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Isolation of the proton-translocating F_0F_1-ATPase from *Rhodospirillum rubrum* chromatophores, and its functional reconstitution into proteoliposomes

Luit Slooten and Saskia Vandenbranden

Vrije Universiteit Brussel, Faculty of Sciences, Laboratory of Biophysics, Brussels (Belgium)

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The proton-translocating F_0F_1-ATPase was isolated from *Rhodospirillum rubrum* chromatophores by extraction with octylglucoside and deoxycholate, and further purified by sucrose gradient centrifugation. The enzyme was reconstituted into sonicated phospholipid vesicles by incubation with cholate, followed by centrifugation through a Sephadex column. ATP-hydrolysis catalyzed by F_0F_1-proteoliposomes is accelerated approx. 5–10 fold and approx. 15–20 fold by protonophorous uncouplers during assays with or without sulfite, respectively. Maximal turnover numbers are approx. 320 and 60 mol/s during hydrolysis with or without sulfite. The reconstituted enzyme is in, or close to, the native state with respect to the kinetics and regulation of ATP-hydrolysis. It generates a proton gradient (ΔpH) during ATPase activity, and the proteoliposomes are capable of ΔpH-driven ATP-synthesis. Gradient centrifugation of the reconstituted vesicles results in separation of lipid without F_0F_1 from proteoliposomes. In the purified proteoliposomes the minimum phospholipid/protein weight ratio is around 3. The distribution profiles of ATPase activity and ΔpH-driven ATP synthesis after gradient centrifugation do not completely overlap. It is inferred that the size of the proteoliposomes decreases slightly with decreasing lipid/protein weight ratios. ATPase-induced 9-aminoacridine fluorescence changes, indicative of the generation of ΔpH, were negative or positive, depending on the absence or presence of fluorescence quenchers in the external solution. The fluorescence changes became more positive when the probe concentration was lowered. The reasons for this are discussed.

Introduction

Energy-transducing membranes in bacteria, mitochondria and chloroplasts possess an enzyme referred to as F_0F_1, which couples proton-translocation to ATP-synthesis or -hydrolysis. F_1 is a water-soluble component consisting of five different subunits referred to as α–ε in decreasing order of magnitude, with a stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$ [1–3]. The primary structure of especially the β-subunit is highly conserved [4], and it is generally believed that this subunit carries the catalytic sites of the enzyme [5–7]. The membrane sector, F_0, functions in proton translocation through the membrane. It contains three different subunits in bacteria [8], probably three or four in chloroplasts [2,3,9], and at least nine in mitochondria [3].

Following the work of Kagawa and Racker [10], a large number of studies have appeared in which F_0F_1 from bacteria [11–18] (including purple bacteria [14–17] and cyanobacteria [18,19]), mitochondria [20–22] or chloroplasts [23–26] was isolated and reconstituted into phospholipid vesicles, often together with other enzymes or enzyme complexes working as proton pumps (see also Refs. 27 and 28 for reviews). These simplified systems are attractive because they allow us to study the mechanism and energetics of ATP-synthesis under well-defined conditions. However, with some exceptions [13,22,26] quantitative studies were hampered by the fact that the turnover rates of F_0F_1 in the reconstituted systems were rather low. This may have been due to a defective reconstitution of F_0F_1 into proteoliposomes [27], as was also the case during reconstitution of F_0F_1 in planar membranes [29]. However, it has also been

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonylfluoride; Mops, 4-morpholinepropanesulfonic acid.

Correspondence: L. Slooten, Vrije Universiteit Brussel, Faculteit der Wetenschappen, Laboratorium voor Biofysica, Pleinlaan 2, 1050 Brussel, Belgium.

suggested that an effective energy transduction between F_0F_1 and other proton pumps may require other, as yet unknown, proteins [28].

Recently, however, it was shown that F_0F_1 from chloroplasts could be reconstituted in such a way that high rates of ATP-synthesis driven by an artificially applied transmembrane electrochemical proton gradient ($\Delta\tilde{\mu}_{H^+}$) are obtained [26]. Thus in principle it is possible to obtain well-coupled proteoliposomes in which a $\Delta\tilde{\mu}_{H^+}$ is kinetically competent to drive ATP-synthesis.

As part of a long-term project we describe here a rapid procedure for isolation of F_0F_1 from chromatophores (pigmented, inside-out vesicles) from the purple bacterium, *Rhodospirillum rubrum,* and its reconstitution into proteoliposomes. *R. rubrum* was chosen because the membrane vesicles isolated from this bacterium have (in comparison with other photosynthetic organisms) a high content of F_0F_1-ATPase, due to the low content of antenna pigments. Our first aim was to develop a procedure in which all added F_0F_1 would be incorporated in such a manner that the system exhibits high turnover rates per enzyme molecule and a good coupling of catalytic activity to protontranslocation. The present results indicate that we have succeeded in both respects. In addition, the reconstituted enzyme appears to be in, or very close to, the native state with regard to the kinetics and regulation of ATPase activity. Finally, some results are presented on the generation of a proton gradient (ΔpH) during ATP-hydrolysis, and on the measurement of that gradient by 9-aminoacridine. Some of these results have been published in a preliminary form [30]. Further results on $\Delta\tilde{\mu}_{H^+}$-driven ATP-synthesis will be published elsewhere [31].

Materials and Methods

Materials. Soybean asolectin (type IV-S), octylglucoside and Triton X-100 were from Sigma. Sodium deoxycholate (Fluka) and sodium cholate (Serva) were used without further purification. ADP (mono-potassium salt), luciferin and firefly luciferase were from Boehringer. Luciferase was dissolved at 1 mg/ml in 0.5 M Hepes/NaOH to pH 7.5, and stored in 0.1-ml aliquots at $-20\,^\circ$C.

Preparation of F_0F_1. R. rubrum was grown, and chromatophores were prepared as described [32], except that sucrose was omitted from the storage medium and the chromatophores were stored on ice. Chromatophores (1 mM bacteriochlorophyll) were incubated for 15 min at $32\,^\circ$C with 15 mM octylglucoside, 2.4 mM deoxycholate and 10 μM PMSF in 43 mM KCl, 175 mM NaCl, 4 mM Hepes (pH 7.9). The suspension was then cooled and all subsequent steps took place at 0–4 °C. The suspension was centrifuged for 1 h at $100\,000 \times g$ and was allowed to decelerate without braking. 3.5-ml portions of the supernatant were immediately layered on top of 35 ml of a linear, stepwise sucrose gradient (0.16–0.52 M, with steps of 0.06 M) in 10 mM Hepes/NaOH to pH 7.9, and centrifuged for 17 h at $60\,000 \times g$ in a swing-out rotor. 1-ml fractions were collected and assayed for ATPase activity. The peak fractions were pooled and concentrated by adding dry Sephadex G-25 (0.25 g/ml) and centrifuging the slurry through a nylon mesh. This step was repeated once or twice. The concentrate (0.3–0.5 mg/ml protein) was stored at $0\,^\circ$C and could be used for approx. 4 weeks. During this storage period the activity declined approx. 30%.

Preparation of liposomes and proteoliposomes. Soybean asolectin was partially purified [10] and stored dry at $-20\,^\circ$C. The dry lipids were hydrated by vortexing in an appropriate buffer solution, usually at 20–30 mg lipid/ml. 2–3 ml of the suspension was sonicated to clarity (20–30 min) with a probe (MSE, 125 W) operated at 3 μm amplitude (1/8 of full output). The mixture was cooled in a water bath at room temperature during sonication.

Proteoliposomes were prepared by mixing 0.16 ml F_0F_1 with 0.245 ml liposomes, 51 μl compensating buffer and 44 μl of 10% (w/v) cholate in distilled water (the compensating buffer was used to set the final buffer and salt concentrations at the desired values). After 30 min incubation at $0\,^\circ$C the suspension was centrifuged through 5-ml columns containing Sephadex G-50 [33] equilibrated with a suitable buffer solution.

Biochemical assays. Unless indicated otherwise, ATP-hydrolysis was measured at $32\,^\circ$C in 2 ml of a medium containing 35 mM K_2SO_3, 133 mM sucrose, 3.2 mM $MgCl_2$, 0.2 mM EDTA, 10 mM Hepes/NaOH to pH 7.9. The reaction was started by addition of 3 mM ATP and was stopped 2 min later by addition of the colorimetric phosphate reagent [34]. It should be noted that in the course of this work we found that chloride has a slight uncoupling effect. From then on (as indicated in the legends) chloride was replaced by sulfate.

All other experiments were carried out at room temperature. 9-Aminoacridine fluorescence was excited with light of 406 nm and was measured at wavelengths around 450 nm (selected with interference filters). ATP-synthesis was measured in a medium containing 25 mM K_2SO_4, 3.2 mM $MgSO_4$, 0.2 mM EDTA, 10 mM sodium phosphate and 10 mM glycylglycin. 2.2 ml of this medium was supplemented with 0.1 mM ADP, 2 μg per ml luciferase, 50 μM luciferin and 0.1 μM valinomycin. The reaction was started by addition of proteoliposomes (suspended in a similar medium) which had been preincubated with 20 mM succinic acid at pH 5.0. The pH of the reaction medium, after this addition, was 8.0. The ensuing luminescence increase, reflecting ATP-synthesis, was recorded. The experiment was terminated by addition of 0.5 nmol ATP, which served

as an internal calibration. As will be published elsewhere, control experiments indicated that ΔpH-driven ATP-synthesis was completely dependent on added ADP and on added phosphate. Furthermore, the reaction was completely inhibited by addition of uncoupler, and was largely inhibited by omission of valinomycin.

Analytical methods. SDS-polyacrylamide gel electrophoresis was carried out in a tube gel apparatus according to Weber et al. [35], with 11% gels. The samples were pretreated for 3 min at 100 °C with 1% (w/v) SDS and 0.1% (v/v) 2-mercaptoethanol. The relative molecular weights of the polypeptides were obtained by comparison with the following standard proteins with their molecular weights in brackets: bovine serum albumin (66000), egg albumin (45000), pepsin (34700), trypsinogen (24000), β-lactoglobulin (18400) and egg white lysozyme (14300). The gels were stained with Coomassie Brilliant Blue 250 R for 20 h.

Protein was determined according to Hess [36], after precipitation according to Peterson [37]. Bacteriochlorophyll was determined using an in vivo extinction coefficient of 140 $mM^{-1} \cdot cm^{-1}$ at 880 nm [38]. For the determination of phospholipids the samples (10–200 μl) were taken to dryness in tubes of 1.4 × 13 cm. 0.22 ml of 96% sulfuric acid was added and the tubes, topped with marbles, were positioned at a depth of 3 cm in an oil bath thermostatted between 165 and 170 °C. After 45 min of charring, 0.22 ml 70% perchloric acid was added and heating was continued overnight. Inorganic phosphorus in these samples was determined according to Bartlett [39], except that we used double volumes of the reagents.

Electron microscopy. Electron micrographs were prepared on the Siemens 102 and Siemens Elmiskop I electron microscopes of the Pasteur Institute Brabant.

Results

Purification of F_0F_1

In order to be able to assess the yield of ATPase activity after the successive purification steps, we measured this activity in the presence of 35 mM sulfite (see Materials and Methods), and (in the case of chromatophores) with 50 μM of the protonophore, CCCP. This allowed us to measure ATPase activity under completely uncoupled conditions, without interference from feedback mechanisms which, in the absence of sulfite, would inactivate the enzyme (cf. Refs. 34 and 40–46).

Detergent extraction of the chromatophores, followed by centrifugation, led to a 4–6-fold purification of F_0F_1-ATPase (Table I, lines 1 and 2). The extract was virtually free from bacteriochlorophyll (less than 2 nmol per mg protein), but was reddish due to the presence of *c*-type cytochromes (the cytochrome bc_1-complex was not extracted). After gradient centrifugation of the extract, 97% of the ATPase activity was recovered in the centrifuge tube, largely in the bottom half. This peak in ATPase activity coincided with a minor protein band, well separated from a major protein band near the top of the tube. The latter band contained the *c*-type cytochromes. After this centrifugation, the small amount of bacteriochlorophyll which was present in the detergent extract, was sedimented.

TABLE I

Purification of R. rubrum F_0F_1

n.d.; not determined.

Fraction	ATPase (units) (1 unit = 1 μmol/min)	Protein (mg)	Specific activity (units per mg protein)	Yield (%)
Chromatophores	894 [a]	312	2.87	100
Detergent extract	483	35.6	13.6	55
Sucrose gradient pool	322	n.d.	n.d.	36
Concentrate	262	4.96	55.8 [b]	29.3

[a] In the presence of 50 μM CCCP.
[b] 89.5% inhibited by 6 μg per ml oligomycin.

The peak fractions of ATPase activity were pooled, concentrated and stored on ice. This activity was, in different preparations, 87–92% inhibited by 6 μg per ml of oligomycin (cf. Table I). A densitometer tracing, obtained after SDS-gel electrophoresis of the concentrate, is shown in Fig. 1. Greek and Roman letters indicate the subunits from F_1 and F_0, respectively, with which the relevant polypeptides are thought to correspond. As indicated by the brackets in Fig. 1, the assignment of bands δ and ε, and of bands a–c is only tentative, and based on a comparison with data obtained with the purified F_1 from *R. rubrum* [47], and with F_0F_1 from the thermophilic bacterium PS3 [48]. A similar assignment was suggested in earlier work on *R. rubrum* F_0F_1 [49]. However, in *Escherichia coli* the δ-subunit of F_1 is larger than the *b*-subunit of F_0 [1].

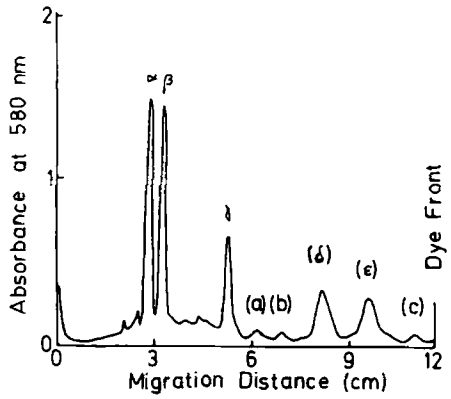

Fig. 1. Densitometer tracing obtained after SDS-gel electrophoresis of *R. rubrum* F_0F_1.

TABLE II

Apparent molecular weights of the polypeptides obtained after SDS-gel electrophoresis of R. rubrum F_0F_1

Brackets indicate polypeptides of which the assignment is tentative (see text).

Band	M_r
α	57000
β	52000
γ	34000
(a)	28500
(b)	25000
(δ)	19000
(ε)	14500
(c)	11000

The apparent molecular weights of the polypeptides are shown in Table II.

Optimization of proteoliposome formation and recovery of ATPase

Proteoliposomes were formed as outlined in Materials and Methods. In order to assess the degree of coupling of the proteoliposomes, we measured the ATPase activity in a medium containing sulfite and determined the coupling ratio, defined as the ratio of ATPase activities measured with and without a saturating concentration of uncoupler (50 μM CCCP [30]). This ratio should be as high as possible.

With respect to salts, the best results were obtained with 70–100 mM of monovalent salts (usually KCl). With regard to divalent cations, the best results were obtained with 10 mM Mg^{2+} during cholate incubation and 3 mM Mg^{2+} during column centrifugation (data not shown).

Fig. 2 shows experiments in which cholate incubation was carried out with one fixed concentration of protein and two fixed concentrations of cholate, whereas the lipid concentration was varied as indicated in the lower scale at the top of the figure. At 3.8 mg per ml cholate (squares), the best reconstitution was obtained with a lipid/protein weight ratio of 47.7 (see upper scale at the top of the figure). At higher lipid concentrations the coupling ratio decreased due to a progressive increase in the rate of hydrolysis measured without uncoupler. A somewhat better functional reconstitution was obtained with 8.8 mg/ml cholate (circles), at lipid/protein weight ratios of 50 and above. The scales at the bottom of the figure give the values of R_e (the effective molar ratio of cholate and lipid in the bilayers, whether micellar or vesicular) during cholate incubation. The values of R_e were calculated according to Almog et al. [50]. We found that an effective reconstitution of F_0F_1 into proteoliposomes requires an R_e-value of at least 0.37 during cholate incubation (Fig. 2). This means [50] that the lipid should occur largely or wholly in the form of micelles at this stage. When the lipid concentration is increased at a low cholate concentration, the proportion of micellar lipid decreases and the reconstitution fails (Fig. 2, squares).

In order to determine the recovery of ATPase activity after cholate incubation and column centrifugation, we compared the activity of an aliquot of F_0F_1 with that of an 'equivalent' amount of proteoliposomes (that is, equivalent if the protein recovery were 100%). The assay was carried out in the presence of 50 μM CCCP in order to fully uncouple the proteoliposomes, and with or without 0.2% Triton X-100. In order to facilitate comparison of the activities observed in the presence of Triton, 'empty' liposomes were added during assay of F_0F_1, in the same lipid/protein weight ratio as used during cholate incubation (the lipids did not affect the activity of F_0F_1 in the absence of Triton – not shown). The results were as follows. In the absence of Triton, 5.8 μg F_0F_1 yielded an activity of 0.196 units; an 'equivalent' amount of proteoliposomes yielded an activity of 0.190 units (1 unit = 1 μmol/min). In the presence of Triton these numbers were 0.176 and 0.173 units, respectively. Thus the recovery of ATPase activity after cholate incubation and column centrifugation was 97% and 98.3% in the absence and presence of Triton, respectively. Since Triton X-100 solubilizes the proteoliposomes, this means that no more than 1.3% of F_0F_1 was incorporated into the proteoliposomes with the F_1-moiety shielded from the external medium. Lipid recovery was less complete: 81–84% of the phospholipid applied to the centrifugation column was recovered from it (data not shown).

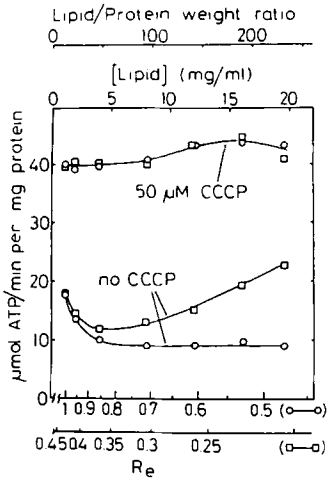

Fig. 2. Effect of cholate and lipid on the functional reconstitution of ATPase activity in F_0F_1-proteoliposomes. Cholate-incubation was carried out with 3.8 (□———□) or 8.8 (○———○) mg/ml of cholate, 84.5 μg/ml F_0F_1 and lipid as indicated in the lower scale on top of the Figure, in a medium containing 133 mM sucrose, 10 mM Hepes, 10.2 mM $MgCl_2$, 0.2 mM EDTA, 70 mM KCl, and NaOH to pH 7.9. The suspension was centrifuged through a column equilibrated with the same buffer, except that $MgCl_2$ was present at 3.2 mM.

Structural and functional properties of F_0F_1-proteoliposomes

The above results indicate that when ATPase is assayed in the presence of 50 μM CCCP but otherwise as described in Materials and Methods, the activity of isolated F_0F_1 is almost equal to the activity of F_0F_1 incorporated in proteoliposomes. In addition the activity of F_0F_1 is not affected by uncouplers. Hence the following relationships are easily established: We define x as the actually measured coupling ratio; c as the coupling ratio which would be observed with fully reconstituted F_0F_1; and p represents the proportion of F_0F_1-molecules which has actually been reconstituted into proteoliposomes. Then

$$p = \frac{1 - x^{-1}}{1 - c^{-1}}$$

and hence $p > 1 - x^{-1}$. So the actually measured coupling ratio yields a minimum estimate for the proportion of F_0F_1-molecules which has been incorporated into proteoliposomes.

The proteoliposomes used for the experiment shown in Fig. 3 exhibited a coupling ratio of 9.67, indicating that over 90% of F_0F_1 had been reconstituted. These proteoliposomes were subjected to density gradient centrifugation, and the distribution of phospholipid (Fig. 3A, triangles), ATPase activity (circles) and ATP-synthesis capacity (crosses) was determined. After centrifugation, an upper band consisting primarily of liposomes without F_0F_1 had been separated from a lower band consisting of proteoliposomes. In assays of ATPase activity the coupling ratio was approximately constant in all fractions (open and solid circles show the ATPase activity with and without 50 μM CCCP). This confirms, as expected, that no separation between reconstituted and non-reconstituted F_0F_1 had taken place. In order to assess the ATP-synthesis capacities (crosses) we determined the amount of ATP synthesized in response to a ΔpH of three units (acid inside). From the data shown in Fig. 3A we determined the phospholipid/protein ratio (Fig. 3B, circles) and the ATP-yield per mg protein (triangles), on the assumptions that (a) the recovery of protein was 100% (in agreement with the recovery of ATPase activity, see legend to Fig. 3A), and (b) the protein concentration in the fractions was proportional to the ATPase activity in the presence of 50 μM CCCP. This is justified as explained at the beginning of this section. The lowest phospholipid/protein ratios were around 4 μmol/mg protein, corresponding with a weight ratio of around 3.3 : 1. The average ATP-yield was about 25 nmol/mg protein, corresponding with about 13 turnovers per mol F_0F_1. This yield exhibited a gradual, 9-fold decrease with increasing depth in the centrifuge tube (aside from the top fraction which, by this criterion, may have contained some non-reconstituted F_0F_1). The ATP-yield in ΔpH-driven ATP-synthesis is dependent on the amount of buffer (i.e. succinic acid at pH 5) within the vesicle, and hence on the internal volume. So the data of Fig. 3B suggest that the average internal volume of the proteoliposomes decreased about 9-fold, and the average internal diameter decreased about 2.2-fold, with increasing depth in the centrifuge tube.

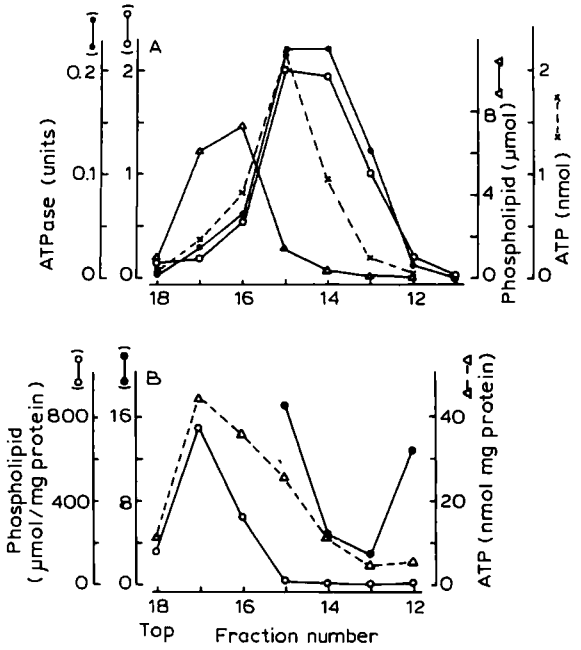

Fig. 3. (A) Recovery of phospholipid (△———△), ATPase activity without uncoupler (●———●) or with 50 μM CCCP (○———○), and ATP-synthesis capacity (×------×) of proteoliposomes after gradient centrifugation. Numbers along the ordinates refer to amounts recovered in the whole fraction. The fraction volume was 2.14 ml. Overall recovery was 89% for phospholipid, 94% for ATPase with CCCP, and 101% for ATPase without CCCP. Proteoliposomes: cholate incubation was carried out in a medium containing 133 mM sucrose, 25 mM K_2SO_4, 10.2 mM $MgSO_4$, 0.2 mM EDTA, 10 mM glycylglycin and NaOH to pH 7.9. The suspension was centrifuged through a column equilibrated with the same buffer, but without sucrose and with 3.2 mM $MgSO_4$ (medium K). Gradient centrifugation: 1.5 ml of column eluate, containing 255 μg F_0F_1 was layered on top of 37 ml of a stepwise sucrose gradient in medium K (7–31%, with steps of 4% w/v). The tubes were centrifuged for 16 h at 70000 × *g*. Assays: ATPase was assayed as in Materials and Methods, except that $MgCl_2$ was replaced by $MgSO_4$. For assay of ATP-synthesis, 0.2 ml of each fraction was mixed with 50 μl of 100 mM succinic acid in medium K; the pH after mixing was 5. See Materials and Methods for further details. (B) Phospholipid/protein ratio (○, ●) and ATP yield per mg protein (△------△) of the fractions shown in (A). See text for details.

Fig. 4B shows an electron micrograph of the proteoliposomes in a fraction with a low ATP-yield per mg protein (fraction 14). The vesicles were fairly uniform in size, with external diameters of 20–30 nm; sometimes they were aggregated. By contrast, Fig. 4A shows that the unfractionated proteoliposomes varied widely in size, ranging from 20 to 120 nm. The large liposomes were found in the top fractions after centrifugation (not

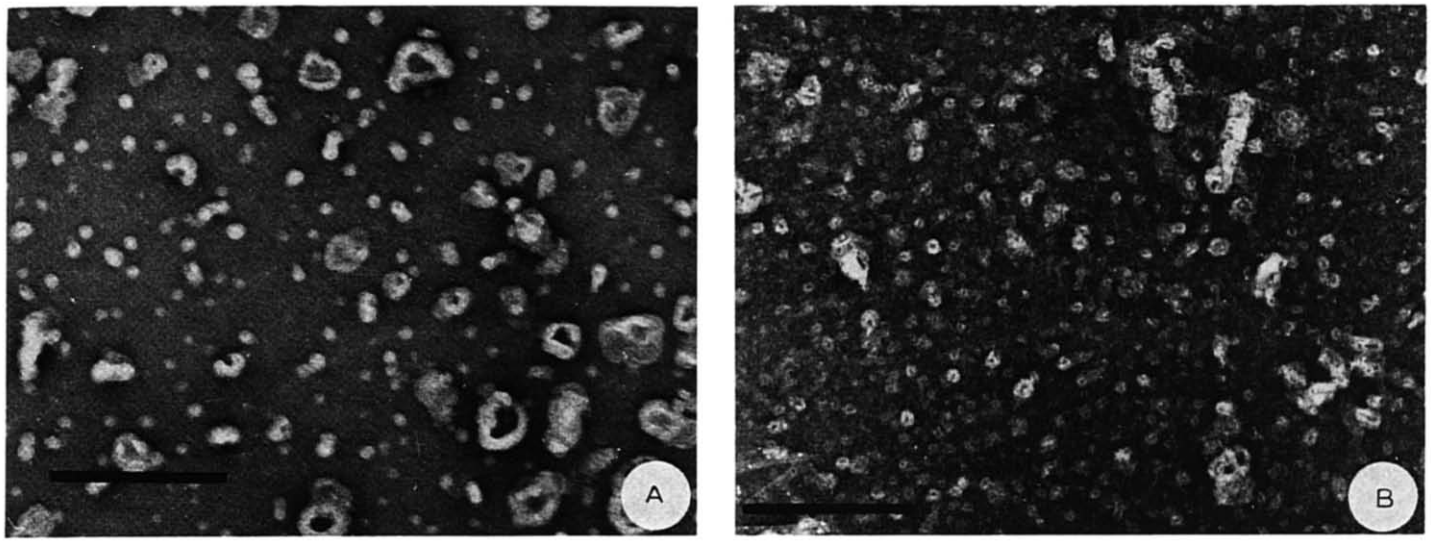

Fig. 4. Parts of original electron micrographs showing proteoliposomes before gradient centrifugation (A), and in fraction 14 after gradient centrifugation (B). The specimens were stained negative with 1% uranylacetate. The electron optical magnification was 25000×. Bars indicate 300 nm.

shown). All other experiments reported below were done with unfractionated proteoliposomes.

When ATP-hydrolysis was measured in the presence of 3 mM ATP but without sulfite, the activity without uncoupler was routinely 15–20 times lower than the activity measured with optimal concentrations of uncoupler [30]. This ratio is even higher than in chromatophores, where it is about 4–7 [34,40,41]. This indicates an excellent coupling between ATPase activity and proton translocation in our proteoliposomes.

TABLE III

Activation of ATPase in proteoliposomes by an artificially applied $\Delta\tilde{\mu}_{H^+}$

Numbers indicate the mean and range of duplicate experiments. Cholate incubation was carried out with 147 μg per ml F_0F_1, 9.8 mg per ml lipid and 8.8 mg per ml cholate in a medium containing 133 mM sucrose, 10 mM Hepes, 10.2 mM $MgSO_4$, 0.2 mM EDTA, 25 mM Na_2SO_4 and NaOH to pH 7.9. The suspension was centrifuged through a column equilibrated with the same medium, but without sucrose and with 3.2 mM $MgSO_4$ (Medium A). Expt. 1: 88 μl proteoliposomes were mixed with 22 μl of 100 mM succinic acid in medium A. The final pH, after mixing, was 5.0. After 3 min, 100 μl of this mixture was added, at $t = 0$, to 1.9 ml of assay medium; this was as medium A, except that it contained K_2SO_4 instead of Na_2SO_4. The final pH, after mixing, was 8.0. Valinomycin (0.1 μM) was added at $t = 5$ s; CCCP (3 μM) was added at $t = 10$ s and ATP (0.3 mM) at $t = 20$ s. The reaction time was 1 min. Expt. 2: As expt. 1 except that succinic acid was added to the assay medium before, instead of together with proteoliposomes. Expt. 3: As expt. 2, except that CCCP was added before, instead of after proteoliposomes.

Expt. no.	Activation	ATPase (μmol per min per mg protein)
1	$\Delta pH + \Delta\psi$	1.01 ± 0.10
2	$\Delta\psi$	0.80 ± 0.12
3	none	0.30 ± 0.12

We have shown earlier that in chromatophores the membrane-bound ATPase is in a low-activity state before ATP-addition, and has to be activated by application of a transmembrane $\Delta\tilde{\mu}_{H^+}$, positive inside. The resulting high-activity state of the enzyme persists for some time after the dissipation of the $\Delta\tilde{\mu}_{H^+}$ by ionophores, even in the absence of ATP [34]. The experiments shown in Table III were done in order to determine whether a similar situation exists in proteoliposomes.

For these experiments the proteoliposomes were prepared in the absence of K^+, and they were then injected into a reaction mixture containing K^+. A K^+-diffusion potential ($\Delta\psi$) of 70–80 mV, positive inside *, was imposed by addition of valinomycin (expts. 1 and 2); in expt. 1 this diffusion potential was combined with a pH-gradient, acid inside, of three units. Then CCCP was added, allowing a rapid collapse of the imposed $\Delta\tilde{\mu}_{H^+}$. Thereafter ATP was added and the rate of hydrolysis was determined. In the control (expt. 3) no ΔpH was applied, and CCCP was added before the proteoliposomes. This should ensure that upon addition of proteoliposomes and valinomycin, K^+ and H^+ equilibrated too rapidly across the membrane to allow the build-up of a significant $\Delta\tilde{\mu}_{H^+}$. Table V shows that after activation by ΔpH and $\Delta\psi$ (expt. 1), and even after activation by $\Delta\psi$ alone (expt. 2), the ATPase activity was considerably higher than in the control. This indicates that F_0F_1-proteoliposomes resemble chromatophores, in that the ATPase is in a low-activity state

* Calculated according to Ref. 51, assuming a membrane thickness of 5 nm, a membrane capacitance of 1 μF/cm² and a proteoliposome internal diameter of 20–30 nm.

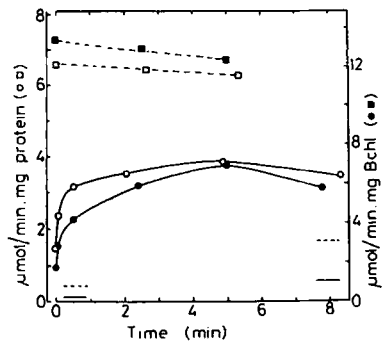

Fig. 5. Self-activation of F_0F_1-ATPase in proteoliposomes (open symbols) and chromatophores (solid symbols). Proteoliposomes were prepared as described in the legend to Fig. 3. ATP-hydrolysis was measured with a pH-electrode in a medium containing, in a final volume of 2.8 ml, 25 mM K_2SO_4, 3.2 mM $MgSO_4$, 0.2 mM EDTA, 1 mM glycylglycin, NaOH to pH 8.0, 0.3 mM ATP, 3 μM CCCP, 0.11 μM nigericin and either proteoliposomes corresponding with 20.7 μg F_0F_1 (open symbols, left-hand scale), or chromatophores corresponding with 11.2 μg bacteriochlorophyll (solid symbols, right-hand scale). Circles: No further additions. Squares: plus 3 mM K_2SO_3. Points at $t = 0$ indicate experiments in which CCCP was added before ATP; rates were measured 9 s after ATP-addition. Other points indicate experiments in which CCCP was added at the indicated time after ATP; rates were measured 9 s after CCCP-addition. Horizontal bars indicate rates of hydrolysis without CCCP, in proteoliposomes (left) and chromatophores (right), in the presence (– – –) or absence of sulfite (———). Bchl, bacteriochlorophyll.

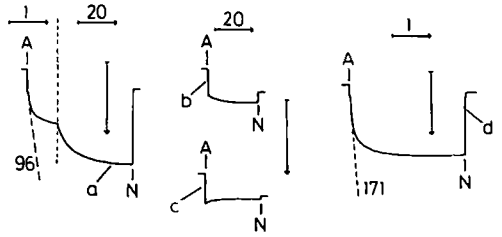

Fig. 6. Time-courses of ATPase-induced changes in 9-aminoacridine fluorescence. Conditions were as in Fig. 5, except that the assay medium contained 10 mM glycylglycin, and the reaction mixture contained 5 μM 9-aminoacridine and proteoliposomes corresponding with 30.7 μg F_0F_1 in a final volume of 2.5 ml. 0.3 mM ATP (A) and 0.1 μM nigericin (N) were added at the indicated time. The vertical dashed line in trace a indicates a change in the speed of the recording paper. The numbers along the horzontal arrows indicate the time scale, in min. Downward pointing arrows indicate a fluorescence change of 25% calculated relative to the fluorescence level observed after addition of nigericin. The numbers along the tangents indicate the time rate of change of the fluorescence, in % per min. Trace a: plus 0.1 μM valinomycin. Trace b: without valinomycin. Trace c: as trace a, but plus 6 μg/ml oligomycin. Trace d: as trace a, but plus 3 mM K_2SO_3.

before ATP-addition, but can be activated in the absence of ATP by a transient $\Delta\tilde{\mu}_{H^+}$, positive inside, of sufficient magnitude.

Another way to activate the ATPase is by 'self-activation'. By this we mean the conversion of the enzyme from the low-activity state into the high-activity state by the $\Delta\tilde{\mu}_{H^+}$ generated during hydrolysis carried out (initially) in the low-activity state. Fig. 5 shows experiments on self-activation of F_0F_1-ATPase in proteoliposomes (open symbols) and chromatophores (solid symbols). (These experiments were done with the pH-electrode technique [34], and nigericin was added in order to prevent proton uptake into the vesicles during hydrolysis). In these experiments, uncoupler (CCCP) was added either before, or at several times after ATP; the circles and squares show the initial rates of hydrolysis in the presence of CCCP, as a function of the time of hydrolysis in the absence of CCCP. (The steady-state rates of hydrolysis in the absence of CCCP are represented by the horizontal bars). When CCCP was added before ATP, the rate of hydrolysis in the absence of sulfite was initially as shown (circles at $t = 0$), and then declined with time (not shown). This confirms that the ATPase-induced $\Delta\tilde{\mu}_{H^+}$ is required for self-activation. The circles show that, in the absence of sulfite, self-activation of ATPase (by a period of hydrolysis without uncoupler) exhibited biphasic kinetics, with a rapid phase ($t_{1/2} \approx 6$ s) and a slow phase ($t_{1/2} \approx 2$ min). The reason for this is probably that self-activation was retarded by product ADP. Rates of hydrolysis measured in the presence of CCCP and 3 mM sulfite (squares) were 1.7–1.9 times higher than the highest rates observed without sulfite; in addition, sulfite eliminated the need for self-activation by a period of hydrolysis in the absence of uncoupler. The main point that concerns us here, is that in all these respects the results obtained with proteoliposomes (open symbols) were similar to those obtained with chromatophores (solid symbols). This indicates that the kinetic and regulatory properties of F_0F_1-ATPase have been preserved during its isolation and reconstitution into proteoliposomes (see 'Discussion').

The generation of a proton gradient (ΔpH) across the proteoliposome membrane during ATP-hydrolysis was monitored qualitatively by changes in 9-aminoacridine fluorescence (Fig. 6). Addition of ATP to a reaction mixture containing proteoliposomes and 9-aminoacridine caused an initial, rapid fluorescence quenching due to a non-enzymic interaction of the probe with ATP. This was followed by a slower, ATPase-dependent fluorescence quenching which was completely abolished by nigericin. As expected, the ATPase-dependent fluorescence change was in the presence of valinomycin (trace a) much more extensive than without valinomycin (trace b), and it was strongly inhibited by oligomycin (trace c). Sulfite caused an approx. 1.8 fold increase in the initial rate of ATPase-induced 9-aminoacridine fluorescence quenching (Fig. 6, trace d), in agreement with the acceleration of ATPase by sulfite (Fig. 5).

In spite of the difficulties which beset the work with 9-aminoacridine (e.g., Ref. 53), this is one of the few probes which can be used as a sensitive probe for a wide range of ΔpH-values, from 0 to 4 units (to be published

TABLE IV

Dependence of the extent of ATPase-induced fluorescence changes on the composition of the medium and the concentration of 9-aminoacridine

The conditions were as described in the legend to Fig. 6, except that during the assay, K_2SO_4 was replaced by the indicated salts and 9-aminoacridine (9AA) was added as indicated. F_r is the fluorescence, in relative units, of 3 μM 9-aminoacridine in the absence of proteoliposomes. Columns 2–4 give the nigericin-sensitive fluorescence changes, in %, induced by addition of ATP. The changes were calculated relative to the fluorescence level observed after addition of nigericin.

Salt	F_r	Fluorescence change (%)		
		3 μM 9AA	1 μM 9AA	0.2 μM 9AA
25 mM K_2SO_4	100	−15.9	−10.0	−4.7
25 mM K_2SO_4 + 3 mM K_2SO_3	82	−14.0	−10.0	+3.3
35 mM K_3SO_3	28	+8.9	+24.5	+35.4
200 mM Mops + 50 mM KOH	17	+8.1	+17.3	+28.6

elsehwere). The experiments shown in Table IV pertain to the mechanism by which 9-aminoacridine fluorescence monitors a transmembrane ΔpH. This table shows the extent of the ATPase-induced fluorescence changes at three different concentrations of the probe (columns 2–4), and in different assay media. In a medium containing sulfate as the main anion, the fluorescence change became less negative when the 9-aminoacridine concentration was lowered from 3 to 0.2 μM (line 1). Addition of 3 mM sulfite did not change the results very much in this respect (line 2). When the sulfite concentration was raised to 35 mM, the fluorescence change was positive, and it became more positive (up to 35.4%) when the 9-aminoacridine concentration was lowered (line 3). This effect was not related to the activation of ATPase by sulfite: a similar effect was observed when sulfite was replaced by 200 mM Mops (line 4). This organic buffer is not an activator of ATPase (not shown). What Mops and sulfite have in common is that they are both strong quenchers of 9-aminoacridine fluorescence at high concentrations (column 1).

One reason for these results is that 9-aminoacridine moves into the vesicles, in response to an ATPase-induced ΔpH (acid inside) according to the mechanism proposed by Schuldiner et al. [52]. Quenching of the fluorescence of the accumulated probe molecules is thought to be dependent on the formation of dimers or multimers [53,54], possibly at the internal membrane surface. Consequently, the proportion of free, fluorescent monomers in the vesicle interior will increase when the total amount of accumulated 9-aminoacridine decreases. This will be the case if, other things being equal, the concentration of 9-aminoacridine added to the external medium is lowered (columns 2–4).

In addition we have shown [30] that liposomes contain a small number of high-affinity 9-aminoacridine binding sites; the fluorescence of 9-aminoacridine is enhanced when (as a result of application of a pH-gradient) the probe is bound to these sites. (This implies that the fluorescence yield of 9-aminoacridine in aqueous solutions is less than 100%). The contribution of this process to be observed, net fluorescence change increases when the lipid/probe ratio increases. At sufficiently high lipid/probe ratios the probe responds to application of a pH-gradient with a net fluorescence increase, even in media without added fluorescence quenchers [30]. Both the free, fluorescent monomers within the vesicle interior and the 9-aminoacridine molecules bound to the high-affinity sites appear to be shielded from the external medium. As a consequence their share in the total fluorescence becomes larger when the fluorescence in the external medium is lowered by the addition of fluorescence quenchers (Ref. 30 and data to be published elsewhere).

All this means that the ΔpH-dependent fluorescence change will become less negative, or more positive, when the concentration of 9-aminoacridine is lowered, or when fluorescence quenchers like Mops or sulfite are added to the external medium. The results presented here and in Ref. 30 indicate that the method used by Schuldiner et al. [52] for calculation of pH-gradients from 9-aminoacridine fluorescence changes, cannot be applied to proteoliposomes. Instead, a calibration curve will have to be measured for each particular application.

Discussion

The simple and rapid two-step procedure for the isolation of F_0F_1 from *R. rubrum* chromatophores results in an approx. 20-fold purification of the enzyme. The densitometer tracings after SDS-gel electrophoresis indicate that contaminants are present in only minor amounts. These tracings are similar to those published earlier [47,49], although all published densitometer tracings differ from one another in the region of the smaller polypeptides. The reason for this is not quite clear, although we found that long staining times are required in order to saturate the staining intensity of the smaller polypeptides. Assuming a subunit stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$ for the F_1 moiety [1–3], and using the assignments shown in Table I, we arrive at a molecular mass of 395 kDa for F_1. This agrees reasonably well with values of around 380 kDa calculated from gene sequences [4,55]. From results obtained recently with chloroplasts [56] and *E. coli* [57,58], a molecular mass of 150 kDa seems a reasonable estimate for F_0 from *R. rubrum*. This would bring the total molecular mass of F_0F_1 to approx. 545 kDa.

After removal of empty liposomes by gradient centrifugation, the proteoliposomes which were most

enriched in ATPase activity appeared to possess a phospholipid/protein weight ratios of around 3.3 : 1, with minimal values of around 2.6 : 1 (data from Fig. 3B). This is in sharp contrast with the required lipid/protein weight ratio during cholate incubation (at least 50 : 1, Fig. 2). We assume that this large excess of lipid is required in order to eliminate competing, 'non-productive' processes, such as formation of detergent-F_0F_1 complexes, or self-aggregation of F_0F_1 [59] during cholate incubation or -removal.

Although the unfractionated proteoliposomes vary widely in size (Fig. 4A), purified proteoliposomes are small and fairly uniform, as indicated by electron microscopy (Fig. 4B) and as suggested by the yield of ATP per mg protein (Fig. 3B). Large liposomes are known to arise out of smaller ones by fusion during removal of cholate [50]. In our experiments, cholate is removed within 2 min during column centrifugation. Hence our data suggest that fusion of small vesicles is retarded somewhat by the presence of F_0F_1 in them. It is probably relevant in this respect that embedding of chloroplast F_0F_1 into liposomes (made of chloroplast lipids) caused a marked increase in membrane viscosity [60].

The proteoliposomes exhibited high turnover rates. In the presence of optimal concentrations of uncoupler, typical turnover rates of the reconstituted enzyme are around 320 mol ATP per s during hydrolysis in the presence of 35 mM sulfite (Fig. 2) and 60 mol ATP per s in the absence of sulfite [30]. All the evidence indicates that the proteoliposomes are well-coupled. (1) They are capable of ATP-synthesis induced by a ΔpH of 3 units. The average ATP-yield of around 13 mol ATP/mol F_0F_1 is similar to values observed with proteoliposomes from chloroplast F_0F_1 and soya lecithin under similar conditions [26]. As will be shown elsewhere [31], much higher yields were obtained under more favorable conditions. (2) The proteoliposomes exhibit a very high (at least 15–20 fold) uncoupler-stimulation of ATP-hydrolysis, during assays without sulfite ([30] and Fig. 5). (3) The ATPase activity of the proteoliposomes generates a transmembrane ΔpH was evidenced by 9-aminoacridine fluorescence changes (Fig. 6). These fluorescence changes give also an indirect indication for the generation of a transmembrane potential ($\Delta\psi$) during hydrolysis: apparently this $\Delta\psi$ has to be collapsed (by valinomycin-mediated K^+-efflux) before a significant ΔpH can be built up.

There are several indications that the kinetic and regulatory properties of the reconstituted ATPase are virtually unmodified in comparison with the chromatophore-bound enzyme. (1) In either case the enzyme is in a low-activity state before ATP-addition, but can be activated by an artificially applied $\Delta\tilde{\mu}_{H^+}$ (positive inside) in the absence of ATP (Table III), or by self-activation in the presence of ATP (Fig. 5). The kinetics of self-activation are similar in chromatophores and protoliposomes. (2) As in chromatophores, the reconstituted enzyme, once activated, is deactivated in the course of hydrolysis in the presence of high concentrations of uncoupler and Mg^{2+} [30]. (3) The ratio of ATPase activities in the presence and absence of the activating anion sulfite, is in proteoliposomes similar to that observed in chromatophores (Fig. 5). The interplay between $\Delta\tilde{\mu}_{H^+}$, which activates the enzyme, and ADP and Mg^{2+} which tend to deactivate the enzyme in the absence of a $\Delta\tilde{\mu}_{H^+}$, has been discussed earlier [39,40]. The activating effect of sulfite has been attributed to a decrease in ADP-affinity [42,45], an increase in the rate of product release [43] and to interference with inhibitory Mg^{2+}-binding [43,46].

Self-activation of the enzyme after ATP-addition is a relatively slow process, because the required $\Delta\tilde{\mu}_{H^+}$ is built up only slowly by the initially inactive enzyme. However, the activation is apparently quite rapid if, instead, an artificial $\Delta\tilde{\mu}_{H^+}$ of sufficient magnitude is applied. This explains why ATP-synthesis after application of a ΔpH of 3 units occurs much more rapidly: in the experiments shown in Fig. 5, the process was complete within 1 s after addition of proteoliposomes (not shown).

There are several reports in the literature that proteoliposomes with cyanobactrial or chloroplast F_0F_1 yield only little uncoupler-stimulation of ATP-hydrolysis [23,61], or fail to exhibit an ATPase-induced quenching of 9-aminoacridine fluorescence [25,62]. Several factors may have contributed to these results. (a) Chloroplast [63] and cyanobacterial F_0F_1 [64] seem to exhibit a specific lipid requirement for the reconstitution of energy-transducing functions; this requirement is apparently not met by soybean asolectin in the case of cyanobacterial F_0F_1 [64], although soybean asolectin is quite effective in the case of chloroplast [26] and *R. rubrum* F_0F_1 (Ref. 15 and this report). (b) In experiments in which the internal volume of the proteoliposomes contains chloride, H^+-influx coupled to ATP-hydrolysis tends to be followed by an electrically silent HCl-efflux, especially in the presence of valinomycin, when the ΔpH is high (cf. Refs. 53 and 65). This has an adverse effect on ATPase-dependent changes in 9-aminoacridine fluorescence (not shown). (c) In liposomes from soybean asolectin (Ref. 30 and Table IV), the changes in 9-aminoacridine fluorescence in response to a proton-gradient (acid inside) are the resultant of processes causing enhancement and quenching of fluorescence. Unless the conditions are well chosen, these processes may approximately cancel, with the result that only small fluorescence changes are observed.

In conclusion, our data indicate that proteoliposomes from *R. rubrum* F_0F_1 are well-coupled, and the enzyme is in, or close to, the native state. Measurements of the rate of ATP-synthesis driven by an artificially applied $\Delta\tilde{\mu}_{H^+}$ will be described elsewhere [31].

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