Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 4233–4237, June 1986
Biochemistry

Steady-state ATP synthesis by bacteriorhodopsin and chloroplast coupling factor co-reconstituted into asolectin vesicles

(membrane-bound proteins/reconstitution/proton gradient)

JOHN KRUPINSKI AND GORDON G. HAMMES

Section of Biochemistry, Molecular, and Cell Biology and Department of Chemistry, Cornell University, Ithaca, NY 14853-1301

Contributed by Gordon G. Hammes, February 24, 1986

ABSTRACT **A method was developed for the co-reconstitution of bacteriorhodopsin and chloroplast coupling factor in asolectin vesicles. First, bacteriorhodopsin was reconstituted from a mixture of octyl glucoside, asolectin, and protein in the presence of ethylenediaminetetraacetic acid by passage through a Sephadex G-50 centrifuge column. Then, the purified coupling factor was reconstituted from a mixture of sodium cholate, bacteriorhodopsin vesicles, and coupling factor in the presence of Mg^{2+} by passage through the centrifuge column. Sucrose density-gradient centrifugation indicated a band of vesicles with slightly different positions in the gradient for maximum vesicle concentration, bacteriorhodopsin vesicle concentration, ATP synthesis, and ATP hydrolysis. The rate of light-driven ATP synthesis reaches a limiting value as the concentration of bacteriorhodopsin and the light intensity are increased. A steady-state rate of ATP synthesis of 1 μmol per mg of coupling factor·min^{-1} has been achieved. Apparently this rate is limited by the heterogeneity within the vesicle population and by the ability of bacteriorhodopsin to form a sufficiently large pH gradient.**

Bacteriorhodopsin (bR) is a light-driven proton pump found in the purple membrane of *Halobacterium halobium* (cf. ref. 1). It is readily reconstituted into phospholipid vesicles in which the interior is acidified upon illumination (cf. ref. 2). Racker and Stoeckenius (3) were the first to demonstrate that vesicles co-reconstituted with bR and the ATP synthesizing complex, initially from mitochondria, synthesized ATP from ADP and inorganic phosphate upon illumination of the vesicles. The observed rates of ATP synthesis were ≈0.1% of the rates for oxidative phosphorylation found in mitochondria. A highly active co-reconstituted preparation of bR and ATP synthase would be an ideal model system with which to perform studies on the mechanism of energy coupling. Consequently, this experimental system has been refined and applied to other ATPases.

The dicyclohexylcarbodiimide-sensitive ATP-synthesizing complex from chloroplasts (DSA) (cf. ref. 4) was first successfully co-reconstituted with bR by Winget *et al.* (5). Subsequently, the co-reconstituted system was used to study the steady-state kinetics of ATP synthesis and hydrolysis (6, 7). The maximum level of light-dependent ATP synthesis in those studies was ≈100 nmol·mg^{-1}·min^{-1}. This value is comparable to that observed in a variety of co-reconstituted preparations (8–12). Recently, van der Bend *et al.* (13) reported synthetic rates of up to 500 nmol·mg^{-1}·min^{-1} with co-reconstituted preparations of the mitochondrial ATP synthase. In the present study, we report the co-reconstitution of bR and DSA from spinach chloroplasts with maximum rates of light-driven ATP synthesis of ≈1 μmol·mg^{-1}·min^{-1}.

MATERIALS AND METHODS

Chemicals. Asolectin was from Associated Concentrates (Woodside, NY). Sephadex G-50 fine was from Pharmacia Fine Chemicals. Pyranine was from Molecular Probes (Plano, TX). Octyl β-D-glucopyranoside was from Calbiochem-Behring. Sodium deoxycholate, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), dicyclohexylcarbodiimide, dithiothreitol, Tricine, and Triton X-100 were from Sigma. All other chemicals were high quality commercial grades.

Reconstitution of bR into Phospholipid Vesicles. bR was reconstituted by using a Sephadex G-50 centrifuge column (14) to remove octyl β-D-glucopyranoside from a mixture of detergent, lipid, and protein. bR was isolated from *H. halobium* strain S9-P and deoxycholate-purified (15). Asolectin liposomes were prepared at a concentration of 10 mg/ml in 150 mM KCl/2 mM EDTA/10 mM Na-Tricine, pH 8.0. In some experiments, the fluorescent pH indicator, pyranine, was substituted for Na-Tricine in the buffer. The asolectin suspension was mixed in a Vortex under nitrogen for 5 min and then sonicated to clarity in a bath-type sonicator (Laboratory Supplies, Hicksville, NY). The asolectin was solubilized by adding 26 μl of 0.5 M octyl β-D-glucopyranoside to 175 μl of vesicles, the mixture was chilled on ice, and the detergent/lipid mixture was added to 175 μl of ice-cold buffer containing 0.05–1.2 mg of deoxycholate-purified bR. Typically, the final concentrations were bR, 1 mg/ml; asolectin, 5 mg/ml; and octyl glucoside, 10 mg/ml. Three milliliters of Sephadex G-50 fine pre-equilibrated in buffer was packed by gravity in a disposable filter column (Fisher Scientific) and excess buffer was removed by centrifugation for 4 min at 900 rpm in an IEC HN-SII tabletop centrifuge (Damon/IEC Division). After a 10-min incubation on ice, the detergent/lipid/bR mixture was applied to the packed Sephadex and centrifuged for an additional 3 min at 900 rpm. For comparison, bR was also reconstituted by forming vesicles as described above and then sonicating the bR and vesicles together for 30 min as described by Bell *et al.* (16).

When necessary, the bR vesicles were concentrated by centrifugation onto a sucrose cushion. The vesicles were transferred to Beckman SW-60 tubes, diluted to 3.5 ml with buffer, and underlaid with 0.2 ml of 40% sucrose (wt/wt). The sample was centrifuged for 60 min at 311,000 × *g* in a Beckman SW-60 rotor maintained at 2°C. Approximately 125 μl of vesicles was collected from the interface.

The concentration of bR in the isolated sample was determined by solubilizing 15 μl of the vesicles in 600 μl of 0.1% Triton X-100 in buffer and measuring the absorbance at 560 nm. The extinction coefficient of reconstituted deoxycholate-purified bR at 560 nm and pH 8 is decreased to ≈1.4

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Abbreviations: bR, bacteriorhodopsin; DSA, dicyclohexylcarbodiimide-sensitive ATP-synthesizing complex from chloroplasts; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

$ml \cdot mg^{-1} \cdot cm^{-1}$. This reflects the pH dependence of the ground state of deoxycholate-purified bR (17).

Reconstitution of DSA into Phospholipid Vesicles. DSA was isolated from commercially available spinach. Both the preparation of crude DSA and the sucrose gradient-purified enzyme were as described (18) except that the concentrations of Na-cholate and octyl glucoside used in the detergent extraction step were reduced to 2.0 mg/ml and 4.4 mg/ml, respectively, and asolectin was omitted from the sucrose gradient. The enzyme was reconstituted into phospholipid vesicles by using a Sephadex G-50 fine centrifuge column to remove Na-cholate from the mixture of protein, detergent, and lipid. Either asolectin vesicles or bR/asolectin vesicles were prepared as described above. Na-cholate (pH 8.0), $MgCl_2$, and DSA were then added in order so that the final concentrations in 350 μl were as follows: Na-cholate, 7 mg/ml (gradient-purified DSA) or 9 mg/ml (DSA, not gradient purified); $MgCl_2$, 9 mM; DSA, ≈0.4 mg/ml; and asolectin with or without bR, 5 mg/ml. The final volume was adjusted to 350 μl with 150 mM KCl/2 mM EDTA/10 mM Na-Tricine, pH 8.0. The centrifuge column reconstitution was carried out as described for bR except that the Sephadex G-50 fine was pre-equilibrated with 150 mM KCl/2 mM EDTA/9 mM $MgCl_2$/10 mM Na-Tricine, pH 8.0. In some experiments, 75 mM K_2SO_4 and 9 mM $MgSO_4$ were substituted for the chloride salts.

The composition of the lipid vesicles was characterized by centrifugation on a sucrose gradient. A 15-ml linear sucrose gradient consisting of 7–40% sucrose (wt/wt) in 150 mM KCl/2 mM EDTA/9 mM $MgCl_2$/0.1 mM ADP/10 mM Na-Tricine, pH 8.0, was layered on top of a 1-ml 60% sucrose cushion. DSA–bR vesicles, which contained pyranine from the bR reconstitution, were applied and centrifuged at 115,000 × g for 12 hr at 2°C in a Beckman SW 27.1 rotor. Fractions of 1.1 ml were collected. DSA was located by using 0.1 ml of each fraction in an ATP–P_i exchange assay (see below). Vesicles were located by solubilizing 0.4 ml of each fraction in an equal vol of 0.2% Triton X-100 in gradient buffer and monitoring the fluorescence of pyranine at 510 nm (465 nm excitation). Fluorescence measurements were made on a Perkin–Elmer MPF 44 spectrofluorimeter. The bR was detected by measuring the absorbance of the solubilized sample at 560 nm. Total protein was determined by the method of Bensadoun and Weinstein (19).

Activity Assays. ATPase and ATP–P_i exchange activities were measured at 37°C as described (5), except that bovine serum albumin was omitted from the reaction medium. Where indicated, dicyclohexylcarbodiimide was added from a 0.25 M dimethyl sulfoxide dry solution and FCCP was added from a 2 mM ethanol solution. For synthesis assays, the exchange reaction medium was used except that ADP was substituted for ATP, and hexokinase and glucose were included at 10 units/ml and 30 mM, respectively. The synthesis assay was performed with 1.2 ml of reaction mixture containing bR–DSA vesicles at a final DSA concentration of ≈25 μg/ml. The reaction was initiated by illumination with a Kodak 600 H slide projector with an ELH lamp (300 W) focused on the sample. For intensity variation, either neutral density filters (Ditric Optics, Hudson, MA) or additional projectors were included. With one projector and no neutral density filters, the light intensity was $\approx 10^6$ $erg/cm^2 \cdot sec^{-1}$ as measured with a YSI-Kettering Model 654 photometer (Radiometer). A test tube (13 × 100 mm) containing the sample was inserted in a clear glass sample holder that permitted 2.5 cm of water to circulate continuously between the projector and the test tube. The water was maintained at 32°C. After 5 min of illumination, half of the sample was removed and added to 70 μl of 50% trichloroacetic acid. The other half of the sample was quenched in the same manner after 10 min of illumination. The synthetic rate was linear for at least 20 min of illumination. The amount of glucose 6-[^{32}P]phosphate was determined by the same extraction procedure used in the exchange assay (5).

RESULTS

The Sephadex G-50 centrifuge column technique used to reconstitute bR and DSA is a rapid means of obtaining a stable preparation of vesicles capable of high levels of light-driven ATP synthesis. Under conditions of near saturating light and maximum incorporation of bR, the vesicles synthesize ATP at a rate of ≈1 $\mu mol \cdot mg^{-1} \cdot min^{-1}$. The preparation maintains 87% of its synthetic activity after storage on ice for 72 hr. However, some protein is lost during the reconstitutions. If bR is used at 1 mg/ml in the original incubation, then ≈30% is lost in the subsequent centrifuge columns. The yield for the DSA is ≈90% as determined either by measuring the total protein in DSA–bR vesicles and subtracting the amount of bR as determined by absorbance or by determining the total protein in DSA vesicles. The activities presented are in terms of total DSA and have not been corrected for this loss.

A sucrose density-gradient profile of the co-reconstituted preparation of bR and DSA is presented in Fig. 1. The data indicate heterogeneity in the vesicle population. The maximum pyranine fluorescence, indicative of the vesicle peak, is observed in fraction 4. The long shoulder on the fluorescence profile suggests different subpopulations of vesicles. The shoulder is not due to the use of pyranine, since the same result was observed when the indicator was omitted and the vesicles were located by light scattering. The bR profile also is asymmetric. The activity profile for ATP–P_i exchange, which peaks in fraction 6, is more symmetric. Despite the lack of homogeneity, a substantial degree of overlap between the various components in the preparation exists. The portion of the gradient that extends from 17–24% sucrose (fractions 4–7) includes 75% of the observed exchange activity, 73% of the bR, and 55% of the pyranine fluorescence. The ATPase activity profile is qualitatively similar to the exchange profile if hydrolysis is assayed in the presence of 10 μM FCCP. If this protonophore is omitted from the assay, then the apparent peak in the hydrolysis activity is shifted from fraction 6 to fraction 7. This result implies that the amount of DSA coupled to the electrochemical proton gradient varies within the vesicle population. The fact that this DSA is associated with bR and lipid suggests that it is improperly reconstituted DSA and not free protein. Also, the lack of any detectable bR at the interface between 40% and 60% sucrose (fractions 14 and 15) indicates that all of the bR is associated with lipid.

The inset of Fig. 1 is an electron micrograph of a typical bR–DSA vesicle preparation. This corroborates the results of the sucrose density-gradient analysis. While bR cannot be visualized at this resolution, DSA is clearly visible as a ball and stalk at the edge of vesicles. Some DSA-free vesicles are also present, consistent with the fact that the peak of pyranine fluorescence in the sucrose gradient is displaced from the exchange activity peak. The electron micrograph indicates that the DSA has a tendency to reconstitute in groups rather than as individual molecules. From other electron micrographs, vesicles were found to range in diameter from ≈25 to 110 nm.

Fig. 2 shows a plot of the rate of light-driven ATP synthesis as a function of the relative light intensity. The most important feature of the data is that the synthetic rate saturates with increasing light intensity. If the data are fit to a rectangular hyperbola, then the extrapolated value for synthesis at infinite light intensity is ≈0.72 $\mu mol \cdot mg^{-1} \cdot min^{-1}$. The data in the figure were obtained with a preparation in which the starting concentration of bR was 1 mg/ml and the DSA (0.4 mg/ml) was not gradient-purified. If gradient-purified DSA is

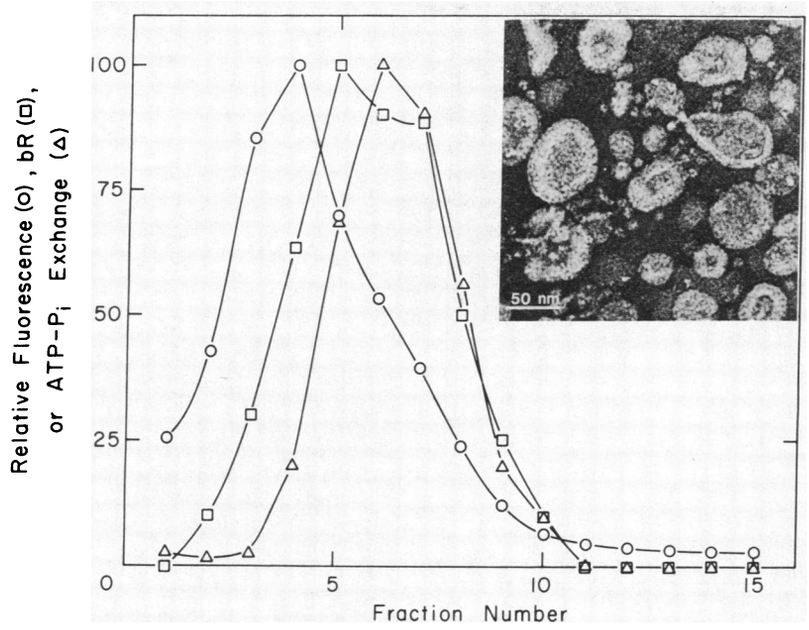

FIG. 1. Sucrose-gradient profile of the bR–DSA vesicles co-reconstituted by the Sephadex G-50 centrifuge column technique. The vesicles contain bR (0.7 mg/ml), DSA (0.4 mg/ml), asolectin (5 mg/ml), and ≈1 μM pyranine in 0.45 ml of 150 mM KCl/2 mM EDTA/9 mM $MgCl_2$/10 mM Na-Tricine, pH 8.0. The conditions of centrifugation and the various assays are described in *Materials and Methods*. The relative amounts of pyranine fluorescence (○) and bR (□), and the relative exchange activity (△) are shown. (*Inset*) Vesicles formed under the same conditions as described for the sucrose gradient, except for the absence of pyranine, were diluted 1:3 with buffer and viewed by negative staining (sodium phosphotungstic acid) in the electron microscope. (Bar = 50 nm.) Electron microscopy was performed courtesy of John Telford, Cornell University.

used, only a modest improvement in the synthetic activity is observed. The synthetic rate at a relative intensity of 3 is increased 20% to 0.7 $\mu mol \cdot mg^{-1} \cdot min^{-1}$. This is in contrast to the specific exchange activity, which is increased by a factor of 2 after gradient purification (ref. 18 and our observations).

Fig. 3 indicates that the concentration of bR used in the co-reconstitution also limits the observed rate of synthesis. bR reconstitutions were performed at the indicated starting concentration, and then gradient-purified DSA was reconstituted at a final concentration of 0.4 mg/ml. For the highest concentration of bR, 3.3 mg/ml, a substantial fraction of the protein was not incorporated in the reconstitution step, as evidenced by residual purple on the Sephadex G-50 after the centrifuge column. The data up to 2 mg/ml show a simple hyperbolic dependence on the bR concentration. If the highest point is eliminated from the analysis, the extrapolated maximum rate of synthesis is $\approx 1.46\ \mu mol \cdot mg^{-1} \cdot min^{-1}$. Clearly, this rate cannot be achieved unless a means of successfully increasing the amount of reconstituted bR can be devised. The maximum specific activity for ATP synthesis observed varies somewhat with the preparations of bR and DSA. However, specific activities of 0.95 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ were routinely obtained; the largest values observed were $\approx 1.2\ \mu mol \cdot mg^{-1} \cdot min^{-1}$.

A variety of different conditions were tested in both the assays and reconstitution. Some typical results are presented in Table 1. A two-step procedure is required for an optimal co-reconstitution, because the two proteins require different conditions for optimal incorporation into phospholipid vesicles. This is evident from several entries in the table. If the bR vesicles are formed by sonication or with 9 mM $MgCl_2$, or if octyl glucoside is substituted for cholate in the DSA reconstitution, the synthetic activity is markedly decreased (Table 1, rows 1–3). If 75 mM K_2SO_4 and 9 mM $MgSO_4$ are substituted for 150 mM KCl and 9 mM $MgCl_2$ in the DSA reconstitution, the specific exchange activity increases, but the synthetic activity decreases (Table 1, rows 4 and 6). The control exchange activities ranged from 250 to 500

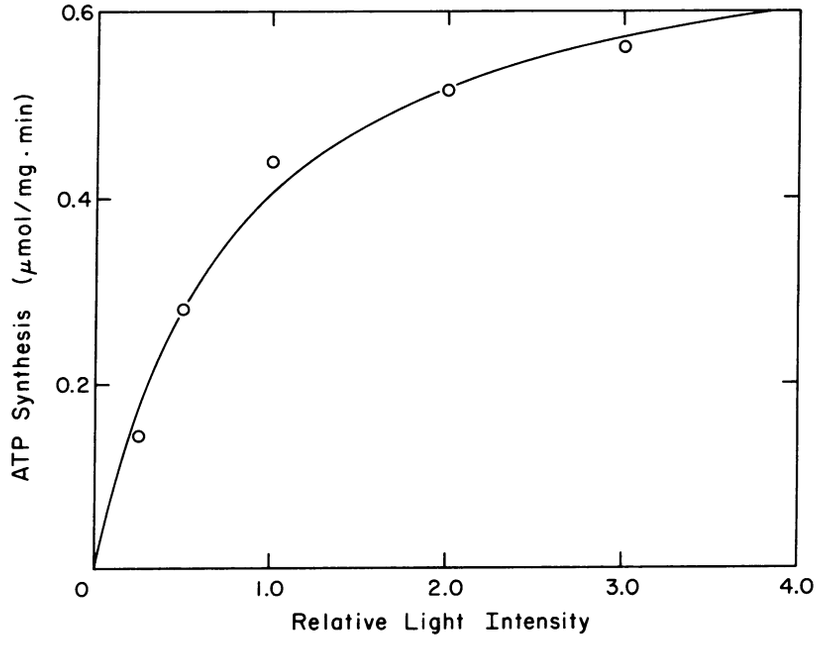

FIG. 2. ATP synthesis as a function of light intensity. bR–DSA vesicles were co-reconstituted as described in *Materials and Methods* with final concentrations of bR at 0.7 mg/ml, DSA at 0.4 mg/ml, and asolectin at 5 mg/ml in 150 mM KCl/2 mM EDTA/9 mM $MgCl_2$/10 mM Na-Tricine, pH 8.0. Synthesis assays were performed at 32°C as described. The assay medium contained 80 mM Na-Tricine (pH 8.0), 12 mM ADP, 12 mM $MgCl_2$, 20 mM inorganic phosphate, $\approx 10^6$ cpm of $^{32}P_i$ per ml, hexokinase (10 units/ml), 30 mM glucose, and bR–DSA vesicles at a final concentration of 25 μg/ml. The intensity was varied by using neutral-density filters or multiple projectors. A relative intensity of 1 corresponds to $\approx 10^6\ erg/cm^2 \cdot sec^{-1}$.

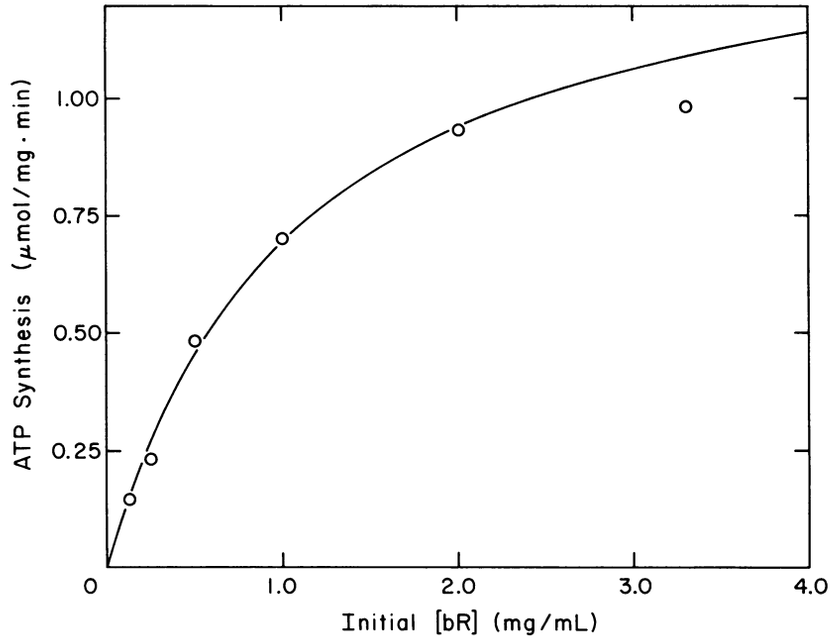

FIG. 3. ATP synthesis as a function of bR concentration. bR was reconstituted as described in *Materials and Methods* with the concentration of bR indicated on the abscissa in the original octyl glucoside incubation. Gradient-purified DSA was then co-reconstituted at a final concentration of 0.4 mg/ml. The assay medium was the same as that described in the legend to Fig. 2. The light intensity was $\approx 3 \times 10^6$ erg/cm^2·sec^{-1}.

nmol·mg^{-1}·min^{-1} in these experiments. If bR is included in the vesicles with DSA, the exchange activity decreases, although it is somewhat stimulated in the presence of light (Table 1, rows 7 and 8). As expected, synthetic and exchange activity are not observed in the presence of FCCP (Table 1, rows 5 and 9), whereas hydrolytic activity is enhanced by both FCCP and dithiothreitol. If 100 μM dicyclohexylcarbodiimide is included in the ATPase assay, hydrolysis is inhibited 50–75%; this suggests that 25–50% of the hydrolysis activity is not coupled to the proton gradient. (The inhibition of hydrolysis by dicyclohexylcarbodiimide is not a quantitative measure of the extent of coupling.)

DISCUSSION

The Sephadex centrifuge column reconstitution procedure used in this study represents a simple means of reconstituting proteins into phospholipid vesicles. This method was first applied to the mitochondrial ATP synthase (13), and we have extended it to bR and DSA from spinach chloroplasts. The method is rapid and yields vesicles capable of a higher rate of light-driven ATP synthesis than any preparation described in the literature. The vesicles formed are heterogeneous (Fig. 1), probably because conditions conducive to the simultaneous incorporation of both DSA and bR at optimal levels could not be achieved. The difficulty is that optimal reconstitution of bR proton pumping activity is achieved in the presence of EDTA (2), whereas DSA requires the presence of Mg^{2+}. The reason for the divalent ion requirement is unknown, but Mg^{2+} may stabilize the DSA during the reconstitution process. This is consistent with the fact that Mg^{2+} is required along with CF_1 to regenerate photophosphorylation in stripped thylakoids (20). The data in Table 1 indicate that the final level of ATP synthesis is sensitive to the method of incorporating bR in the first reconstitution step. Previously we have observed that the percentage fluorescence quenching of the entrapped pH indicator, pyranine, is increased by more than an order of magnitude if bR is reconstituted with octylglucoside instead of using a sonication procedure (15).

Table 1. Activities of reconstituted preparations

Activity assayed	Conditions	Activity, % control
Synthesis	bR by sonication	14
	bR with $MgCl_2$	40*
	DSA with octyl glucoside	6*
	SO_4^{2-} instead of Cl^-	67
	FCCP (20 μM)	0
Exchange	SO_4^{2-} instead of Cl^-	150
	bR vesicles	52
	bR vesicles plus light	115
	FCCP (2 μM)	0
Hydrolysis	FCCP (10 μM)	133
	Dithiothreitol (50 mM)	133
	FCCP and dithiothreitol	178

The reconstitutions and activity assays were carried out as described in *Materials and Methods*. Changes relative to the control are given in the second column. Gradient-purified DSA was used except for those entries with an asterisk. The vesicles used contained bR except for rows 6 and 9.

The electron micrograph (Fig. 1 *Inset*) indicates that the DSA distribution in the vesicles is irregular. The reason DSA reconstitutes in groups is unknown, but a similar phenomenon has been noted in the reconstitution of the ATP synthase from *Wolinella succinogenes* (12). Even within the subpopulation of vesicles that contains DSA, variations in the degree of coupling between the proton gradient and enzyme activity occur. This is evident from the shift in the peak of hydrolytic activity observed when the sucrose gradient fractions are assayed in the presence of FCCP. Attempts to obtain a more uniform distribution of vesicles by increasing the detergent concentration resulted in overall decreases in the synthetic activity (data not shown). In recent experiments, we have noted some improvement by running small-scale sucrose gradients with the same amount of sample as was applied to the gradients in Fig. 1. By substitution of an SW-60 rotor for the SW-27.1 rotor, the purified vesicles can be isolated in 3 hr.

Another important problem in trying to maximize the synthetic activity of the co-reconstituted preparation is the leakiness of the membrane to protons. The overall FCCP stimulation of the ATPase activity is 1.3-fold. The relative stimulation of hydrolysis by protonophore may be lipid dependent: only 1.2-fold stimulation was obtained when the mitochondrial ATP synthase was reconstituted in asolectin

vesicles, whereas a 4.2-fold stimulation was observed in phosphatidylcholine vesicles (13). Despite the apparent increase in the leakiness of the asolectin vesicles, the observed synthetic activity was still superior to that found in the phosphatidyl choline vesicles (13). This may reflect the increased proton-pumping capacity of bR reconstituted in asolectin (21). The addition of bR to the vesicles results in a decrease in exchange activity (Table 1, row 7). The additional protein may increase the leakiness of the vesicles, either through the presence of residual detergent or by interfering with the proper reconstitution of DSA. Illumination of the bR–DSA vesicles permits the induced leakiness to be overcome, but very little additional stimulation of exchange activity by light occurs. In fact, the additional exchange in the light could also represent net ATP synthesis. The combined results of Figs. 2 and 3 indicate that the ability of bR to form a large enough pH gradient appears to be the limiting factor in the maximum attainable level of synthetic activity. No attempt has been made to quantify the magnitude of the pH gradient in the bR–DSA vesicles because heterogeneity within the population will necessarily lead to the observation of lower bounds rather than exact values (6). Estimates for the electrochemical proton gradient for bR vesicles have been ≈150 mV, while artificially imposed electrochemical proton gradients of ≈280 mV were required to obtain ATP synthesis at a similar rate to that observed in oxidative phosphorylation (12).

In summary, the co-reconstitution of bR and DSA by the two-step centrifuge column technique results in a vesicle population that is capable of light-driven ATP synthesis at a rate that is improved by more than an order of magnitude relative to the best preparation that was available previously (7). The rate is a factor of 2 larger than the best co-reconstituted preparation of bR and the mitochondrial ATP synthase (13). However, the estimated rate of ATP synthesis in oxidative phosphorylation (13) is a factor of 5 larger, and the maximum levels of photophosphorylation estimated in chloroplasts are 50 times larger (22). The results presented suggest that the rate in synthetic vesicles is limited by the presence of heterogeneity within the vesicle population and the ability of bR to form a sufficiently large proton gradient.

We are grateful to Ms. Lisa Bosch for her assistance in the preparation of DSA. This work was supported by a grant from the National Institutes of Health (GM 13292).

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