Soybean phospholipid + oligomycin [d]			4	
Expt. 2				
Dioleoylphosphatidylcholine	26	2.7	99	n.d.
Dilinoleoylphosphatidylcholine	33	3.5	117	n.d.
Soybean phospholipid	14	1.2	401	n.d.
Expt. 3				
Egg yolk phosphatidylcholine	11	2.7	35	38.4 (0.2) [e]
Yeast phosphatidylinositol	8	1.0	29	37.8 (0.5) [e]
Bovine phosphatidylserine	7	1.0	31	37.8 (0.5) [e]
Soybean phospholipid	8	1.0	169	41.4 (0.1) [e]
Expt. 4				
Egg yolk phosphatidylcholine	16	2.1	59	39.4 (0.1) [e]
Phosphatidylcholine/ phosphatidylethanolamine (3 : 1)	18	2.4	74	40.7 (0.1) [e]
Phosphatidylcholine/ phosphatidylethanolamine (1 : 1)	12	2.1	82	41.7 (0.1) [e]
Phosphatidylcholine/ phosphatidylethanolamine (1 : 3)	11	1.6	74	41.0 (0.1) [e]
Soybean phospholipid	11	1.0	200	41.8 (0.1) [e]

[a] In the presence of FCCP, ATP hydrolysis was 60% or more oligomycin-sensitive.

[b] Between brackets, standard deviations are given, calculated for three experiments in which the second step of co-reconstitution was performed independently.

[c] 2 μl FCCP solution (2.5 mg/ml in ethanol) was added during illumination.

[d] 4 μl oligomycin (1 mg/ml in methanol) was added during illumination.

[e] The difference is given between two samples taken at different times when steady-state conditions (constant ATP/ADP ratio) were reached during illumination.

was found. This explains why still the same ATP-P_i exchange activities were found for soybean phospholipid, compared with the other phospholipids (Table IV).

Relationship between ATP synthesis and light-driven proton pump activity

In order to test the activity of bacteriorhodopsin after co-reconstitution in the different phospholipids, light-driven proton pump activities were measured. In order to compare initial proton pump activities with ATP synthesis activities, the dependence of both activities on light intensity was measured. When the reciprocal values of the light intensities were plotted against the reciprocal values of the initial proton pump activities or ATP

TABLE IV

DEPENDENCE OF ATP-P_i EXCHANGE ACTIVITY ON THE TYPE OF PHOSPHOLIPID

The 'Rott-Nelson' ATP synthase preparation was used.

Phospholipid	ATP-P_i exchange ($nmol \cdot min^{-1} \cdot mg^{-1}$) [a]
Egg yolk	182
Phosphatidylcholine/phosphatidylethanolamine (1 : 1)	214
Yeast mitochondrial phospholipid	186
Soybean phospholipid	149

[a] Performed after reconstitution of ATP synthase (0.2 mg/ml) in phospholipid (10 mg/ml) by the cholate (1.1%) gel filtration method as described in Materials and Methods.

synthesis activities (Fig. 3), straight lines were obtained. In Fig. 3, the results of three experiments are shown: two with soybean phospholipids and one with egg yolk phosphatidylcholine. In Table V, initial proton pump activities and ATP synthesis activities at infinite light intensity, calculated from the data presented in Fig. 3, are given. It can be seen that both activities correspond with each other. In Table V, also H^+/ATP ratios calculated from both activities are given. Although the two soybean phospholipid preparations differed in activities, similar H^+/ATP ratios

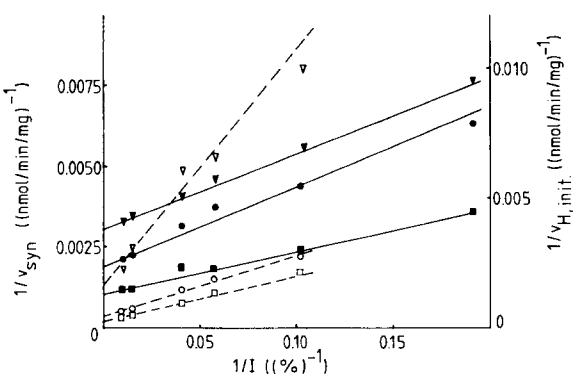

Fig. 3. Dependence of light-driven initial proton pump activities and ATP synthesis activities on light intensity. (———) Proton pump activities; (- - - - - -) ATP synthesis activities. ■, □, Soybean phospholipid preparation 1; ●, ○, soybean phospholipid preparation 2; ▼, ▽, egg yolk phosphatidylcholine preparation.

were found in both experiments. For egg yolk phosphatidylcholine liposomes, this value was 2–3-times higher. Extent values (measured at 100% light intensity) gave the same relation to the ATP synthesis activities (Table V). Instead of calculating H^+/ATP ratios from initial proton pump activities, we also calculated these ratios from initial proton pump activities, we also calculated these ratios from proton pump activities estimated for bacteriorhodopsin at steady-state conditions. As a measure for this, we took the efflux of protons when light was switched off under steady-state conditions. Performing the experiment at 100% light intensity, values of 2–3 were found with the soybean phospholipid preparation; for the egg yolk phosphatidylcholine preparation, again a higher value was found (Table V). Higher H^+/ATP ratios were found when the experiment was done at lower light intensities for both the soybean phospholipid and egg yolk phosphatidylcholine preparations (results not shown). The H^+/ATP ratios were only rough estimates, taking into consideration that the proton pump and ATP synthesis activities were not measured in the same cuvette (although the same light source was used) and that no ADP and P_i was present during measurement of the proton pump activity.

In order to study the effect of variation in proton permeability of the membrane on ATP synthesis activity, the dependence of the latter on light intensity was measured at different protonophore concentrations. From the plots obtained (Fig. 4), it can be seen that for both egg yolk phosphatidylcholine and soybean phospholipid liposomes, the ATP synthesis activity was independent of protonophore concentration at infinite light intensity (this was found in more experiments).

Co-reconstitution at different protein/phospholipid ratios

One way to achieve better coupling between bacteriorhodopsin and ATP synthase might be to lower the phospholipid concentration, keeping the protein concentration constant. The results of an experiment to test this are illustrated in Fig. 5. ATP synthesis reached a maximal value at phospholipid concentrations of 10 mg/ml or more. At low phospholipid concentrations, lower activities were found, although even at the lowest concentra-

TABLE V

RELATION BETWEEN LIGHT-DRIVEN PROTON PUMP ACTIVITY AND ATP SYNTHESIS ACTIVITY

Values were calculated by linear regression from Fig. 3.

Preparation	Soybean phospholipid (1)	Soybean phospholipid (2)	Egg yolk phospholipid choline
Initial proton pump activity (nmol/min per mg) [a]	735	394	266
Extent (nmol/mg) [b]	193	154	96
ATP synthesis activity (nmol/min per mg) [c]	417	286	63
H^+/ATP [d]	9	7	21
H^+/ATP [e]	3	2	8

[a] Initial proton pump per mg bacteriorhodopsin at infinite light intensity.
[b] Extent per mg bacteriorhodopsin at 100% light intensity.
[c] ATP synthesis per mg ATP synthase at infinite light intensity.
[d] H^+/ATP ratios calculated from initial proton pump activity and ATP synthesis at infinite light intensity.
[e] Calculated from bacteriorhodopsin proton pump activities at steady-state conditions during illumination at 100% light intensity.

tion tested (0.5 mg/ml), still activities of more than 100 nmol ATP · min^{-1} · mg^{-1} were found. ΔG_p behaved qualitatively in the same manner as ATP synthesis activity.

Also the bacteriorhodopsin concentration was varied. In the concentration range tested, no maximal value was reached for the light-driven ATP synthesis activity (not shown). At a bacteriorhodopsin concentration of 1 mg/ml (normally used), however, an ATP synthesis activity close to the maximal value was reached.

When ATP synthase was titrated in the concentration range 0.02–0.4 mg/ml, the specific ATP synthesis activity decreased only by a factor 1.7 when going from low to higher ATP synthase concentrations (not shown).

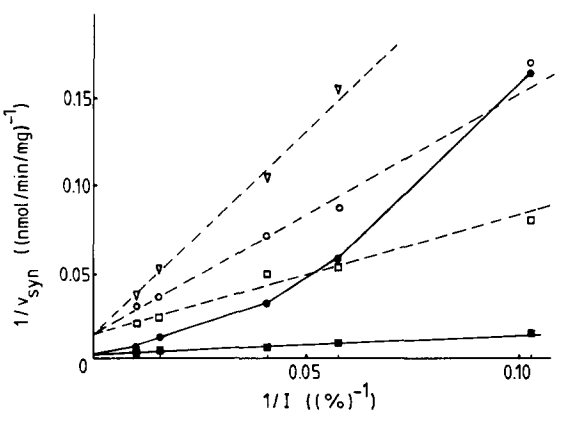

Fig. 4. Dependence of the ATP-synthesis activity on protonophore concentration (FCCP) at different light intensities. Filled symbols, soybean phospholipids used; open symbols, egg yolk phosphatidylcholine used. ■, no FCCP; ●, 205 nM FCCP; □, no FCCP; ○, 10 nM FCCP; ▽ 20 nM FCCP.

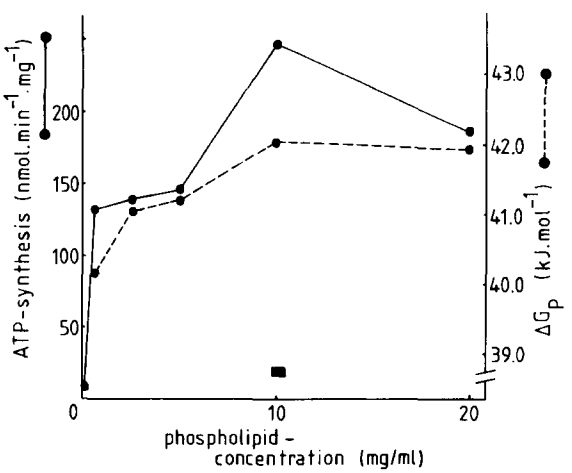

Fig. 5. Variation in phospholipid concentration. The bacteriorhodopsin and ATP synthase concentrations were kept constant (Materials and Methods). The bar indicates the concentration normally used.

Fractionation of liposomes after co-reconstitution

Sucrose density gradient centrifugation. In Fig. 6, a sucrose density gradient pattern is show for liposomes containing bacteriorhodopsin and ATP synthase obtained after co-reconstitution under the optimal conditions described in Materials and Methods (1.1% cholate and soybean phospholipid). It can be concluded that heterogeneity was present, as judged from the bacteriorhodopsin, ATPase and phospholipid distributions. At the top of the gradient, liposomes are found which contain little or no protein. At 30–35% sucrose, bacteriorhodopsin could be found which probably is still present in the form of sheets, considering the high density and the low concentration of phospholipid in these fractions. At about 20–25% sucrose, most of the bacteriorhodopsin was found in a peak together with ATP synthase. This result indicated that most bacteriorhodopsin is incorporated in the same liposomes as the ATP synthase. To test this further, the pattern of Fig. 6 was compared with a pattern (not shown) obtained after co-reconstitution at 0% cholate, a condition in which a much lower functional coupling was found (Fig. 2). The two patterns did not differ much with respect to the bacteriorhodopsin, ATPase and phospholipid distributions. This result could mean that only a small fraction (not detectable) of bacteriorhodopsin and ATP synthase were coupled in the same liposomes, or that liposomes containing both enzymes also arrived at the same sucrose concentration as the liposomes with only one of the proteins.

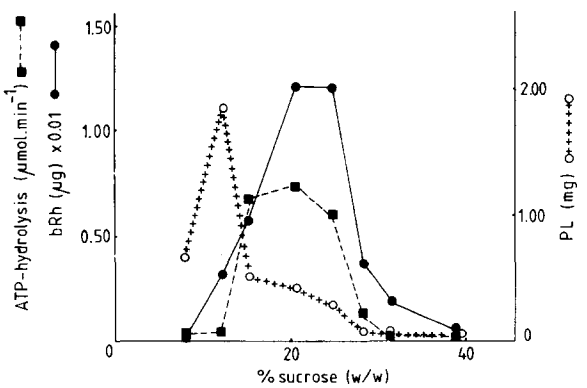

Fig. 6. Sucrose density gradient centrifugation for bacteriorhodopsin (bRh) and ATP synthase co-reconstituted in soybean phospholipid (PL) (using the 'Rott-Nelson' preparation). Co-reconstitution was according to the sonication-gel filtration method. Bacteriorhodopsin, phospholipid and ATP synthase are given in total amount per fraction (2 ml). Recovery for bacteriorhodopsin, phospholipid and ATPase activity was 60, 80 and 80%, respectively.

In order to obtain a better separation between liposomes containing bacteriorhodopsin or ATP synthase only from those containing both enzymes, centrifugation was performed for 62 h instead of 15 h. In this case, a real equilibrium pattern was found; the same pattern was found when liposomes were brought in the gradient near the bottom before centrifugation was started (not shown). However, no better separation was obtained between bacteriorhodopsin and ATP synthase than in the experiment of Fig. 6. Both proteins appeared in a combined peak, now at 25–30% sucrose.

In order to determine the density of non-incorporated ATP synthase, ATP synthase alone was loaded onto a sucrose gradient. After 62 h of centrifugation most of the ATPase complex could be found at a higher density (not shown) than that found for preparation obtained after co-reconstitution using the same centrifugation time. From this, we conclude that all ATP synthase had become associated with phospholipid after co-reconstitution and therefore was incorporated in liposomes.

The ATP synthesis activity per amount of ATP synthase (for which we took the ATP hydrolysis activity in the presence of FCCP as a measure) in different fractions in Fig. 6 was constant (not shown). Thus, no indications were found by sucrose gradient centrifugation that part of the ATP synthase had become functionally better coupled to bacteriorhodopsin after co-reconstitution than the rest.

Sepharose 2B-CL gel filtration. From a sucrose density gradient (obtained after 15 h centrifugation) like the one shown in Fig. 6, the ATPase-containing peak fractions were pooled and concentrated by vacuum dialysis (Schleicher and Schüll, Dassel, F.R.G.; using Ultrahulzen apparature type UH 100/2B and Ultrahulzen type UH 100/25). The concentrated fraction was loaded onto a Sepharose 2B-CL column and eluted. In Fig. 7, the elution profile of the ATPase activity is

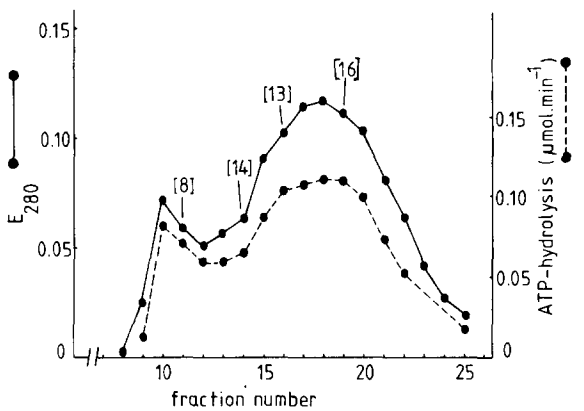

Fig. 7. Sepharose 2B-CL gel filtration of pooled and concentrated sucrose density gradient fractions. ATP synthase is given in total amount per fraction (2.5 ml). Figures between brackets represent ATP synthesis activity per amount ATP hydrolysis activity (nmol·μmol^{-1}). Recovery of ATPase activity was 71%.

given together with the absorbance at 280 nm. The bacteriorhodopsin and phospholipid concentrations were too low to detect in this case. A small amount of the ATPase activity could be found in the void volume fractions, the rest in a retarded broad peak. In Fig. 7, the figures between brackets represent the ATP synthesis activity per ATP hydrolysis activity, measured in the presence of FCCP. There was no large variation in this parameter, although for the smaller liposomes, higher values were found than for the liposomes in the void volume fractions. The liposomes in the retarded broad peak had the same values as the starting preparation that was loaded onto the column. On the basis of this criterion, no large heterogeneity could be detected in the liposomes involved in the ATP synthesis reaction during Sepharose 2B-CL gel filtration.

Discussion

In order to be able to do quantitative studies on energy transduction by proton pumps, homogeneous liposome suspensions are needed in which bacteriorhodopsin and ATP synthase are maximally coupled in a functional way. For this reason, several aspects of the co-reconstitution technique were studied.

Overall procedure

A two-step procedure (sonication-gel filtration method) gave the best results in our hands (Table II). We found 2–3-times higher ATP synthesis activities (up to 500 nmol · min^{-1} · mg^{-1}) than reported in the literature (up to 200 nmol · min^{-1} · mg^{-1} [3–8]). ATP synthesis was completely blocked in the presence of an uncoupler or oligomycin. Also, no ATP synthesis was found after incubation in the dark. This is in accordance with ATP synthesis taking place by ATP synthase [1] and not by, for example, myokinase that might still be present in the ATP synthase preparation.

Maximal light-driven ATP synthesis was found when about 1% cholate was used during co-reconstitution, as was also found by Ryrie et al. [5] who used dialysis to remove the cholate. Using the cholate-dilution technique, they found the highest activity at a cholate concentration of 0.3%. The lower activities at higher cholate concentrations which they found in the latter case might be explained by the relatively high cholate concentration after dilution which may disturb the process of ATP synthesis. Using the gel filtration method to remove cholate, we found 2–3-times higher ATP synthesis activities (with the 'Rott-Nelson' preparation) than Ryrie obtained with the cholate-dilution technique.

Purification of the ATP synthase

An optimal co-reconstitution of bacteriorhodopsin and ATP synthase may be largely dependent on the quality of the ATP synthase. For bacteriorhodopsin this seems to be no problem, as it is stable under rather denaturing conditions [9,10]. For the ATP synthase, which is rather sensitive to the conditions it is exposed to, purification and reconstitution may give problems. We purified ATP synthase in two ways. Both preparations obtained were similar in oligomycin-sensitive ATP hydrolysis activity and similar SDS-polyacrylamide gel patterns were found. However, ATP-P_i exchange and light-driven ATP synthesis activities were quite different. The reasons for this discrepancy are not clear, but may be related to the type of detergent used for extraction, or to proteolytic breakdown of essential, but not further identified, subunits: the purification method according to Rott and Nelson is twice as rapid as that of Ryrie.

Effect of different types of phospholipid

With soybean phospholipid, the best functional coupling of bacteriorhodopsin and ATP synthase was achieved. The reason for the lower results found with other phospholipids (Table IV), even with yeast mitochondrial phospholipid which forms the original environment for the ATP synthase, is not clear. Apparently, the reason is not the presence of large amounts of unsaturated fatty acid residues (especially linoleic acid [30]) in soybean phospholipid or the presence of negatively charged phospholipids which may be essential for the functioning of ATP synthase [31]. Also, it seemed not to depend on the phospholipid dependence of the intrinsic activity of ATP synthase itself, comparing ATP hydrolysis and ATP-P_i exchange activities for the different phospholipids. Even lower values were obtained for these parameters with soybean phospholipid. In this case, it may be considered that the ATPase inhibitor subunit [32] binding may be different for the different phospholipids. We could, however, not demonstrate the presence of this subunit through trypsin activation of ATP hydrolysis activity [16] in either of our ATP synthase preparations.

A third possibility is a difference in the bacteriorhodopsin proton pump activity. For dioleoylphosphatidylcholine, it has been reported [9] that lower initial proton pump activities and extent values were found than for soybean phospholipids. Comparing initial proton pump activities and extent values for egg yolk phosphatidylcholine and soybean phospholipids (Fig 3, Table V), we found that bacteriorhodopsin was less active in the egg yolk phosphatidylcholine vesicles. The lower, light-driven ATP synthesis activities in these vesicles can be explained by this finding. Differences in the initial proton pump activities and extent values are probably due to an effect of the phospholipids on bacteriorhodopsin itself, or to a not optimal protein distribution, orientation among and in the liposomes, respectively.

Membrane leakiness for protons seems to have less effect, if one considers that the FCCP stimulation of ATP hydrolysis (Table III) was much higher for, e.g., egg yolk phosphatidylcholine than for soybean phospholipids. In the experiment of Fig. 4, the effect of membrane leakiness for protons on the light-driven ATP synthesis activity was further investigated. It was found that at infinite light intensity, the ATP synthesis reaction was not sensitive to variation in membrane leakiness. The same holds for the proton gradient measured in bacteriorhodopsin liposomes [33]. Apparently, at very high light intensity, bacteriorhodopsin has an overcapacity in proton pump activity compared to the membrane leakiness for protons. In this an explanation may be found for the fact that soybean phospholipids still give high ATP synthesis activity, although they give rather leaky liposomes, as found from the low FCCP stimulation factor of the ATP hydrolysis activity (Table III).

Another reason for the difference in ATP synthesis activity for the different phospholipids may be that there is a difference in protein distribution among the liposomes. This is supported by H^+/ATP ratios calculated from initial proton pump activities and light-driven ATP synthesis activities at infinite light intensity (Table V). For different soybean phospholipid preparations about the same ratio was found, but for egg yolk phosphatidylcholine liposomes, this value was significantly higher. This can be understood as the pumped protons are less efficiently used in the latter liposomes. As can be concluded from Tables III and IV, the ATP synthase itself had the same activity in both egg yolk phosphatidylcholine and soybean phospholipid liposomes. An explanation for the difference in H^+/ATP ratio may be that with egg yolk phosphatidylcholine, less bacteriorhodopsin is incorporated in liposomes together with ATP synthase. ΔG_p values are running parallel with ATP synthesis activity, which corresponds to both aspects mentioned above: a difference in bacteriorhodopsin activity and a difference in protein distribution.

ΔG_p values give information about the electrochemical proton gradient sensed by ATP synthase, as well as about the fraction of ATP synthase that is not coupled to bacteriorhodopsin in the same liposomes but only participates in the ATP hydrolysis reation. For the phosphatidylcholine/ethanolamine experiment, the discrepancy between ATP synthesis and ΔG_p can be explained by the assumption that more ATP synthase is coupled to bacteriorhodopsin in the same liposomes, but that bacteriorhodopsin has a lower activity (relative to soybean phospholipid vesicles).

Efficiency of energy transduction in the co-reconstituted preparation

One of the limiting factors in the light-driven ATP synthesis reaction was the light intensity under the conditions normally used (100%, Fig. 3). Therefore, the initial proton pump activity of bacteriorhodopsin and ATP synthesis activity were compared after extrapolation to infinite light intensity (Fig. 3). For the optimal preparation with highest light-driven ATP synthesis activity, an H^+/ATP ratio of 7–9 was found. Since it is generally assumed that the H^+/ATP ratio for the ATP synthase is 2–4 (cf. for instance Ref. 34), it can be concluded that there is an overcapacity of bacteriorhodopsin proton pump activity. The initial proton pump activity, however, will be higher than the proton pump activity during ATP synthesis, as bacteriorhodopsin will be slowed down by the presence of an electrochemical proton gradient [18,35]. When H^+/ATP ratios were calculated from bacteriorhodopsin proton pump activities under steady-state conditions (Table V), ratios of 2–3 were found for soybean phospholipid preparations at maximal light intensity, which, although they were only rough estimates, are about the same as expected for the ATP synthase [34].

The ATP synthesis activity in intact mitochondria may be 5000 nmol/min per mg ([36], based on a mitochondrial ATP synthase content of about 10%, w/w [37]. For the optimal co-reconstituted system, only 10% of the activity in vivo (500 nmol/min per mg) was found, which is, however, not too bad considering that this means a 100-times improvement in comparison with the first reports on the co-reconstitution [2]. Although light intensity is a limiting factor, low activities are found even after extrapolation to infinite light intensity (Table V). The ATP synthesis activities found at infinite light intensity were independent of variation in proton permeability (Fig. 4) and the bacteriorhodopsin proton pump capacity seems to be also not a limiting factor considering the observed H^+/ATP ratios (Table V). One explanation may be that still not enough bacteriorhodopsin is coupled to ATP synthase in the active liposomes. A way to accomplish this may be varying the concentrations of protein and phospholipid during co-reconstitution. Variation in bacteriorhodopsin or ATP synthase concentrations did not result in much higher activities (results not shown). Lowering the phospholipid concentration also did not result in higher ATP synthesis activities (Fig. 5). In the latter case, however, it could be that relatively less protein becomes incorporated in the liposomes at lower phospholipid concentrations.

Structural analysis is necessary to achieve a better understanding of the role of heterogeneity in protein distribution and orientation among and in the liposomes. Analysis performed so far by sucrose gradient centrifugation indicated that for the optimal preparation most of the bacteriorhodopsin and ATP synthase were coupled in the same liposomes. At the moment, we are testing antibody techniques, trying to obtain more reliable information about the protein distribution among the liposomes. This information will also be important for further thermodynamic and kinetic studies.

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References

1 Mitchell, P. (1966) Biol. Rev. 41, 445–502
2 Racker, E. and Stoeckenius, W. (1973) J. Biol. Chem. 249, 662–663
3 Dewey, T.G. and Hammes, G.G. (1981) J. Biol. Chem. 256, 8941–8946
4 Takabe, T. and Hammes, G.G. (1981) Biochemistry 20, 6859–6864
5 Ryrie, I.J., Critchley, C. and Tillberg, J.E. (1979) Arch. Biochem. Biophys. 198, 182–194
6 Alfonzo, M., Kandrach, M.A. and Racker, E. (1981) J. Bioenerg. Biomem. 13, 375–391
7 Sone, N., Takeuchi, Y., Yoshida, M. and Ohno, K. (1977) J. Biochem. 82, 1751–1758
8 Oren, R., Weiss, S., Garty, H., Caplan, S.R. and Gromet-Elhanan, Z. (1980) Arch. Biochem. Biophys. 205, 503–509
9 Huang, K.-S., Bayley, H. and Khorana, H.G. (1980) Proc. Natl. Acad. Sci. USA 77, 323–327
10 Huang, K.-S., Bayley, H., Liao, M.-J., London, E. and Khorana, H.G. (1981) J. Biol. Chem. 256, 3802–3809

11 Kagawa, Y. and Racker, E. (1971) J. Biol. Chem. 246, 5477–5487
12 Rouser, G. and Fleischer, S. (1967) Methods Enzymol. 10, 385–406
13 Stutterheim, E., Henneke, M.A.C. and Berden, J.A. (1980) Biochim. Biophys. Acta 592, 415–430
14 Oesterhelt, D. and Stoeckenius, W. (1974) Methods Enzymol. 31A, 667–678
15 Oesterhelt, D. and Hess, B. (1973) Eur. J. Biochem. 37, 316–326
16 Ryrie, I.J. (1977) Arch. Biochem. Biophys. 184, 464–475
17 Rott, R. and Nelson, N. (1981) J. Biol. Chem. 256, 9224–9228
18 Hellingwerf, K.J., Arents, J.C., Scholte, B.J. and Westerhoff, H.V. (1979) Biochim. Biophys. Acta 547, 561–582
19 Penefsky, H.S. (1979) Methods Enzymol. 56, 527–530
20 Berden, J.A. and Henneke, M.A.C. (1981) FEBS Lett. 126, 211–214
21 Bergmeyer, H.U. (ed.) (1970) Methoden der enzymatischen Analyse, 2nd edn., Verlag Chemie, Weinheim
22 Rosing, J. and Slater, E.C. (1972) Biochim. Biophys. Acta 267, 275–290
23 Böttcher, C.J.F., Van Gent, C.M. and Pries, C. (1961) Anal. Chim. Acta 24, 203–204
24 Peterson, G.L. (1977) Anal. Biochem. 83, 346–356
25 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254
26 Laemmli, U.K. (1970) Nature 227, 680–685
27 Racker, E. (1979) Methods Enzymol. 55, 527–530
28 Van Dijck, P.W.M. and Van Dam, K. (1982) Methods Enzymol. 88, 17–25
29 Hjelmeland, L.M. (1980) Proc. Natl. Acad. Sci. USA 77, 6368–6370
30 Kagawa, Y., Kandrach, A. and Racker, E. (1973) J. Biol. Chem. 248, 676–684
31 Brown, R.E. and Cunningham, C.C. (1982) Biochim. Biophys. Acta 684, 141–145
32 Ebner, E. and Maier, K.L. (1977) J. Biol. Chem. 252, 671–676
33 Arents, J.C., Hellingwerf, K.J., Van Dam, K. and Westerhoff, H.V. (1981) J. Membrane Biol. 60, 95–104
34 Van Dam, K., Westerhoff, H.V., Krab, K., Van der Meer, R. and Arents, J.C. (1980) Biochim. Biophys. Acta 591, 240–250
35 Quintanilha, A.I. (1980) FEBS Lett. 117, 8–12
36 Kovac, L., Bednarova, H. and Greksak, M. (1968) Biochim. Biophys. Acta 153, 32–42
37 Kagawa, Y. and Racker, E. (1966) J. Biol. Chem. 241, 2475–2482