

Coupling Factor Adenosine-5'-triphosphatase from *Rhodospirillum rubrum*: A Simple and Rapid Procedure for Its Purification

Friedrich-Karl Lücke and Jobst-Heinrich Klemme

Institut für Mikrobiologie der Universität Bonn

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When photosynthetic membranes from *Rhodospirillum rubrum*, devoid of loosely bound small molecules and proteins, were passed through a French-pressure cell, the enzyme adenosine-5'-triphosphatase (EC 3.6.1.3.) (ATPase) was released into the soluble fraction.

The solubilized ATPase was purified to homogeneity. In many respects it behaved like the enzyme purified by other workers, but it also hydrolyzed Mg-ATP with a small, but significant rate. Furthermore, it was much more stable.

Maximal restoration of photophosphorylation in ATPase-depleted membranes was achieved by addition of about 1 mg purified ATPase per mg bacteriochlorophyll. For reconstitution of NAD^+ -photoreduction, about half of this amount was saturating.

Introduction

The function of the membrane-bound ATPase in bacterial photosynthesis has been studied during the last few years in various laboratories^{1–4}. Recently, the ATPase from *Rhodospirillum rubrum* membranes was purified to homogeneity^{5, 6}, but the pure enzyme turned out to be unstable, and a rather harsh treatment was employed to solubilize the enzyme. Furthermore, the purification was rather laborious.

Quite different values for the specific ATPase activity in *Rhodospirillum rubrum* photosynthetic membranes can be found in the literature, ranging from 17 (ref. 7) to 200 (ref. 8) $\mu\text{mol/h} \cdot \text{mg}$ BChl under comparable assay conditions. These findings led us to reinvestigate some structural and kinetic properties of the membrane bound and solubilized ATPase of this organism. In this communication, we wish to report on a simple preparation procedure yielding pure and stable coupling factor ATPase. The electrophoretically homogeneous enzyme reconstitutes cyclic photophosphorylation and NAD^+ -photoreduction in depleted membranes.

Requests for reprints should be sent to Prof. Dr. J.-H. Klemme, Institut für Mikrobiologie, Meckenheimer Allee 168, D-5300 Bonn 1.

Abbreviations: BChl, bacteriochlorophyll a; FCCP, carbonyl-cyanide-*p*-trifluoromethoxyphenylhydrazone; ATPase, adenosine-5'-triphosphatase.

Materials and Methods

Growth of bacteria

Rhodospirillum rubrum S1 (originally obtained from Dr. H. Gest, Dept. of Microbiology, Indiana University, Bloomington, Ind.) was grown anaerobically at 2000 lx illumination (100 W tungsten lamps) and 30 °C in completely filled, flat screw-capped bottles with a capacity of about 500 ml. The medium contained 30 mM sodium malate, 7.6 mM $(\text{NH}_4)_2\text{SO}_4$, 30 μg biotin/l and mineral salts as specified in ref. 9. The cultures were started with 3% (v/v) inoculum and were grown for about 32 hours until the late exponential phase (optical density at 660 nm about 1.9; final pH about 7.9). The cells were harvested by centrifugation ($10\,000 \times g$, 15 min) at 4 °C, washed once in 50 mM Tris-HCl, pH 8.0, containing 1 mM MgCl_2 , and resuspended in 50 mM glycylglycine-NaOH, pH 7.5, plus 1 mM MgCl_2 , at about 1 g wet weight per 4 ml. They could be stored at –18 °C for at least two months.

Preparations of membranes and purified ATPase

Details of the purification procedure are described below (see "Results"). All purification steps were performed at 0–5 °C. The purity of the enzyme preparations was checked by electrophoresis on 5% polyacrylamide gels. The alkaline system No. 1¹⁰ was used without stacking gel. Electrophoresis was run at 4 mA per gel in a Desaga apparatus (Desaga, Heidelberg; no. 72207); gels were stained with 1% amido black 10B in 7% acetic acid for 20 min and destained with 5% acetic acid.

Assay of ATPase

ATPase was assayed in the presence of 5 mM ATP (Na-salt), 100 mM Tris-HCl, pH 8.0, and 2.5 mM MgCl_2 ("Mg-ATPase") or 10 mM CaCl_2 ("Ca-ATPase"). The reaction was initiated by addition of ATP and terminated after 5 min at 30 °C with an equal volume of ice-cold 0.6 M trichloroacetic acid. Denatured protein was centrifuged off, and liberated inorganic phosphate was determined colorimetrically¹¹. For determination of ADP, the reaction was stopped by addition of an equal volume of 0.6 M HClO_4 , and ADP in the neutralized supernatant was assayed spectrophotometrically by following the NADH-oxidation at 366 nm in a reaction mixture containing 40 mM Tris-HCl, pH 7.2, 10 mM MgCl_2 , 50 mM KCl, 1 mM phosphoenolpyruvate, 0.3 mM NADH, and 6 units each of pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27). The rate of ATP-hydrolysis was proportional to protein concentration and remained constant until about 0.3 $\mu\text{mol P}_i$ per ml were liberated.

Photophosphorylation and NAD^+ -photoreduction

The reactions were carried out at 30 °C and 4000 lx in cuvettes under nitrogen. The increase in absorbance at 366 nm was read every two minutes in an Eppendorf-spectrophotometer. Dark control rates were subtracted. The reactions were initiated by addition of the membranes or by switching on the light.

ATP-formation (anaerobically in the presence of small amounts of succinate) was coupled to NADP^+ -reduction by addition of glucose, hexokinase (EC 2.7.1.2) and glucose-6-phosphate-dehydrogenase (EC 1.1.1.49)¹². The assay mixture contained 67 mM Tris-HCl, pH 8.0, 12 mM MgCl_2 , 2 mM ADP, 5 mM NaH_2PO_4 , 0.3 mM Na-succinate, 20 mM glucose, 0.5 mM NADP^+ , 12 units each of hexokinase and glucose-6-phosphate-dehydrogenase, 10–20 μg BChl and water to make 3 ml (*cf.* ref. 8). In early experiments, NADP^+ and glucose-6-phosphate-dehydrogenase were omitted, and the glucose-6-phosphate formed was determined after stopping the reaction with HClO_4 . Both methods gave identical results. Desalting of the auxiliary enzymes proved to be unnecessary.

The reaction mixture for NAD^+ -photoreduction contained 40 mM Tris-HCl, pH 8.0, 2 mM MgCl_2 , 1.5 mM NAD^+ , 4 mM Na-succinate, 2.4 mg bovine serum albumine, 30–60 μg BChl and water to make 3 ml (see ref. 13). Omission of bovine serum albumine gave only 20% of the original activity.

Bacteriochlorophyll (BChl) was determined using the *in vivo* extinction coefficient of 140 mM $^{-1}\text{cm}^{-1}$

(ref. 14). Protein was assayed by the Lowry method¹⁵ (using bovine serum albumine as the standard) after precipitation with trichloroacetic acid. Membrane proteins were subsequently incubated in 0.5 N NaOH at 60 °C for 1 hour before addition of the reagents (*cf.* ref. 16).

Chemicals

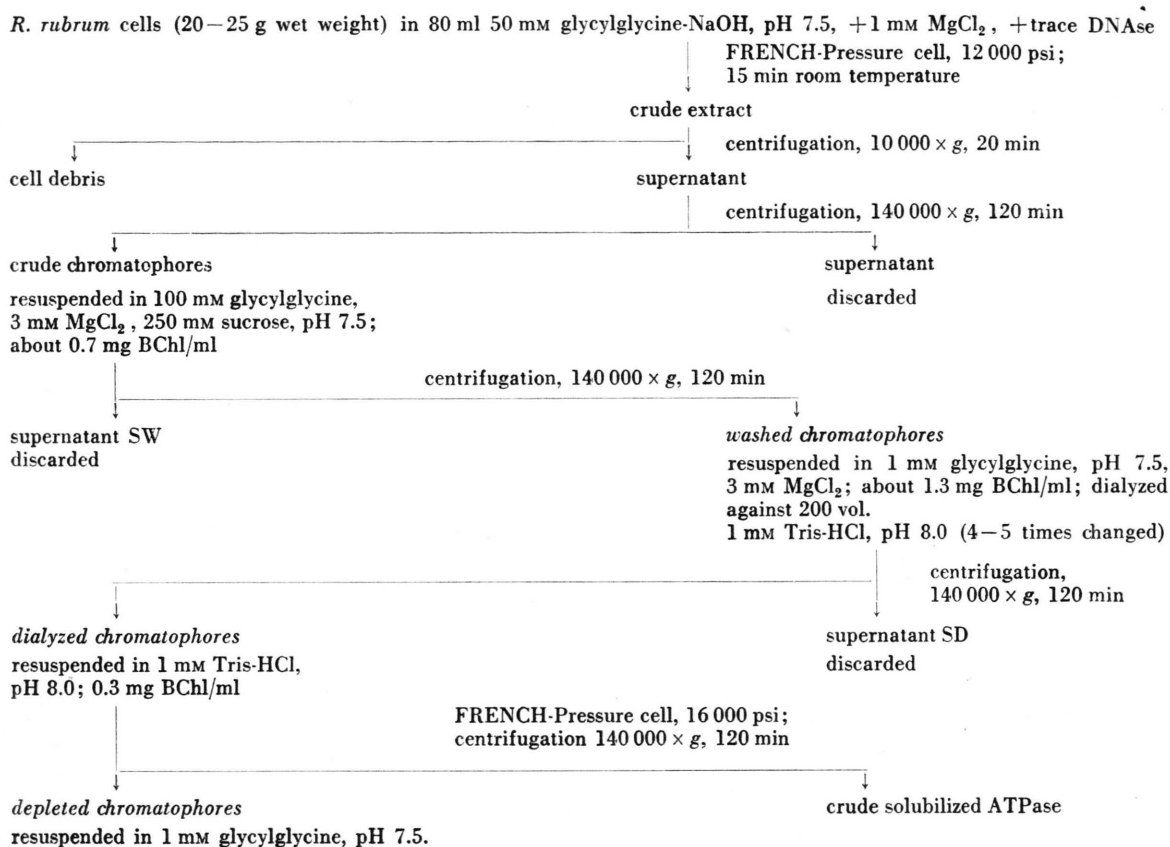
Biochemicals, enzymes, and FCCP were from Boehringer AG, Mannheim; oligomycin (components A:B:C = 60:30:10), bovine serum albumine, and chemicals for gel electrophoresis were from Serva, Heidelberg; dicyclohexylcarbodiimide was from Fluka, Buchs (Switzerland), and Sepharose 6B from Deutsche Pharmacia, Frankfurt. Other chemicals (reagent grade) were from Merck AG, Darmstadt.

Results

Preparation of the ATPase

Most bacterial membrane ATPases can be solubilized by extensive washing of the membranes with Mg^{2+} -free buffer of low ionic strength (see ref. 17 for review). However, when the photosynthetic membranes from *Rhodospirillum rubrum* were washed twice with 50 mM, and subsequently five times with 1 mM Tris-HCl, pH 8.0, no significant ATPase activity appeared in the supernatants after high-speed centrifugation. Addition of 1 mM EDTA or 1 mM ATP to the washing buffer or an osmotic shock treatment¹⁸ did not solubilize the enzyme either. Furthermore, there was no increase in the total ATPase activity and no significant loss of its sensitivity to inhibition by oligomycin and activation by the uncoupler FCCP. This indicates that neither some "masking" substances nor some other "coupling factors" were removed by these treatments. When these washed membranes were passed through an Aminco French-pressure cell at 16000 psi, the ATPase was partially solubilized. Washing of the membranes with dilute buffer could be replaced by dialysis against the same buffer. Scheme 1 summarizes the procedure finally used to solubilize the ATPase from the photosynthetic membranes. The membrane preparations could be stored for two weeks at 0 °C or for longer time at –18 °C in the presence of 50% glycerol.

For further purification of the enzyme, the solubilisate was made 40 mM in Tris-HCl, pH 7.5, and then fractionated with solid ammonium sulfate. The fraction precipitating between 30 and 60% satura-

Scheme 1. Preparation of ATPase-depleted membranes and crude solubilized ATPase from *Rhodospirillum rubrum*.

tion was dissolved in 6 ml of 50 mM Tris-HCl, pH 8.0, containing 1 mM ATP and 0.5 mM EDTA, centrifuged ($140\,000 \times g$, 60 min) to remove insoluble material, and then applied to a Sepharose 6 B column (2.5×65 cm) equilibrated with the same buffer. Fractions of 3.1 ml were collected and those which showed a constant, high specific Ca-ATPase activity ($12-13 \mu\text{mol}/\text{min} \cdot \text{mg}$ protein) were pooled. The enzyme was eluted at the 1.7-fold void volume (determined with dextran blue). If compared with the elution volumina of ferritin, catalase and lactate dehydrogenase, this value indicated a molecular weight of the ATPase of about 360 000 (see also ref. 5). It was precipitated with ammonium sulfate (60% saturation) and dissolved again in a small volume of 50 mM glycylglycine, pH 7.5, containing 1 mM ATP and 0.5 mM EDTA. Desalting of this preparation proved to be unnecessary for the experiments described; in the text, it is referred to as "purified ATPase".

Electrophoresis on 5% polyacrylamide gels showed that the solubilisation procedure applied was rather selective for the ATPase. When the crude solubilisate ($44 \mu\text{g}$ protein) was applied to the gel only a small amount of material which had not or only scarcely entered the gel, and the band showing ATPase activity could be detected — visually or densitometrically — after staining. The purified enzyme displayed only one band in the gel when up to $82 \mu\text{g}$ protein were applied. In no case was any heterogeneity of ATPase activity observed, judged from the distribution of the "activity stain"¹⁹ in polyacrylamide gels and from the elution behaviour during Sepharose 6 B gel filtration.

A representative purification protocol is given in Table I. It should be noted that about 2.3 units of Ca-ATPase appear in the solubilisate for each unit Mg-ATPase removed, and that there is no further increase of the total units during the purification of the solubilized enzyme. The considerable

Table I. Purification of the coupling factor ATPase. One unit is defined as 1 μmol P_i liberated per min. *Mg-ATPase*: 5 mM ATP, 2.5 mM MgCl_2 . *Ca-ATPase*: 5 mM ATP, 10 mM CaCl_2 . Volumes indicated were corrected for aliquots taken. For designation of fractions, see Scheme 1.

Fraction	Volume [ml]	Total-protein [mg]	Total-BChl [mg]	Mg-ATPase		Ca-ATPase	
				[units/mg protein]	Total	[units/mg protein]	Total
washed chromatophores	28.7	1004	31.9	0.143	143.4	0.094	94.4
dialyzed chromatophores	24.0	722	27.8	0.187	134.7	not determined	
depleted chromatophores	29.6	604	25.0	0.063	37.9		
crude solubilisate	73.0	55	0.84	0.36	19.8	4.13	226.4
30–60% ammonium sulfate saturation, centrifuged	5.5	18	0.001	0.65	11.7	7.42	132.5
Sephacrose 6 B fractions no. 53–61	28.8	8.35	0	0.85	7.1	12.2	103.0
fractions 53–61 concentrated with ammonium sulfate	2.0	5.7	0	0.77	4.4	12.5	71.2

loss of activity during ammonium sulfate fractionation is probably due to reassociation with membrane fragments or phospholipids present in the crude solubilisate, since the purified enzyme is not affected by ammonium sulfate.

Properties of the purified enzyme

In the presence of 10 mM CaCl_2 , the purified ATPase showed a specific activity of about 13 μmol $\text{P}_i/\text{min}\cdot\text{mg}$ protein, and a K_m for Ca-ATP of 1.4 mM. As also found for the membrane-bound ATPase, equimolar amounts of ADP and phosphate were formed. Contrary to the membrane-bound enzyme, the solubilized ATPase was not inhibited by oligomycin or phospholipase A treatment^{5,8} and only slightly affected by dicyclohexylcarbodiimide [DCCD] (88% of the control activity in the presence of 50 μM DCCD). In contrast to previous findings⁵, Mg-ATP is also hydrolyzed, but with only 6–7% of the rate obtained with Ca^{2+} as divalent cation. The concentrations of the two cations which were optimal for activity are similar for the solubilized and the membrane-bound ATPase: Free Mg^{2+} ions apparently inhibit the ATPase in both states (Fig. 1). Like the Ca-ATPase, the Mg-ATPase activity of the purified enzyme was not inhibited by oligomycin and phospholipase A treatment. The substrate saturation curve for Mg-ATP, however, did not follow Michaelis-Menten-kinetics while the curve for Ca-ATP did (data not shown). If both

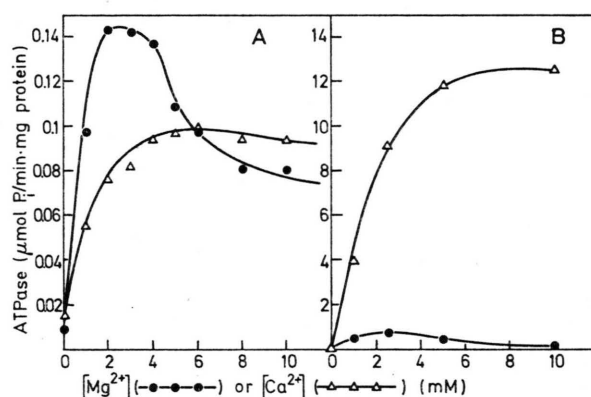


Fig. 1. Dependence of *Rhodospirillum rubrum* ATPase on divalent metal cations. A, Photosynthetic membranes (10 μg BChl). B, Purified ATPase (3 μg [Δ] and 55 μg [$\bullet$] protein, respectively). ATP-concentration was 5 mM.

Mg^{2+} and Ca^{2+} were present in the assay mixture, ATP-hydrolysis was inhibited (about 85% inhibition with 10 mM CaCl_2 plus 1 mM MgCl_2) in a similar fashion to that already reported⁵.

The purified enzyme was remarkably stable: Storage of the precipitate obtained by addition of an equal volume of saturated ammonium sulfate solution for 6 months at 4 °C resulted in the loss of only about 10% of the Ca-ATPase and recoupling activity. When this aged preparation was subjected to polyacrylamide gel electrophoresis, no faster moving protein band could be detected (cf. ref. 6).

Table II. Reconstitution of photophosphorylation and NAD^+ -photoreduction in different preparations. Activities are expressed as $\mu\text{mol/h} \cdot \text{mg BChl}$. A saturating amount of purified ATPase or 20 μg oligomycin per 3 ml were added, where indicated. For designation of fractions, see Scheme 1. n.d., not determined.

Activity tested	Photophosphorylation			NAD^+ -photoreduction			Mg-ATPase		
	1	2	3	1	2	3	1	2	3
Preparation — No.									
dialyzed chromatophores	249	327	275	12.9	26.9	40.0	286	336	347
depleted chromatophores	56	97	12.5	0.0	20.9	4.4	91	222	129
depleted chromatophores + purified ATPase	185	188	130	3.1	26.0	13.2	n.d.	n.d.	356
depleted chromatophores + oligomycin	1	n.d.	<0.5	4.1	26.8	14.7	n.d.	n.d.	19

Reconstitution experiments

As a measure for the rebinding of the coupling factor ATPase to the membranes, reconstitution of photophosphorylation and of Mg-ATPase are usually assayed, whose dependence on the presence of this enzyme is obvious^{3, 5}. The photoreduction of NAD^+ in *Rhodospirillum rubrum* is also dependent on the presence of the coupling factor ATPase, but its role seems to be different since oligomycin is able to — at least partially — replace it². Therefore, reconstitution of NAD^+ -photoreduction was also analyzed. Preincubation of membranes and purified ATPase in the presence of 20 mM MgCl_2 gave poor reconstitution of the activities tested. However, satisfactory results were obtained when membranes and ATPase were incubated together in the complete assay mixture for ten minutes in the dark. Therefore, in the experiments described in this section, the assay mixtures were routinely preincubated in the dark for 20 min at 30 °C before

starting the reaction by switching on the light or (if the reconstituted Mg-ATPase activity was to be tested) by addition of ATP.

Table II shows the extent of reconstitution of photophosphorylation and NAD^+ -photoreduction obtained with different preparations of ATPase and ATPase-depleted membranes. If the membranes had lost less than 50% of their original Mg-ATPase activity (as in preparation 2), NAD^+ -photoreduction was only slightly affected. This is in good agreement with the data shown in Fig. 2: While maximal restoration of photophosphorylation was achieved on the addition of about 1 mg purified ATPase per mg BChl, about 0.5 mg/mg BChl were sufficient for NAD^+ -photoreduction. The “theoretical equivalence point” indicated in Fig. 2 was calculated by dividing the total Ca-ATPase activity in the crude solubilisate by the specific activity of the purified enzyme and relating the obtained value to the BChl-content of the dialyzed membranes.

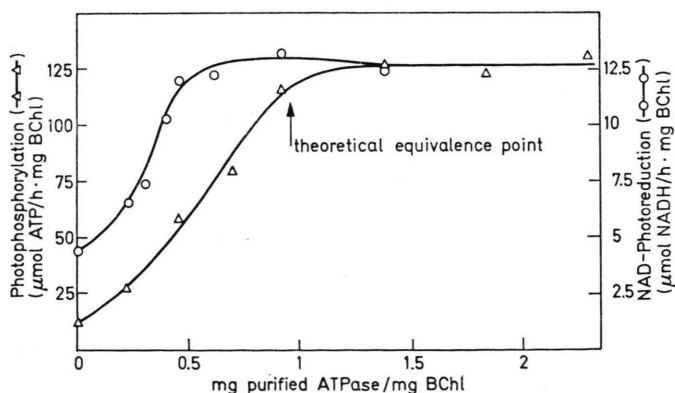


Fig. 2. Reconstitution of photophosphorylation (Δ) and NAD^+ -photoreduction ($\circ$) in ATPase-deficient membranes by addition of varied amounts of purified ATPase. The assay mixtures contained 9.3 μg BChl for photophosphorylation and 37–55 μg BChl for NAD^+ -photoreduction. Activities in the dialyzed membranes were 275 $\mu\text{mol ATP/h} \cdot \text{mg BChl}$ and 40 $\mu\text{mol NADH/h} \cdot \text{mg BChl}$.

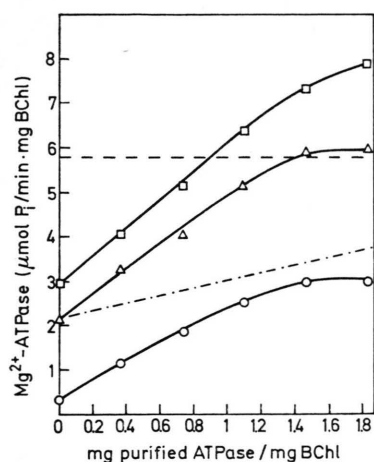


Fig. 3. Reconstitution of Mg^{2+} -dependent ATPase activity in depleted membranes by addition of varied amounts of purified ATPase. The assay mixture (1 ml) contained $9.3 \mu g$ BChl. After preincubation (20 min at $30^\circ C$) in the presence of $2.5 \text{ mM } MgCl_2$, the reaction was started with 5 mM ATP .

$\triangle$ — $\triangle$, $+0.01 \text{ ml ethanol}$ (control);
 $\circ$ — $\circ$, $+10 \mu g$ oligomycin;
 $\square$ — $\square$, $+2 \text{ nmol FCCP}$;
 — — —, activity of the dialyzed membranes;
 - · - · -, theoretical activity calculated by addition of Mg -ATPase activity of depleted membranes and of purified enzyme present in the respective assay mixture.

Fig. 3 shows the result of an experiment aimed at restoring the "energy transducing" (that is, oligomycin-sensitive and uncoupler-stimulated) Mg^{2+} -dependent ATPase activity of the membranes. It can be seen that the Mg -ATPase was fully restored by an amount of purified enzyme comparable to that applied for photophosphorylation. This activity, however, was only partially (up to about 50%) inhibited by $10 \mu g$ oligomycin per ml, and was stimulated — as was the residual Mg -ATP activity of the depleted membranes — only about 1.3-fold by $2 \mu M$ FCCP. Similar results (not shown) were obtained with $10 \text{ mM } MgCl_2$ (instead of 2.5 mM) although in this case, there was 1.8-fold stimulation by FCCP. It should be pointed out that in this preparation (No. 3 in Table II) only about 50% of the original photophosphorylation rate (completely sensitive to oligomycin) could be restored by a saturating amount of purified ATPase.

If more than 50% of the membrane-bound ATPase are removed by solubilization, the relative extent of reconstitution of NAD^+ -photoreduction by purified ATPase or oligomycin can be obtained

Table III. Photoreduction of NAD^+ by *Rhodospirillum rubrum* chromatophores. Purified ATPase was added in saturating amounts (1.15 mg/mg BChl). The concentration of oligomycin was $20 \mu g$ per 3 ml or about $0.5 \text{ mg per mg BChl}$. For designation of fractions, see Scheme 1. n.d., not determined.

Fraction	Additions	$\mu\text{mol NADH/}$ $\text{h} \cdot \text{mg BChl}$	
		Minus oligo- mycin	Plus oligo- mycin
dialyzed chromatophores	none	40	40
dialyzed chromatophores	$3.7 \mu M$ FCCP	0	0
depleted chromatophores	none	4.4	14.7
depleted chromatophores	purified ATPase	13.2	14.7
depleted chromatophores	boiled ATPase	4.0	n.d.

with good reproducibility and in good agreement with ref. 2 although the absolute values differ (Table II). As reported by Jones and Vernon⁷, the activity was sensitive to the uncoupler FCCP. De-natured ATPase was ineffective (Table III).

Some attempts were made to improve the extent of reconstitution of NAD^+ -photoreduction. Replacing the Tris buffer by 40 mM glycylglycine, pH 7.5, increasing the Mg^{2+} concentration to 5 mM , inclusion of 250 mM sucrose into the assay mixture, or use of an equivalent amount of the crude solubilisate instead of the purified enzyme did not result in a higher extent of reconstitution.

Discussion

Contrary to the majority of chemotrophic bacteria¹⁷ and to the phototrophic sulfur bacterium *Chromatium D*²⁰, the nonsulfur purple bacteria studied so far contain an oligomycin-sensitive membrane-bound ATPase which can not be solubilized by simply washing the membranes with buffer of low ionic strength^{1, 4, 5, 21}. The purification method described in this paper takes advantage of the fact that the ATPase is rather tightly bound to the photosynthetic membranes. The enzyme can be selectively released from the membranes by a mechanical treatment with the French pressure cell (*cf.* ref. 21)

after the loosely bound proteins have been removed by extensive washing with diluted, Mg^{2+} -free buffer. The solubilization procedure increases the total ATPase activity by a factor of about 2.5 (provided the enzyme is assayed under optimal conditions, *i.e.* in the presence of 5 mM ATP and 10 mM Ca^{2+} or 2.5 mM Mg^{2+} , respectively; compare Fig. 1). A similar increase in total activity is observed if the membrane-bound Mg-ATPase is tested in the presence of a saturating amount of uncoupler (2 μ M FCCP). Contrary to the findings of Johansson *et al.*^{5,22}, the results of the present paper do not provide any evidence for the presence of a protein which inhibits or "masks" ATPase activity. However, since Johansson *et al.*⁵ also reported a considerably lower specific Mg-ATPase activity of the membranes, the existence of such "masking" protein(s) can not be excluded. This problem is now under investigation in our laboratory.

From the specific activity of the membrane-bound Mg-ATPase in the presence of uncoupler, and of the purified Ca-ATPase, one can calculate a ratio of 1 mol ATPase per 390 mol BChl (assuming molecular weights of 360,000 and 911, respectively) in our preparation. For *Rhodopseudomonas sphaeroides* chromatophores, a ratio of 1 : 300 was calculated⁴.

The purified *Rhodospirillum rubrum* ATPase hydrolyzed Mg-ATP with about 6% of the rate obtained with Ca-ATP. The hydrolysis of Mg-ATP by an enzyme solubilized from *Rhodospirillum rubrum* membranes has been reported by Konings and Guillory²³; the rates published by these authors are very similar to ours.

The purified enzyme was active in restoring photophosphorylation in "depleted" membranes up to 75% of the original activity (Table II). The failure to reconstitute the photophosphorylation capacity completely could be due to a partial loss of a component comparable to the mitochondrial "oligomycin sensitivity conferring protein"²⁴. This conclusion was based firstly on the fact that oligomycin sensitive Mg-ATPase could only be restored up to values of 50–60% of the original activity, and secondly on the fact that the restored Mg-ATPase was only weakly stimulated by FCCP (Fig. 3).

The results of our experiments on the NAD^{+} -photoreduction were comparable to those reported

by Gromet-Elhanan², although the latter author used a less well defined coupling factor preparation and membranes depleted of ATPase-activity by treatment with 2 M LiCl. Thus, it seems unlikely that the incomplete restoration of the photophosphorylation and NAD^{+} -photoreduction activities is due to the loss of components of the electron transport system or unfavorable conditions for rebinding. Instead, removal of more than 50% of the coupling factor ATPase seems to cause some alterations of the structure and/or conformation of membrane components that can only partially be reversed by readdition of the enzyme or oligomycin.

The evidence is overwhelming now that in *Rhodospirillum rubrum*, NAD^{+} is reduced by succinate via a "reversed electron flow"⁷ driven by the "high-energy intermediate" which — in terms of Mitchells²⁵ chemiosmotic hypothesis — is a "proton-motive force" (proton gradient plus membrane potential). In chloroplasts, removal of the coupling factor ATPase results in a strong increase of the permeability of the membrane to protons; dicyclohexylcarbodiimide — which acts in an analogous manner as oligomycin — reverses this effect²⁶. In mitochondria^{24,27} and in *Escherichia coli* membranes²⁸, such a dual (catalytical and structural) function of the coupling factor ATPase has also been demonstrated. The inhibition of the NAD^{+} -photoreduction in *Rhodospirillum rubrum* chromatophores by removal of the ATPase could therefore be due to a similar mechanism. The "ATPase-depleted membranes" described in this paper seem to have a somewhat increased proton permeability, judged from the weak stimulation by uncouplers of their residual Mg-ATPase. However, attempts to measure proton gradients and electrical potentials across native and "depleted" membranes of photosynthetic bacteria failed to show such a "structural role" for the coupling factor ATPase^{1,2,4}. Thus, it is not yet clear why its removal from the membranes causes such a severe inhibition of NAD^{+} -photoreduction.

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