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# Reconstitution of beef heart mitochondrial $F_0F_1$ in reverse phase evaporation vesicles

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**Abstract**

Beef heart mitochondrial $F_0F_1$ was reconstituted in proteoliposomes by a new procedure. $MF_0F_1$ was inserted in preformed reverse phase evaporation vesicles of large diameters prepared from asolectin ($MF_0F_1$–REV). Reconstitution was mediated by Triton X-100, which was subsequently removed by treatment with Bio-Beads. Parameters which resulted in optimal reconstitution were described. The $MF_0F_1$–REV proteoliposomes catalyzed an exchange between $P_i$ and ATP and were capable of proton pumping. Both reactions were inhibited by oligomycin and uncoupler of oxidative phosphorylation. The range of $P_i$–ATP exchange activity of the proteoliposomes (70–110 nmol $min^{-1}$ $mg^{-1}$) compared favorably with activities obtained in vesicles reconstituted by cholate dialysis or cholate dilution. The most important aspect of this method is that, unlike other reconstitution methods, exogenous $F_1$ and other coupling factors are not required to obtain high $P_i$–ATP exchange activity by $MF_0F_1$–REV. This simple and rapid reconstitution procedure should be useful for future studies dealing with functional analysis of $MF_0F_1$. © 1997 Elsevier Science B.V.

*Keywords:* Mitochondrial $F_0F_1$; ATP synthase; Reconstitution; Reverse phase evaporation vesicle; Proteoliposome; $P_i$–ATP exchange

## 1. Introduction

ATP synthases ($F_0F_1$) from bacteria, chloroplasts and mitochondria contain a proton channel in $F_0$ whereas the catalytic sites are located on $F_1$ [reviewed in [1–6]]. This complex of proteins and phospholipids is responsible for ATP synthesis coupled to electron transport in vivo. The structure of $F_1$ with its five non-identical subunits has been studied extensively, culminating in a recent report of its X-ray structure [7]. In contrast to $F_1$, the peptide composition of $F_0$ can differ depending on its source. Bacterial $F_0$ consists of only three subunits, all of them integral membrane proteins, whereas mammalian mitochondrial $F_0$s contain 4–8 additional peptides [8–11], some of which may form part of the stalk.

Because of the relative simplicity of the peptide composition of bacterial $F_0F_1$s, the ease with which they can be dissociated into individual subunits and then reconstituted, and the possibility of genetic ma-

Abbreviations: REV, reverse phase evaporation vesicles; TX-100, Triton X-100; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SMP, submitochondrial particles; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; $TF_0F_1$, $F_0F_1$ of thermophilic *Bacillus* PS3; $CF_0F_1$, chloroplast $F_0F_1$; $MF_0F_1$, mitochondrial $F_0F_1$

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nipulation, studies of the $F_0F_1$ of *E. coli* and thermophilic *Bacillus* PS3 have contributed significantly to our knowledge of the role of respective subunits and their interaction. Of the eukaryotic $F_0F_1$s, that of mammalian mitochondria is the most complex in peptide composition. Considerable efforts have been made in obtaining $F_0F_1$ preparations containing a minimal number of essential peptide components. Several procedures for such preparations have been described [8–11]. The biological activity of purified preparations of either bacterial or eukaryotic $F_0F_1$ was usually studied in reconstituted systems where $F_0F_1$ was incorporated into phospholipid vesicles. Successful reconstitution was indicated if uncoupler- or oligomycin-sensitive ATP driven proton pumping and/or $P_i$–ATP exchange reaction could be demonstrated [12–14], while ATP synthesis in these vesicles could only be obtained by additional manipulation. For example, ATP synthesis driven by an artificially imposed pH gradient has been demonstrated in proteoliposomes reconstituted from $TF_0F_1$ and $CF_0F_1$ [15–17]. Alternatively, $F_0F_1$ could be co-reconstituted with bacteriorhodopsin in the same vesicles. Upon light activation of bacteriorhodopsin, a proton gradient is generated to drive ATP synthesis [10,18–23].

Beef heart mitochondrial $F_0F_1$ was the first $F_0F_1$ to be successfully reconstituted either alone [12] or with bacteriorhodopsin [18] using the method of cholate dialysis. Recently, several reports have appeared describing reconstitution by detergent dialysis or column filtration of beef heart $F_0F_1$ preparations of greater purity [9,10,20,24]. However, $P_i$–ATP exchange activity and ATP synthesis by these vesicles were low, response to inhibitors and uncouplers was variable, and the results were generally unsatisfactory. It is possible that the detergent dialysis method is not suited for reconstitution of highly purified beef heart $F_0F_1$ preparations.

In this paper, we describe a procedure in which reconstitution of beef heart mitochondrial $F_0F_1$ was accomplished by the insertion of $F_0F_1$ in preformed liposomes of large diameter in the presence of TX-100. The method was adapted from a procedure developed for reconstitution of bacteriorhodopsin [25]. $MF_0F_1$ proteoliposomes prepared in this way catalyze a high rate of $P_i$–ATP exchange and proton pumping and both reactions are inhibited by oligomycin and uncoupler. The procedure has several advantages over other reconstitution methods and should be useful in future studies of the biological activity of individual $F_0$ components as well as mechanism of energy coupling.

## 2. Materials and methods

### 2.1. Materials

Asolectin (soybean phospholipids) was obtained from Associated Concentrates and partially purified by acetone extraction [12]. Previous analysis of such preparations showed it to be composed of phosphatidylcholine (40%), phosphatidylethanolamine (33%), phosphatidyl-inositol (14%), lyso-phosphatidylcholine (5%) and cardiolipin (4%) [26]. Cholic acid (Sigma) was recrystallized from 70% ethanol [12]. TX-100 was purchased from Pierce. Acridine orange (9-amino-6-chloro-2-methoxyacridine) was obtained from Molecular Probes, Eugene, OR. $^{32}P_i$ was from ICN. Bio-Beads SM2 were from Bio-Rad.

### 2.2. Preparations

$F_0F_1$ was prepared from beef heart submitochondrial particles (SMP) according to Serrano et al. [13] and suspended in 50 mM sucrose, 10 mM Tris–$SO_4$, pH 8.0, 0.5 mM EDTA, 1 mM $MgSO_4$ and 0.5 mM dithiothreitol at 30–35 mg protein $ml^{-1}$. Recovery of ATPase activity in $MF_0F_1$ was approximately 80%, and the protein yield was approximately 15%. The specific ATPase activity of this phospholipid-deficient $MF_0F_1$ preparation was 0.6–1 μmol $P_i$ $min^{-1}$ mg $protein^{-1}$, which was increased to 5–6 μmol $P_i$ $min^{-1}$ $mg^{-1}$ in the presence of added phospholipids. Whereas $P_i$–ATP exchange activity of SMP prepared from heavy layer mitochondria (approximately 250 nmol $min^{-1}$ mg $protein^{-1}$) was higher than that of SMP prepared from light layer mitochondria (approximately 100 nmol $min^{-1}$ mg $protein^{-1}$), there was little difference in the exchange activity of proteoliposomes of $MF_0F_1$ prepared from either type of SMP.

### 2.3. Preparation of reverse phase evaporation vesicles (REV)

REV were prepared based on the principle described by Szoka and Papahadjoupolous [27]. Purified asolectin (60 mg) in chloroform:methanol (2:1) was placed in an $18 \times 200$ mm glass tube. Solvent was removed under a stream of nitrogen. The dried lipids were dissolved in 2–3 ml ether and dried under nitrogen again. Ether (4.5 ml) and 1.5 ml REV buffer (50 mM KCl, 20 mM Tris–$SO_4$, pH 8, 0.2 mM EDTA) were added to the tube, which was covered with aluminum foil and sonicated 3–5 min at 10–15°C in a cylindrical bath type sonicator (Laboratory Supply, Hicksville, NY). The milky suspension was immediately transferred to a 100 ml round-bottomed flask. Ether was removed by rotary evaporation at 23°C. After 5 min evaporation, the flask was disconnected and 0.5 ml REV buffer was added to the viscous mixture. Evaporation was continued for 20–30 min until all the ether was removed. The turbid but homogeneous suspension of asolectin REV was then dialyzed overnight against 1 l of REV buffer at room temperature. The asolectin REV were stable for at least 3 days without visible precipitation if maintained at 4°C.

### 2.4. Reconstitution of $MF_0F_1$ proteoliposomes by insertion of $MF_0F_1$ in REV

The parameters for optimal reconstitution, yielding a high rate of $P_i$–ATP exchange, are described in the text. The standard reconstitution procedure, carried out at room temperature, is as follows. In a flat-bottomed tube (1.3 cm diameter and 4.5 cm in height) and in a final volume of 0.6 ml, the following materials were added in the order described: 0.3 ml asolectin REV (approximately 30 mg $ml^{-1}$), 0.1 ml REV buffer, water, and $MF_0F_1$ (final concentration, 1 mg $ml^{-1}$) in a volume of 0.54 ml. While the mixture was vigorously stirred, 60 μl of 10% TX-100 was added. leading to a decrease in the turbidity of the mixture. The mixture was then stirred slowly for 10 min before addition of 200 mg Bio-Beads, after which stirring was continued for 3 h. As TX-100 was being removed by the Bio-Beads, an increase in the turbidity of the mixture could be noticed. The slightly turbid proteoliposomes were collected with a pipette tip to avoid picking up any of the beads. The reconstituted proteoliposomes were stable for several hours when kept at 0°C.

### 2.5. Assays

ATPase activity of $MF_0F_1$ was assayed at 30°C in the absence or presence of added phospholipids in 0.5 ml of a reaction mixture described by Pullman et al. [28] using 20–40 μg of protein. Phospholipids were added from a stock solution (50 mg $ml^{-1}$ in 2% detergent) to a final concentration of 200–400 μg $ml^{-1}$. After 5 min at 30°C, the reaction was terminated by the addition of 50 μl of 50% trichloroacetic acid. Denatured proteins were precipitated by centrifugation in a microfuge. Aliquots (0.1–0.4 ml) of the supernatant solution were added to 4 ml of ammonium molybdate reagent for colorimetric determination of inorganic phosphate by a modified method of Heinonen and Lahti [29]. The advantage of this method over other methods of colorimetric determination of $P_i$ is that the ammonium molybdate reagent (2 volumes of acetone, 1 volume of 2.5 M $H_2SO_4$, 1 volume of 10 mM ammonium molybdate) dissolved any residual phospholipids which were not completely sedimented by centrifugation and yielded a clear yellow solution upon reaction with $P_i$, the absorbance of which could be determined at 355 nm.

$P_i$–ATP exchange activity was determined as follows. $MF_0F_1$ proteoliposomes (75–100 μl) were preincubated in 0.9 ml solution containing 50 μmol KCl, 40 μmol Tris–$SO_4$, pH 8, 4 μmol $MgSO_4$, 1 μmol EDTA for 5 min at 30°C. The reaction was initiated by the addition of 0.1 ml solution containing 0.1 M $MgSO_4$, 0.1 M ATP, 0.2 M potassium phosphate (200 000 cpm μmol $P_i^{-1}$), pH 7.4. After 10 min at 30°C, the reaction was stopped by 0.1 ml 50% trichloroacetic acid. After centrifugation to remove the denatured proteoliposomes, 0.3 ml supernatant was extracted to separate $^{32}P_i$ and [$^{32}$P]ATP [30]. An aliquot of the aqueous phase containing [$^{32}$P]ATP was counted. Decrease of specific radioactivity of $^{32}P_i$ due to ATP hydrolysis was less than 10%.

Phospholipid concentration was determined by the amount of $P_i$ released after digestion with $Mg(NO_3)_2$ [31].

### 2.6. Proton pumping

ATP driven proton pumping was determined using acridine orange as a pH sensitive probe. The reaction mixture (2 ml) contained 200 mM KCl, 20 mM Tris–$SO_4$, pH 7.4, 10 mM $MgSO_4$, 2 μM acridine orange, 0.5 ng valinomycin and 50–200 μg proteoliposomes. The reaction was initiated by the addition of 1 mM ATP. Changes in the fluorescence of acridine orange were monitored at 530 nm with an excitation wavelength of 470 nm [32].

### 2.7. Determination of internal volume of asolectin REV

Asolectin REV (2 ml) were prepared in the presence of [$^{14}$C]inulin (3.32 μCi $mg^{-1}$) at 0.33 mg inulin $ml^{-1}$. An aliquot (1.5 ml) was applied to a Sepharose Cl-6B column (1.6 cm × 26 cm) equilibrated in REV buffer. Vesicles were eluted in the void volume and were completely separated from free inulin. The amount of [$^{14}$C]inulin in the sample applied to the column and in the pooled REV fractions from the column were determined by scintillation counting as well as the phospholipid concentration of the pooled REV was determined. The internal volume of asolectin REV, expressed as μl mg phospholipids$^{-1}$, was calculated from the amount of [$^{14}$C]inulin incorporated into the vesicles, utilizing assumptions about membrane thickness and surface area per phospholipid molecule [33,34].

## 3. Results

### 3.1. Properties of $MF_0F_1$ used for reconstitution

The $MF_0F_1$ preparation used in the present study was obtained by ammonium sulfate fractionation of submitochondrial particles solubilized by cholate [13]. Analysis by SDS polyacrylamide gel electrophoresis showed that the peptide composition was similar to that described in the original report. In addition to the peptides designated as components of beef heart $F_0F_1$ [9,10,23], the only detectable contaminants were four peptides in the range of 60–110 kDa and a peptide of 28 kDa (data not shown). The former were probably residual cytochrome components and the latter most likely adenine nucleotide translocase [13]. This preparation has been shown to have a low phospholipid content (0.08 μmol mg protein$^{-1}$) and low ATPase activity which could be fully activated by phospholipids added as mixed phospholipid-detergent micelles [13]. We found that at a ratio of phospholipids to protein of 5 to 1, the ATPase activity of the $F_0F_1$ preparation used in this study was increased 10-fold to 6 μmol $min^{-1}$ $mg^{-1}$ by soybean phospholipids (asolectin) whereas activation by PC or PE alone was less. Similar ATPase activities were obtained whether asolectin was dispersed in TX-100, octylglucoside, n-dodecylmaltoside, or cholate. Inhibition of the ATPase activity by oligomycin (4 μg $ml^{-1}$) was greater than 95% either in the absence or presence of phospholipids.

### 3.2. Reconstitution of $MF_0F_1$ by insertion in reverse-phase evaporation vesicles

The basic procedure for preparation of proteoliposomes by insertion of $MF_0F_1$ in REV involves the following steps: (1) Preparation of REV, (2) incubation of $MF_0F_1$ with REV in the presence of suitable amount of a selected detergent in order to allow insertion, and (3) removal of detergent by Bio-Beads. We investigated the effect of various parameters with the goal of obtaining optimal functional reconstitution as monitored by $P_i$–ATP exchange activity of the proteoliposomes. These include types of lipids used to prepare the REV, the most suitable detergent for mediating the insertion of $MF_0F_1$, detergent/phospholipids ratios, phospholipid and protein concentration, optimal preincubation time, and contact time with Bio-Beads needed to obtain tightly sealed proteoliposomes.

In the selection of phospholipids for reconstitution of $MF_0F_1$ proteoliposomes, purified asolectin and various combinations of PC, PE, PA, and cholesterol, previously used in other reconstitution studies [17,19,25], were used with TX-100 as the detergent. Certain combinations, e.g., egg PC and bovine liver PE (1:1) failed to form vesicles when subjected to the procedure of REV preparation. Proteoliposomes reconstituted from asolectin REV exhibited the highest activity.

The effect of detergent concentration on reconstitution of $MF_0F_1$ in asolectin REV was then investi-

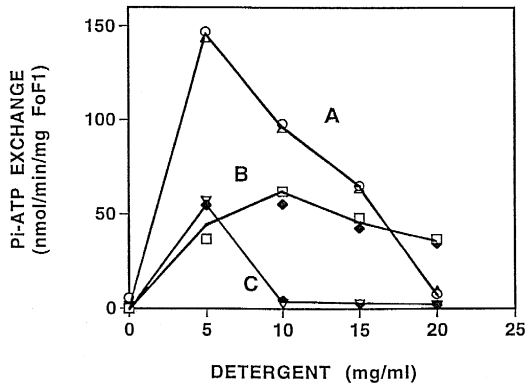


Fig. 1. Titration of detergents for optimal reconstitution of $F_0F_1$ proteoliposomes. REV of asolectin (30 mg $ml^{-1}$) was prepared in 20 mM Tris–$SO_4$, pH 8, 50 mM KCl, 1 mM DTT, 0.1 mM ATP and 0.2 mM EDTA as described in Section 2. $F_0F_1$ (10 mg $ml^{-1}$) was suspended in 0.5 M sucrose, 20 mM Tris–$SO_4$, pH 7.4, 0.5 mM EDTA, 0.1% asolectin, 1% TX-100 and 0.5 mM ATP. The reconstitution mixture (0.6 ml) contained 9 mg asolectin, 0.6 mg $F_0F_1$, 2 mM $MgSO_4$ and the indicated concentration of detergent. The mixture was incubated with stirring for 1 h at room temperature, then 48 mg Bio-Beads were added. Bio-Beads (48 and 96 mg) were added again after 90 and 180 min. Proteoliposomes were collected 60 min after the last addition of Bio-Beads. Aliquots (75 μl) were used to determine $P_i$–ATP exchange activity. Detergents used were cholate, curve A (○, △), TX-100, curve B (□, ◆), and octylglucoside, curve C (▽, ▲). The different symbols for each curve represent duplicate measurements.

gated with TX-100, cholate and octylglucoside. Composite data shown in Fig. 1, obtained from 3 separate experiments, illustrate the different efficacy of the 3 detergents. In these experiments, asolectin REV, $MF_0F_1$, and detergent were preincubated for 1 h followed by three successive addition of Bio-Beads (see legend of Fig. 1). At an asolectin concentration of 15 mg $ml^{-1}$, $P_i$–ATP exchange activity in proteoliposomes reconstituted with 10 mg $ml^{-1}$ TX-100 was greater than with 5 mg $ml^{-1}$ TX-100, but the activity remained essentially the same when TX-100 was increased to 20 mg $ml^{-1}$ (Fig. 1, curve B). In contrast, $P_i$–ATP exchange activity declined sharply in proteoliposomes when reconstituted at a detergent concentration greater than 5 mg $ml^{-1}$ for cholate (Fig. 1, curve A). The range of concentration of octylglucoside suitable for reconstitution was even narrower (Fig. 1, curve C). While the experiments presented in Fig. 1 indicated that significantly higher activity was obtained in proteoliposomes reconstituted with cholate, greater variability was observed with this detergent in seven different experiments. Under the standard reconstitution condition described in Section 2, similar $P_i$–ATP exchange activity were obtained with either TX-100 (10 mg $ml^{-1}$) or cholate (varying between 3.3–6.6 mg $ml^{-1}$) (Table 1). Table 1 also showed again that little activity was seen in vesicles reconstituted in the presence of 10 mg $ml^{-1}$ octylglucoside. Because effective reconstitution was obtained over a wider range of TX-100 concentration and the results were more consistent, TX-100 was chosen as the detergent for the remaining of the study.

In contrast to reconstitution with bacteriorhodopsin [25], we found that prolonged preincubation of asolectin REV, $MF_0F_1$, and TX-100 was not necessary. The same $P_i$–ATP exchange activity was obtained in the reconstituted vesicles when preincubation time varied between 10 to 60 min before the addition of Bio-Beads (Fig. 2A). There was also little difference in the activities of the $MF_0F_1$–REV whether Bio-Beads were added 3 separate times (a total of 320 mg $ml^{-1}$ reconstitution mixture) as was performed for the experiments in Fig. 1 or if the total amount was added at the beginning (data not shown), which had been shown to remove TX-100 at a faster rate [25]. This result indicated that, in contrast to bacteriorhodopsin reconstitution, the $P_i$–ATP exchange activity of the reconstituted $F_0F_1$–REV was less sensitive to the rate of detergent removal. The

Table 1
$P_i$–ATP exchange activities of $F_0F_1$–REV prepared at different detergent concentrations

| Detergent | Concentration (mg $ml^{-1}$) | $P_i$–ATP exchange activity (nmol $min^{-1}$ mg $protein^{-1}$) |
|---|---|---|
| TX-100 | 10 | 104 |
| Octylglucoside | 5 | 96 |
| | 10 | 4.9 |
| Cholate | 3.3 | 73.6 |
| | 5 | 101 |
| | 6.6 | 115 |

Recontitution was carried out as described in Section 2 with 15 mg $ml^{-1}$ asolectin REV, 1 mg mg $F_0F_1^{-1}$ and the indicated concentrations of detergents. The three components were preincubated in a volume of 0.6 for 10 min before the addition of 200 mg Bio-Beads. After 3 h, the vesicles were recovered for determination of $P_i$–ATP exchange activity.

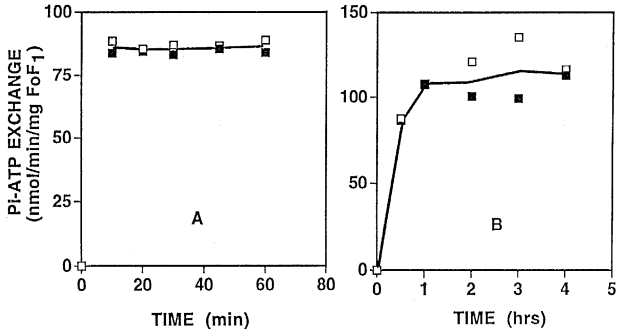


Fig. 2. (A) Effect of time of preincubation of asolectin REV, $F_0F_1$ and TX-100 on reconstitution. Experimental condition was the same as that described in the legend of Fig. 1 using TX-100 at 10 mg $ml^{-1}$. A separate reconstitution mixture was prepared for each time point. Bio-Beads were added at the indicated time after addition of TX-100. Open and filled squares represent duplicate measurements from the same experiment. (B) Contact time with Bio-Beads for optimal reconstitution. A reconstitution mixture (2.4 ml) containing asolectin REV (15 mg $ml^{-1}$), $F_0F_1$ (1 mg $ml^{-1}$) and TX-100 (16.7 mg $ml^{-1}$) was incubated for 15 min. The time of addition of Bio-Beads (770 mg) was considered time 0. At the indicated times, aliquots of the mixture were taken and the proteoliposomes collected for $P_i$–ATP exchange activity determination. The open and filled squares represent duplicate measurements in the same experiment.

practical advantage of only one addition of Bio-Beads becomes obvious when a large number of reconstitution mixtures need to be prepared simultaneously. Fig. 2B shows that significant exchange activity was obtained 30 min after addition of Bio-Beads. Maximal activity, however, required longer contact with the beads. A contact time of 3 h was chosen for most reconstitution experiments.

The protocol established as described above was used to determine the optimal $MF_0F_1$ and phospholipid concentration required for reconstitution. At 15 mg phospholipid $ml^{-1}$ and a TX-100 to phospholipids ratio of 1.23, the concentration range of $MF_0F_1$ which resulted in maximal activity was 0.5–1 mg $ml^{-1}$ (Fig. 3A). Premixing of $MF_0F_1$ with TX-100, asolectin, and ATP, a condition used for reconstitution of $CF_0F_1$ [17], was not necessary (data not shown). At 1 mg $F_0F_1$ $ml^{-1}$, but at a lower TX-100 concentration (10 mg $ml^{-1}$ instead of 16.7 mg $ml^{-1}$ as for the experiment in Fig. 3A), proteoliposomes with a phospholipid concentration of 6.8 mg $ml^{-1}$ (and a TX-100/phospholipid ratio of 1.47) yielded higher activity (Fig. 3B). These results indicate that detergent/phospholipids ratios may be more important than phospholipid concentration per se in determining the activity of the proteoliposomes. The lower limit of phospholipid/protein ratio (approximately 7) which yielded proteoliposomes of high $P_i$–ATP exchange activity (Fig. 3B) was significantly lower than used in other reconstitution procedures, some exceeding 100 [17,19,20].

### 3.3. Comparison with other reconstitution procedures

In previous reports on reconstitution of $MF_0F_1$, proteoliposomes were prepared by the procedures of

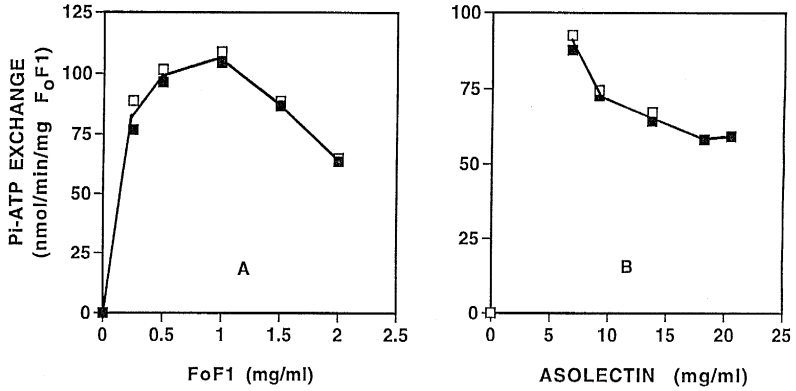


Fig. 3. Effect of variation of parameters on the activity of the reconstituted proteoliposomes. (A) Effect of varying $F_0F_1$ concentration: reconstitution was carried out with asolectin (13.6 mg $ml^{-1}$), TX-100 (16.7 mg $ml^{-1}$) and the indicated concentrations of $F_0F_1$. (B) Effect of varying asolectin concentration: reconstitution was carried out with $F_0F_1$ (1 mg $ml^{-1}$), TX-100 (10 mg $ml^{-1}$) and the indicated concentrations of asolectin. The open and filled squares represent duplicate measurements in the same experiment.

Table 2
$P_i$–ATP exchange activities of $F_0F_1$ proteoliposomes reconstituted by detergent dialysis, detergent dilution and insertion in REV

| Reconstitution method | Detergent, phospholipids | $P_i$–ATP exchange activity (nmol $min^{-1}$ mg $F_0F_1^{-1}$) | |
|---|---|---|---|
| | | $-F_1$ | $+F_1$ |
| Dialysis | Cholate, asolectin | 16.1 | 16.8 |
| | Cholate, PC:PE | 73.5 | 105.4 |
| Dilution | Cholate, asolectin | 24.1 | 57.4 |
| | Cholate, PC:PE | 16.3 | 70.5 |
| Insertion in REV | TX-100, asolectin | 105.7 | 116.2 |
| | Cholate, asolectin | 67.5 | 72.5 |

Reconstitution of $F_0F_1$ proteoliposomes by cholate dialysis and cholate dilution was carried out under conditions previously described for optimal reconstitution [9,10]. Phospholipids used were either asolectin or egg PC:bovine liver PE (1:1). The phospholipid and $F_0F_1$ concentration used in the three procedures were 40 and 4 mg $ml^{-1}$ for cholate dialysis, 30 and 1–2 mg $ml^{-1}$ for cholate dilution and 15 and 1 mg $ml^{-1}$ for insertion in REV. For the last procedure, TX-100 was present at 10 mg $ml^{-1}$ and cholate was present at 11.25 mg $ml^{-1}$ before the addition of Bio-Beads. $P_i$–ATP exchange activity was determined as described in the absence or presence (20 μg) of $F_1$.

cholate dialysis [12,13] or cholate dilution [13,14]. In the experiment shown in Table 2, the same $MF_0F_1$ preparation was reconstituted by these two methods using either asolectin or a mixture of egg PC: bovine liver PE (1:1). Since PC/PE failed to form REV, only the data obtained with asolectin $MF_0F_1$–REV prepared using either TX-100 or cholate are presented.

The maximal $P_i$–ATP exchange activities obtainable with proteoliposomes reconstituted by the three procedures were similar (70–110 nmol $min^{-1}$ mg $protein^{-1}$) except for $MF_0F_1$–asolectin vesicles reconstituted by cholate dialysis which exhibited only 1/5 of the activity as the $MF_0F_1$–PC/PE proteoliposomes. Of the three reconstitution methods, vesicles reconstituted by cholate dilution showed a marked dependency on excess $F_1$ for the attainment of maximal activity. Addition of exogenous $F_1$ increased the $P_i$–ATP exchange activity of these vesicles by 2–5 fold. Enhancement of activity by $F_1$ in vesicles reconstituted from PC/PE by cholate dialysis was also significant (50%). In contrast, exogenous $F_1$ had negligible effect on the activity of $MF_0F_1$–REV whether reconstitution was mediated by cholate or TX-100. $P_i$–ATP exchange activities of all three types of proteoliposomes were completely abolished by the uncoupler, FCCP.

A characteristic of vesicles prepared by reverse phase evaporation was their large diameters, which can vary with the type of phospholipids used [27]. Internal volume of asolectin REV was assessed by the incorporation of $^{14}$C-inulin. An average internal volume of 8.2 μl $mg^{-1}$ phospholipids was calculated from the results, which corresponded to a diameter of 90 nm of the proteoliposomes. Similar values were obtained for the $MF_0F_1$ proteoliposomes. The average size of these vesicles was considerably larger than that obtainable with the cholate dialysis or cholate dilution methods, which are typically in the range of 25–35 nm [35]. We made no efforts to obtain vesicle population of uniform size since the proteoliposomes were not used for kinetic analysis.

### 3.4. Proton pumping

Proteoliposomes reconstituted from insertion of $MF_0F_1$ in asolectin REV were capable of ATP driven proton pumping. This was demonstrated with the pH sensitive dye acridine orange, whose fluorescence decrease reports a decrease of pH [36]. Fig. 4 shows that addition of ATP to the proteoliposomes caused a decrease of fluorescence of acridine orange which could be reversed by oligomycin, an inhibitor of $F_0$ (lower curve of Fig. 4), or FCCP (upper curve of Fig.

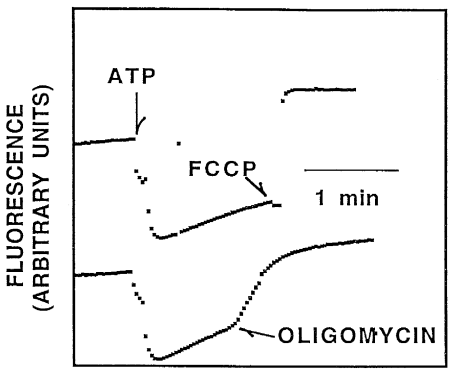


Fig. 4. ATP driven proton pumping in $F_0F_1$ proteoliposomes. For each assay, 50 μg of $F_0F_1$–REV protein were used. 1 mM ATP was added at the arrow indicated. The effect of oligomycin (5 μg) is shown in the lower curve. The effect of FCCP (2.5 μM) is shown in the upper curve.

4), a proton ionophore and uncoupler of oxidative phosphorylation.

## 4. Discussion

Beef heart mitochondrial $F_0F_1$ was the first membrane protein complex to be successfully reconstituted in phospholipid vesicles. Proteoliposomes prepared from asolectin and partially purified $MF_0F_1$ by cholate dialysis were shown to be capable of carrying out $P_i$–ATP exchange reaction and ATP-driven proton pumping, both of which require an intact membrane structure [12]. Subsequently, several other procedures were developed for reconstitution of membrane associated functions of a variety of membrane proteins. These include detergent dilution, sonication, freeze–thaw sonication, incorporation in the absence and presence of detergent, and fusion [35,37]. Methods for incorporation of transport and channel proteins in planar lipid bilayers were also established [38,39].

Several of these methods have been employed successfully in the reconstitution of various types of $F_0F_1$ yielding important information of this energy coupling system. For example, it has been shown that (1) the proton pumping activity of the *E. coli* $F_0$ could be restored upon assembly of the individual $F_0$ subunits in proteoliposomes [40], (2) rate of ATP synthesis approaching that of chloroplast was obtainable in reconstituted $CF_0F_1$ proteoliposomes upon acid–base transition [16], and (3) ATP induced current generation in $TF_0F_1$ reconstituted in planar bilayer membranes [41].

Most of the proteoliposome preparation methods mentioned above produce small vesicles with high surface curvature [35]. Furthermore, the actual mechanism regarding the interaction of protein, phospholipids, and detergent during reconstitution is not well understood. More recently, reconstitution procedure producing larger vesicles was developed and the mechanism better elucidated [see 42 for review]. The method utilized reverse-phase evaporation vesicles (REV) which were first described by Szoka and Papahadjopoulos [27]. The diameters of these REV are in the range of 100–200 nm with internal volumes 10–30 times greater than that of sonicated unilamellar lipid vesicles [27,43] and have obvious advantage for the purpose of delivery of drugs and other therapeutic agents. Rigaud et al. extended the use of REV for reconstitution purposes. Using bacteriorhodopsin as a model system, they developed a detergent mediated reconstitution procedure which appeared to have general applicability for most membrane proteins. The behavior of three detergents in particular, TX-100, octylglucoside and cholate, and their interaction with bacteriorhodopsin has been studied in great detail and shown to be different [25]. Upon mixing of REV with increasing concentration of detergents, the REV are first saturated with detergents, followed by solubilization of REV until only mixed micelles of phospholipids and detergents exist [25,42]. The optimal detergent concentrations where incorporation of monomeric bacteriorhodopsin in preformed REV occurred, however, differed depending on the detergent used. In the presence of octylglucoside, incorporation of bacteriorhodopsin in REV occurred before the REV were saturated with octylglucoside, whereas reconstitution in the presence of cholate did not occur until the detergent, lipids and protein were in the form of ternary micelles. These and other studies have increased our understanding of the reconstitution process significantly [42], however, it is also important to realize that parameters for optimal reconstitution could vary markedly with different membrane proteins.

Reconstitution in REV has been applied to $CF_0F_1$ [17] and $TF_0F_1$ [19–21], the latter was co-reconstituted with bacteriorhodopsin. In these studies, the different parameters for optimal reconstitution were thoroughly explored. Preliminary results from one of these studies indicated that conditions suitable for $CF_0F_1$ and $TF_0F_1$ were not directly transferable to reconstitution of a pig heart $MF_0F_1$ preparation [19].

Because of the desirable features of the REV proteoliposomes [42], we explored the possibility of its application to beef heart $F_0F_1$. The $MF_0F_1$ preparation used in this study contains five peptides in addition to those considered to be essential for its function [8–11]. However, since most of the previous successful reconstitution was obtained with such preparations [13,37], any differences in reconstitution between the present and the previous procedures can be contributed to the method itself rather than variability in $MF_0F_1$ preparations. Thus, it is possible to

recognize two unique features of the present method for beef heart $MF_0F_1$ reconstitution: (1) Triton X-100, a detergent which has been used in the preparation of purified $MF_0F_1$ [9], is the most suitable detergent for $MF_0F_1$ reconstitution in REV, (2) additional coupling factors are not required for the $MF_0F_1$–REV to exhibit maximal $P_i$–ATP exchange activity.

While octylglucoside, cholate, and TX-100 could all mediate incorporation of $F_0F_1$ in asolectin REV, the effective concentration range of octylglucoside and cholate were limited when compared to TX-100. We have also found that at high detergent/protein ratio, octylglucoside and cholate caused loss of ATPase activity of submitochondrial particles whereas TX-100 was not harmful. Although the loss of ATPase activity was not a serious concern under the reconstitution conditions, the most consistent results were obtained with the use of TX-100. Optimal reconstitution of $MF_0F_1$ in asolectin REV was obtained at TX-100/phospholipids ratios of 0.7–1.5. $F_0F_1$ insertion most likely proceeded by transfer of the protein complex present in phospholipids–detergent–proteins micelles into destabilized REV saturated by TX-100 [42]. This mode of reconstitution would favor the unidirectional insertion of an asymmetric membrane protein complex such as $F_0F_1$ with at least two thirds of its mass being hydrophilic.

In two previous studies in which $CF_0F_1$ [17] and $TF_0F_1$ and bacteriorhodopsin [19,20] were reconstituted in REV of PC:PA (9:1), the optimal weight ratio of phospholipids/protein was 133 for $TF_0F_1$ [19], and 80 for $CF_0F_1$ [14]. These values translate into protein concentrations in the range of 0.03–0.05 mg $ml^{-1}$ in these vesicles. In the present study, we obtained high $P_i$–ATP exchange activity in proteoliposomes with a phospholipids/$MF_0F_1$ ratio of 7 and $MF_0F_1$ concentrations of 0.5–1 mg $ml^{-1}$. The large discrepancy in phospholipids/protein ratios for optimal reconstitution in the other two reports and the present report is not understood but may be related to the fact that different phospholipid mixtures were used for reconstitution. The $P_i$–ATP exchange activities of the asolectin $MF_0F_1$–REV compares favorably with values previously obtained with $F_0F_1$ proteoliposomes reconstituted by other methods (see Table 2 and Ref. [12–14,37]) and was at least 10 times higher than the activity obtained in vesicles reconstituted from PC:PA–REV and a purified beef heart $F_0F_1$ preparation [23], which were prepared under experimental conditions significantly different from that described in this report.

Reconstitution in REV overcomes several disadvantages of the two classical and widely used reconstitution procedures. In the cholate dialysis procedure, prolonged dialysis against large volumes of reconstitution buffer is necessary. In the cholate dilution procedure, dilution of phospholipids–detergent–proteins mixture into large amount of buffer is required to reduce the detergent concentration sufficiently for vesicle formation. This necessitates high initial concentration of lipids and $F_0F_1$ for activity measurement. Furthermore, reproducible results can only be obtained by using cholic acid recrystallized from commercial reagent. In comparison, the procedure described here is simpler with respect to time, reagent and execution, and may prove to be a more suitable method for reconstitution of purified $F_0F_1$ preparations than cholate dialysis. The most significant improvement of the present method over other methods is that addition of extraneous $F_1$ (Table 2) or oligomycin sensitivity conferring protein (data not shown) is no longer necessary for the expression of full $P_i$–ATP exchange activity. These results suggest that the $MF_0F_1$ preparation was not deficient in any of the protein components required for functional coupling. The requirement of additional coupling factors for $MF_0F_1$ proteoliposomes prepared by cholate dialysis and cholate dilution was apparently the result of loss of activity suffered by these protein components during reconstitution, which did not occur during reconstitution of $F_0F_1$–REV. This particular feature should be useful when analyzing the $F_0F_1$ subunits in functional assembly. Lastly, the larger diameter of these vesicles facilitate the non-random insertion of $F_0F_1$ and will also be useful when incorporation of large amounts of desired solutes or probes in the intravesicular space is desirable.

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