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Purification of the Energy-Transducing Adenosine Triphosphatase Complex from *Rhodospirillum rubrum*†

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ABSTRACT: The oligomycin- and *N,N'*-dicyclohexylcarbodiimide-sensitive adenosine triphosphatase complex extracted with Triton X-100 from the chromatophores of *Rhodospirillum rubrum* was extensively purified. The purification procedure included (diethylamino)ethylcellulose chromatography and glycerol gradient centrifugation. The specific activity of Mg^{2+}-dependent ATP hydrolysis in the purified preparation increased about 11-fold, while that of Ca^{2+}-dependent ATP hydrolysis increased 50-fold as compared with chromatophores. The purified adenosine triphosphatase complex dissociated into a maximum of eight different polypeptides upon electrophoresis in the presence of sodium dodecyl sulfate. The estimated subunit molecular weights were as follows: 56 000 (α), 50 000 (β), 33 000 (γ), and those ranging from 17 000 to 9400 for the remaining smaller subunits. The purified preparation was incorporated into phospholipid vesicles by using the freeze–thaw technique. The reconstituted vesicles catalyzed [^{32}P]ATP exchange, which was almost completely inhibited by both oligomycin and *N,N'*-dicyclohexylcarbodiimide as well as by a protonophorous uncoupler, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone.

Proteolipid vesicles containing purified coupling factor ATPase complexes (CF_0–F_1)[1] seem to be very attractive models for studying mechanisms of oxidative- and photophosphorylation (Kagawa, 1972).

Numerous attempts have been undertaken in recent years to isolate and purify from various sources ATPase complexes capable of energy conversion.

Regarding mammalian mitochondria, a number of oligomycin-sensitive ATPase preparations containing 12–15 polypeptide bands on $NaDodSO_4$ gels have recently been described (Kagawa & Racker, 1966; Tzagoloff et al., 1968; Swanljung et al., 1973; Sadler et al., 1974; Serrano et al., 1976; Stigall et al., 1978; Berden & Voorn-Brouwer, 1978). Of those listed above, the preparation isolated by Serrano et al. (1976) seems to be most extensively studied with respect to energy-transfer activities. Vesicles, which catalyzed [^{32}P]ATP exchange and ATP-driven proton translocation, were reconstituted from the purified ATPase complex and phospholipids; in addition, site III phosphorylation was demonstrated when cytochrome oxidase was added to the reconstituted vesicles. However, these energy-transfer activities were assayed in the presence of purified F_1 and OSCP proteins added to the reaction mixtures.

More purified ATPase complexes, containing eight to ten polypeptides, were isolated from yeast mitochondria (Tzagoloff & Meagher, 1971; Ryrie, 1975a,b, 1977; Ryrie & Blackmore, 1976). One of these ATPase preparations (Ryrie, 1975b, 1977; Ryrie & Blackmore, 1976) was capable of energy-linked activities when incorporated into liposomes.

Highly purified DCCD-sensitive ATPase complexes have recently been isolated and extensively studied from aerobic thermophilic bacterium PS3 (Sone et al., 1975, 1977a,b) and from spinach chloroplasts (Pick & Racker, 1979). Both of these complexes dissociated into eight polypeptides on $NaDodSO_4$ gels and, after incorporation into phospholipid vesicles, were capable of ATP-driven proton translocation, [^{32}P]ATP exchange, and pH-jump phosphorylation. When bacteriorhodopsin was included in the vesicles, light-induced

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[1] Abbreviations used: F_0–F_1, energy-transducing ATPase complex sensitive to energy-transfer inhibitors, consists of water-soluble F_1 subunits and hydrophobic proton-translocating subunits (F_0); DCCD, *N,N'*-dicyclohexylcarbodiimide; $NaDodSO_4$, sodium dodecyl sulfate; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; Tricine, tris(hydroxymethyl)methylglycine; Tris, tris(hydroxymethyl)aminomethane; Triton X-100, octylphenoxypoly(ethoxyethanol); OSCP, oligomycin sensitivity conferring subunit of the ATPase complex.

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ATP synthesis was demonstrated. It was later shown by Sone et al. (1978) that F_0–F_1 containing only seven subunits was capable of [^{32}P]ATP exchange when incorporated into the vesicles.

The above described highly purified Mg^{2+}-ATPase complexes, isolated from evolutionarily distant organisms, all required vesicular structure for the energy-transfer activities. When incorporated into phospholipid vesicles, these ATPase complexes could act as H^+ translocators upon addition of ATP. Furthermore, the ATPase complexes, isolated from the membranes of thermophilic bacterium and spinach chloroplasts, could drive ATP synthesis at the expense of artificially imposed proton gradients. These experimental findings are of valuable support to the chemiosmotic theory of energy coupling (Mitchell, 1966).

It seems apparent that isolation and purification of reconstitutively active ATPase complexes from additional sources, such as photosynthetic bacteria, would contribute to our knowledge of the energy-coupling mechanisms and of the molecular structure of this remarkable enzyme.

Oren & Gromet-Elhanan (1977) have recently reported the isolation and partial purification of the oligomycin- and DCCD-sensitive ATPase complex from the chromatophores of the photosynthetic bacterium *Rhodospirillum rubrum*. The preparation contained 13 major polypeptide bands on $NaDodSO_4$ gels (Oren & Gromet-Elhanan, 1979). Light-induced ATP synthesis was demonstrated in the vesicles reconstituted from the crude ATPase preparation, phospholipids, and bacteriorhodopsin.[2]

This report describes purification of the *R. rubrum* ATPase complex consisting of a maximum of eight polypeptides on $NaDodSO_4$ gels and reconstitution of proteoliposomes active in [^{32}P]ATP exchange.

Experimental Procedure

Materials. *R. rubrum* cells of strain S_1 were grown as described by Briller & Gromet-Elhanan (1970). Soybean phospholipids (commercial grade) were purchased from Sigma and were partially purified by the procedure of Kagawa & Racker (1971). ATP (grade I), defatted bovine albumin, oligomycin, FCCP, Triton X-100, Tricine, Tris, and valinomycin were purchased from Sigma. DEAE-cellulose (DE-22) was obtained from Whatman Biochemicals. Acrylamide, methylenebis(acrylamide), and glycerol were purchased from BDH. $NaDodSO_4$ was purchased from Bio-Rad Laboratories, and DCCD was obtained from Fluka. All chemicals and organic solvents used were of analytical grade.

Preparations. Chromatophores were prepared according to Gromet-Elhanan (1970) and stored in liquid air at a concentration of 60 mg of protein/mL.

Liposomes containing the purified ATPase complex were prepared by using essentially the freeze–thaw technique (Kasahara & Hinkle, 1977; Pick & Racker, 1979). Phospholipids (80 mg) were suspended in 2 mL of a solution containing 50 mM Tricine–NaOH (pH 8) and 1 mM EDTA and sonicated to clarity, usually for 10–15 min. The purified ATPase preparation (1 mL of a solution after step III, containing 0.3 mg of protein) was dialyzed for 4 h at 4 °C against a medium containing 50 mM Tricine–NaOH (pH 8) and 0.05% Triton X-100 (v/v). The medium was replaced every hour during dialysis. The dialyzed protein solution was combined with the sonicated phospholipids (2 mL), mixed on a Vortex mixer, and frozen in liquid air. After being thawed at room temperature, the liposomes were sonicated for 30–60 s in an ice bath.

Analytical Methods. Electrophoresis in the presence of $NaDodSO_4$ was performed according to Weber & Osborn (1969). Gels were fixed, stained, and destained as described by Nelson et al. (1973). Phosphorylase *a* (M_r 94 000), pyruvate kinase (M_r 57 000), pepsin (M_r 35 000), and α-chymotrypsin (M_r 25 000) with its subunits (M_r 13 000 and 11 000) were used as standards for the determination of the subunit molecular weights of the purified ATPase complex. Protein concentration in the samples was determined by the method of Lowry et al. (1951). Protein in the samples (up to 100 μL) was precipitated with 1 mL of acetone–methanol (5:2) and dissolved in 0.5 mL of 1% $NaDodSO_4$ before addition of Lowry reagents. The concentration of bacteriochlorophyll in chromatophores was calculated by using a molar extinction coefficient of 140 000 at 880 nm given by Clayton (1963).

Mg^{2+}- or Ca^{2+}-dependent ATPase activity was measured for 5–15 min at 35 °C in a reaction medium which contained, in a final volume of 1 mL, 50 mM Tris-HCl (pH 8), 2 mM $MgCl_2$ (or 4 mM $CaCl_2$), and either chromatophores (150–200 μg of protein) or ATPase preparations (15–30 μg). Oligomycin and DCCD (when used) were added as solutions in methanol and were incubated with the ATPase preparations for 10 min at 35 °C before starting the reaction. The reaction was initiated by the addition of 4 mM ATP and terminated by adding 0.1 mL of 50% trichloroacetic acid. The released inorganic phosphate was determined colorimetrically by the method of Taussky & Shorr (1953). For the Triton-containing protein samples, 60 μL of 10% $NaDodSO_4$ was added to each test tube after termination of the reaction. Activity of [^{32}P]ATP exchange in the reconstituted liposomes was measured for 15–20 min at 35 °C in a reaction medium which contained, in 1 mL, 80 mM Tricine–NaOH (pH 8), 2.5 mg of defatted bovine albumin, 12.5 mM KH_2PO_4 (containing 2×10^7 cpm), and 0.3 mL of proteoliposomes which contained 30 μg of the purified ATPase complex. Energy-transfer inhibitors and uncouplers were added as solutions in methanol and were incubated with the reaction mixture for 10 min at room temperature before starting the reaction. The exchange was initiated by adding 10 mM each of ATP and $MgCl_2$ and terminated by addition of 0.1 mL of 70% perchloric acid. Organic ^{32}P was determined according to Avron (1960).

Results

Purification of Oligomycin- and DCCD-Sensitive Coupling Factor ATPase from the Chromatophores of R. rubrum

All purification steps were performed at 4 °C.

Step I: Extraction with Triton X-100. Chromatophores (~300 mg of protein) were thawed and suspended in a solution containing, in a final volume of 100 mL, 50 mM Tris-HCl (pH 8), 20% (v/v) glycerol, and 0.3% (v/v) Triton X-100. After incubation in ice for 1 h, the solution was centrifuged in a Beckman 60 Ti rotor at 60 000 rpm for 2 h. About 60–70% of the ATPase activity and 20–30% of the chromatophore protein were recovered in the supernatant after centrifugation (Table I). The rest of the ATPase activity appeared in the pellet, which was discarded. It was possible to solubilize up to 90% of the membranal ATPase by using higher detergent concentrations and inclusion of 0.15 M KCl in the extraction medium. However, under these conditions many other nonspecific proteins were released from the membrane. In-

[2] R. Oren, H. Garty, S. R. Caplan, and Z. Gromet-Elhanan, unpublished experiments.

Table I: Purification of the ATPase Complex from the Chromatophores of *R. rubrum*[a]

preparation	protein (mg)	μmol of P_i released/(mg of protein/min)		yield (%)	
		Mg^{2+}-ATPase	Ca^{2+}-ATPase	Mg^{2+}-ATPase	Ca^{2+}-ATPase
chromatophores	330	0.16	0.07		
Triton extract	100	0.36	0.27	68	117
DEAE-cellulose fractions	17	1.16	0.88	37	65
glycerol gradient fractions	3	1.83	3.33	10	43

[a] Assay of the ATP hydrolysis was performed as described under Experimental Procedure.

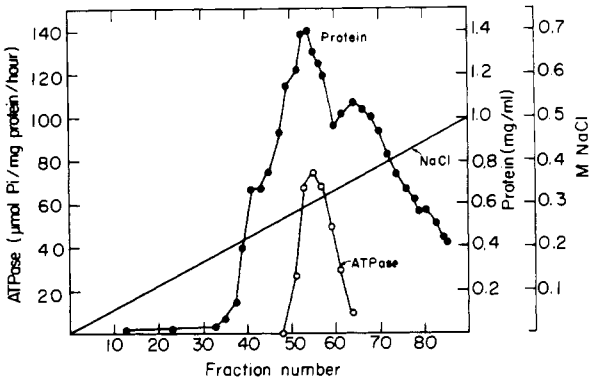

FIGURE 1: Distribution of the ATPase activities in different fractions eluted from the DEAE-cellulose column. DEAE-cellulose chromatography of the Triton extract was performed as described under Results (step II of the purification procedure). Fractions of 1.8 mL were eluted from the column with a NaCl gradient of 0–0.6 M. Assay of the ATPase activities and of protein concentrations in the fractions was performed as described under Experimental Procedure.

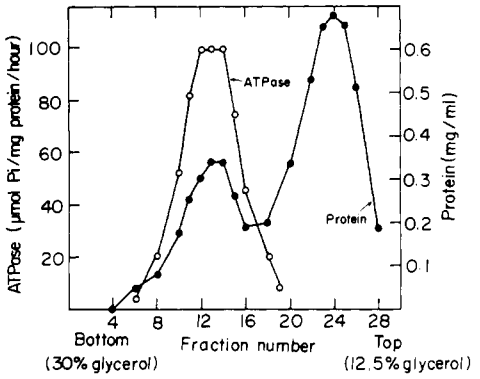

FIGURE 2: Glycerol gradient centrifugation of the ATPase fraction eluted from the DEAE-cellulose column. Fractions of 38 mL active in ATP hydrolysis (containing 1.3 mg of protein/mL), collected from three DEAE-cellulose columns, were concentrated on a XM-300 Diaflo membrane to a protein concentration of 2 mg/mL in a final volume of 16 mL. Four milliliters of the above protein solution was applied on each of four gradients of 12.5–30% glycerol, containing 50 mM Tris-HCl (pH 8), 0.1% Triton X-100, and 5% methanol, in a final volume of 34 mL. The gradients were centrifuged in a SW 27 Spinco rotor for 38 h at 26000 rpm. Fractions of 1.3 mL were collected and assayed for ATPase activity and protein concentration as described under Experimental Procedure.

clusion of 20% glycerol in the extraction medium was necessary in order to prevent considerable (up to 50%) inactivation of the solubilized ATPase.

Step II: DEAE-cellulose Chromatography. The supernatant from step I (80–100 mg of protein) was diluted twofold with 50 mM Tris-HCl (pH 8) and applied to a DEAE-cellulose column (2 × 18 cm) which was preequilibrated with a solution containing 50 mM Tris-HCl (pH 8) and 0.1% Triton X-100. The column was flushed with 100 mL of a solution containing 50 mM Tris-HCl (pH 8), 0.1% Triton X-100, and 10% glycerol. The adsorbed material was eluted with a linear gradient of 0–0.6 M NaCl (100 mL in each chamber) in the same buffer. The peak of ATPase activity was eluted at ~0.3 M NaCl (Figure 1). Fractions with activity over 1 μmol of P_i released/(mg of protein/min) were combined and stored frozen in liquid air until use.

Approximately 20-fold reduction of total protein was achieved in this step, and the specific activity of ATP hydrolysis increased sevenfold (Table I). The presence of 10% glycerol in the washing and eluting buffer was necessary in order to obtain satisfactory yields of the ATPase activity.

Step III: Glycerol Gradient Ultracentrifugation. Active fractions from the DEAE column were thawed and loaded (1.5 mL, containing 1.8 mg of protein in each tube) on the top of a linear gradient of 12.5–30% glycerol in 50 mM Tris-HCl (pH 8), 0.1% Triton X-100, and 5% methanol. The gradients were centrifuged in a SW 41 Spinco rotor for 17 h at 40000 rpm. Fractions with ATPase activity over 1.6 μmol of P_i/(mg of protein/min) were combined and could be stored frozen in liquid air for several months without any considerable loss of activity. For large-scale preparations, active DEAE-cellulose fractions from three columns (~50 mg of protein) were thawed, concentrated two- to threefold on a Diaflo XM-300 membrane, and centrifuged in the glycerol gradients of the above described composition in SW 27 Spinco rotor at 26000 rpm for 38 h. It should be kept in mind, however, that ~30% of the ATPase activity was usually lost on the Diaflo membrane during ultrafiltration.

Results of a typical glycerol gradient centrifugation are shown in Figure 2. Two protein peaks were separated after centrifugation, the minor one coinciding with the peak of ATPase activity in the mid-part of the gradient tube. The broad protein peak collected from the upper part of the gradient was not active in ATP hydrolysis.

After the last purification step there was a 100-fold reduction of total protein and the specific activity of Mg^{2+}-ATPase increased 11-fold, as compared with chromatophores.

As was the case for the previously obtained partially purified ATPase preparation (Oren & Gromet-Elhanan, 1979), considerable activation of Ca^{2+}-ATPase over Mg^{2+}-ATPase was observed in the course of purification of the ATPase complex described here (Table I).

The purified preparation appeared to contain six distinct polypeptides when analyzed on $NaDodSO_4$ gels (Figure 3). The samples were usually concentrated by lyophilization, and the gels were overloaded with protein (note 150 μg of protein in Figure 3). When smaller amounts of protein were applied on the gels (100 μL of the purified preparation without concentration, containing ~30 μg of protein), two very close protein bands could be seen in each of the positions 4 and 5. Therefore, it has been assumed that the total number of different polypeptides in the preparation is eight, as indicated in Figure 3 by inclusion of numbers 4a and 5a. A typical overloaded gel has been nevertheless chosen for presentation here because on such gels the small subunits were stained intensively. Application of 30 μg of protein on the gel results

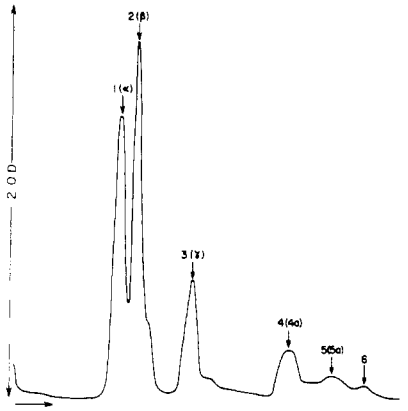

FIGURE 3: $NaDodSO_4$ gel electrophoresis pattern of the purified ATPase complex. Electrophoresis in the presence of $NaDodSO_4$ was performed as described under Experimental Procedure. The purified preparation was concentrated by lyophilization to 2 mg/mL, and 75 μL was applied on the gel. The gel, stained with Coomassie Brilliant Blue, was scanned at 520 nm.

Table II: Oligomycin and DCCD Sensitivity of Mg^{2+}-ATPase Catalyzed by Different Preparations of the *R. rubrum* ATPase Complex[a]

preparation	inhibition (%) oligomycin, 5×10^{-5} M	DCCD, 10^{-4} M
chromatophores	90	95
Triton extract	80	80
DEAE-cellulose fractions	80	40
glycerol gradient fractions	70	35

[a] Oligomycin and DCCD were prepared as 10% solutions in methmethanol. Final concentration of methanol in the reaction mixture for ATP hydrolysis was 0.2% upon addition of energy-transfer inhibitors. Methanol alone added in concentrations up to 1% did not inhibit ATP hydrolysis in any of the ATPase preparations. The ATPase activity was assayed as described under Experimental Procedure.

in less than 2 μg of protein in each of the low molecular weight bands, assuming they are present each in one copy per complex. Such an amount gives quite faint staining on the gels used here (tubes of 7 mm in diameter).

The estimated molecular weights for the three larger subunits of the purified ATPase complex were as follows: 56000 (α), 50000 (β), and 33000 (γ). The molecular weights of the remaining subunits were found to be in the range of 17000–16000, 11000–10000, and 9400 for the subunits 4–4a, 5–5a, and 6, respectively.

Addition of phospholipids to the assay did not increase the specific activity of ATP hydrolysis in the purified preparation. However, when sonicated phospholipids (1 mg/mL) were included in the glycerol gradient buffer (Pick & Racker, 1979), which contained 0.2% Triton X-100 and 50 mM Tris-HCl (pH 8), the yield of the ATPase activity in the preparation increased up to twofold (preliminary experiment).

The ATPase activity was sensitive to both oligomycin and DCCD throughout the purification procedure (Table II). However, as is evident from this table, only 35% inhibition of Mg^{2+}-ATPase by DCCD could be obtained after step III of purification. No stronger inhibition could be achieved when higher DCCD concentrations were used. On the contrary, insensitivity and sometimes slight stimulation of the ATPase were observed with higher DCCD concentrations (up to 2.5×10^{-4} M; data are not shown). Similar effects of DCCD were reported by Ryrie (1977) for the ATPase complex extracted with Triton X-100 from yeast mitochondria and by Serrano et al. (1976) for the ATPase preparation isolated from bovine heart mitochondria by using lysolecithin in the last purification step. Such insensitivity may be caused by Triton X-100 (or by lysolecithin), which might interfere with binding of DCCD to the enzyme. Our data confirm that DCCD may be a poor indicator of the intactness of the energy-converting ATPase complex under some conditions (Ryrie, 1977). While the DCCD sensitivity of Mg^{2+}-ATPase activity of the purified enzyme was rather low, the Ca^{2+}-ATPase of the latter preparation was found to be completely insensitive to DCCD used in concentrations inhibitory for Mg^{2+}-ATPase (not shown).

Table III: [^{32}P]ATP Exchange Catalyzed by Liposomes Containing Purified ATPase Complex Isolated from the Chromatophores of *R. rubrum*[a]

system	nmol of $AT^{32}P$ formed/(mg protein/min)	inhibition (%)
experiment I		
complete	23.3	
10^{-4} M oligomycin	2.8	88
2.4×10^{-4} M DCCD	3	87
5×10^{-5} M FCCP	0.3	98.6
−phospholipids	0	
experiment II		
complete	10.8	
5×10^{-5} M FCCP	0.3	96
100 mM KCl	8.6	20
100 mM KCl plus 10^{-5} M valinomycin	1.4	87

[a] Reconstitution of proteoliposomes and assay of [^{32}P]ATP exchange were performed as described under Experimental Procedure. Energy-transfer inhibitors and uncouplers were added as solutions in methanol. Concentrations of methanol in the reaction mixture, as a result of these additions, did not exceed 0.7%. When added alone in the above indicated concentrations, methanol did not influence the rate of [^{32}P]ATP exchange.

The purified ATPase complex catalyzed [^{32}P]ATP exchange when incorporated into liposomes consisting of soybean phospholipids (Table III). The only successful technique out of several tried by us for reconstitution of active proteoliposomes was the freeze–thaw technique (Kasahara & Hinkle, 1977; Pick & Racker, 1979). Neither cholate dilution nor cholate dialysis techniques (Racker et al., 1975) were successful, probably because of high Triton X-100 concentrations in the purified ATPase preparation. The [^{32}P]ATP exchange was highly sensitive to both oligomycin and DCCD and was completely inhibited by the protonophorous uncoupler of phosphorylation FCCP (Table III). Addition of valinomycin at high potassium concentration also appeared to be inhibitory for the above reaction.

Discussion

A procedure has been described for purification of the oligomycin- and DCCD-sensitive ATPase complex from the chromatophores of the photosynthetic bacterium *R. rubrum*. The latter procedure yielded a highly purified ATPase preparation capable of [^{32}P]ATP exchange in the reconstituted liposomes. The purified ATPase complex dissociated on the $NaDodSO_4$ gels into a maximum of eight different polypeptides. Similar subunit numbers were reported for F_0–F_1 ATPase preparations isolated from the membranes of thermophilic bacterium (Sone et al., 1975) and from spinach chloroplasts (Pick & Racker, 1979). Three larger polypeptides of the ATPase complex described here were identified as α, β, and γ subunits with molecular weights of 56000, 50000,

and 33 000, respectively. The above numbers closely correlate with those reported by Johansson & Baltscheffsky (1975) for the relevant subunits of F_1 ATPase isolated from *R. rubrum* chromatophores. The identities and individual functions of the five smaller subunits of the purified ATPase complex, with molecular weights ranging from 17 000–to 9400, remain to be elucidated.

[^{32}P]ATP exchange in the dark was first demonstrated in the chromatophores of *R. rubrum* by Horio et al. (1965). As in mitochondria and chloroplasts, [^{32}P]ATP exchange seems to be a partial reaction of ATP synthesis in the chromatophores [see Gromet-Elhanan (1977) for review]. It has been shown here that purified ATPase complex, upon incorporation into liposomes, is capable of carrying out [^{32}P]ATP exchange, which is highly sensitive to energy-transfer inhibitors as well as to the protonophorous uncoupler FCCP (Table III). This seems to indicate that purified ATPase complex contains a functional energy-coupling device (F_0 subunits).

In the chromatophores a comparatively large light-induced electric transmembrane potential has been observed, which seems to be closely linked with the terminal steps of photophosphorylation [for review, see Gromet-Elhanan (1977)]. The inhibition of the [^{32}P]ATP exchange in the reconstituted proteoliposomes by valinocymin plus K^+, described here (see Table III), appears to be in line with these findings. Similar action of valinomycin on the [^{32}P]ATP exchange reaction has also been observed in the vesicles reconstituted from phospholipids and ATPase complex isolated from the membranes of thermophilic bacterium (Sone et al., 1975). However, valinomycin plus K^+ was reported to have stimulated twofold the [^{32}P]ATP exchange in the phospholipid vesicles which contained ATPase complex isolated from yeast mitochondria (Ryrie, 1975b). These differences in response to valinomycin may reflect variable relative contributions of the electric potential and of the pH gradient to the transmembrane electrochemical proton gradient in different energy-transducing membranes.

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