Purification, Subunit Structure, and Kinetics of the Chloroform-Released F_1ATPase Complex from *Rhodospirillum rubrum* and Its Comparison with F_1ATPase Forms Isolated by Other Methods

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A stable and homogeneous adenosine-5′-triphosphatase (ATPase, EC 3.6.1.3) has been solubilized from *Rhodospirillum rubrum (R. rubrum)* chromatophores by chloroform extraction. Purification of the Ca^{2+}-dependent ATPase activity was 200-fold. Ca^{2+} can be replaced by Mg^{2+}, Cd^{2+}, and Mn^{2+}. The K_m for Ca-ATP (0.17 mM) is increased about 5-fold during solubilization of the enzyme, whereas the K_m values for Mg-ATP (0.029 mM) and Cd-ATP (0.014 mM) are not affected.

The chloroform-released ATPase has a molecular weight of $400{,}000 \pm 30{,}000$ and consists of the following subunits (molecular weights in parenthesis): α(58,000), β(53,500), γ(39,000), δ(18,500), and ε(14,000). The amino acid composition and the fluorescence spectra are presented.

Besides the chloroform-released ATPase complex three other Ca^{2+}-dependent ATPase forms have been isolated from *R. rubrum* chromatophores by other methods for comparison. Ultrasonication of the membranes leads to the release of an ATPase complex which is mainly composed of α, β, and γ-subunits. From an acetone powder extract an ATPase complex could be purified by affinity chromatography which is composed of four kinds of subunits (α, β, γ, δ). The same acetone powder yields an ATPase consisting of only three different types of subunits (α, β, γ) if the final purification step is preparative disc electrophoresis on 6% polyacrylamide gels instead of affinity chromatography.

Introduction

Coupling factor ATPases from mitochondria, chloroplasts, and bacteria exhibit many similarities, in particular with respect to catalytic function, subunit structure and molecular weight. The enzymes may be released by a variety of techniques. The catalytic properties of the membrane-bound ATPase complexes are affected in various ways by the solubilization procedure (for review see refs. [1, 2]).

For the photosynthetic bacterium *Rhodospirillum rubrum* changes in bivalent cation specificities have been observed after solubilization [3–6].

In the present study we describe the preparation of a purified ATPase complex from *R. rubrum* which appears to be closely related to the membrane-bound enzyme with respect to its bivalent cation specificity. It will also be demonstrated by comparative investigations how subunit composition and kinetic properties of this coupling factor ATPase are affected by the kind of solubilization and purification technique applied.

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+ Herrn Professor Dr. Henry Albers zum 75. Geburtstag gewidmet.

Materials and Methods

Reagents

Sepharose CL-6B was obtained from Deutsche Pharmacia, Freiburg. The reagents for electrophoresis were purchased from Serva, Heidelberg, and protein standards were from Boehringer, Mannheim. All other chemicals used in this study were of analytical grade purchased from E. Merck, Darmstadt.

Measurement of ATPase activity

ATPase activity was assayed continuously at 37 °C by measuring the liberated inorganic phosphate according to Arnold *et al.* [7]. One unit (U) of ATPase activity is defined as the number of μmol of inorganic phosphate liberated during 1 min. Protein concentration was estimated by the Lowry method [8].

Bacteriochlorophyll determination

Bacteriochlorophyll concentration was estimated by measuring the absorbance of the chromatophores at 880 nm. The extinction coefficient of Clayton [9] was used.

Analytical disc gel electrophoresis

Analytical disc gel electrophoresis was carried out in 5% polyacrylamide gels according to system 1a described by Maurer [10]. Dissociation into subunits was performed at 100 °C for 1 min in the presence of 2% sodium dodecyl sulfate and 5% β-mercaptoethanol.

Sodium dodecyl sulfate disc gel electrophoresis was carried out in 12% polyacrylamide gels containing 0.1% sodium dodecyl sulfate according to Laemmli [11]. Protein bands were detected by staining with Coomassie brilliant blue G 250. The gels were scanned at 578 nm in an Eppendorf photometer equipped with a gel cuvette. Molecular weights were estimated on calibrated gels using bovine serum albumin, lactat dehydrogenase, trypsin inhibitor, myoglobin, and cytochrom c as standards.

Preparative disc gel electrophoresis

Preparative disc gel electrophoresis was carried out in an apparatus from Shandon (Camberley, Great Britain). The gels, containing 6% acrylamide and 0.57% N,N′-methylenebisacrylamide were polymerized with 0.08% N,N,N′,N′-tetramethylethylendiamine and 0.1% ammonium peroxydisulfate in 0.28 M Tris-HCl buffer at pH 8.7. The length of the separating gel was 6 cm. Sample gels were not used. Tris-glycine (6.06/4 g/l), pH 8.8, was used as upper gel buffer. The lower gel buffer contained 0.4 M Tris-HCl, pH 8.0, and elution was carried out with 0.1 M Tris-HCl at pH 8.0. Electrophoresis was run at 300 V/45 mA for 6–10 h at 4–7 °C.

Absorption and fluorescence spectra

Absorption spectra were obtained by means of a Cary spectrophotometer, model 15.

Fluorescence measurements were carried out with a Perkin Elmer spectrofluorimeter, model MPF-4A, at room temperature. The emission spectra were corrected for the wavelength dependent response of the instrument. The samples were diluted to 5 μg/ml to prevent self-absorption of the fluorescence light.

Amino acid analysis

Amino acid analysis was performed after acidic hydrolysis (6 N HCl, 110 °C, 24 h under vacuum) with a Biotronik BT 6000 amino acid analyzer (München, BRD), controlled by a computer programming system [12]. A micro bore column 0.6 × 25 cm containing Durrum DC-4A resin was used. Tryptophan was determined after basic hydrolysis according to Drèze and Reith [13, 14] with the same amino acid analyzer.

Preparation of crude ATPase

Chromatophores from *R. rubrum*, strain FR1 (DSM-No. 1068), were prepared according to a published procedure [15].

A crude ATPase activity has been released from the chromatophores by chloroform treatment. The present method is a modification of the chloroform-extraction procedure of mitochondrial ATPase by Beechey *et al.* [16]. Our procedure is related to the method of Webster *et al.* [6].

The chromatophores were washed in 10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and resuspended in the same buffer to obtain a final bacteriochlorophyll concentration of 0.2 mM. The suspension was vigorously mixed with 0.5 × vol. of added analytical grade chloroform for 30 s at room temperature and centrifuged at low speed to seperate the two phases. The aqueous layer thus obtained was centrifuged at 24 °C for 75 min at 90,000 × *g*. The clear supernatant was dialyzed against 30 × vol. of 45 mM Tris-HCl, pH 7.5, containing 10% glycerol (v/v) for 24 h at 4 °C to remove residual chloroform.

Purification of chloroform-released ATPase

The crude enzyme was subjected to fractionated ammonium sulfate precipitation. The precipitate obtained at 30% to 60% ammonium sulfate saturation was resuspended in 45 mM Tris-HCl, pH 7.5, containing 10% glycerol (v/v) and dialyzed against 2 l of the same buffer for 18 h at 4 °C. Homogeneity of the ATPase was achieved by gel filtration on a Sepharose CL-6B column (2.5 × 100 cm), equilibrated with 50 mM Tris-HCl, pH 7.5. 4-ml fractions

were collected and the 4 to 6 tubes containing about 60% of the eluted ATPase activity were combined. All purification steps were carried out at 4 °C.

Preparation of ATPase by acetone powder extraction and sonication

Alternative preparations of ATPase complexes from *R. rubrum* chromatophores were carried out by acetone powder extraction [3] and ultrasonication [17]. The crude ATPase fractions were further purified by ammonium sulfate precipitation, gel filtration, and affinity chromatography as described earlier [18, 19]. In some experiments the affinity chromatography was replaced by preparative disc gel electrophoresis as final purification step.

Table I. Release of ATPase activity from *R. rubrum* chromatophores by treatment with various organic solvents. The conditions were the same as described for chloroform treatment in Materials and Methods.

Organic solvent	Total ATPase activity [U]	Specific ATPase activity [U/mg]
chloroform	12.0	3.2
diethyl ether	26.9	2.4
n-butyl acetate	13.4	1.8
ethyl acetate	12.8	1.9
carbon tetrachloride	13.2	1.4
benzol	10.4	0.9
isoamylethanol	6.7	0.4
n-butanol	0.1	0.0
1-propanol	0.0	0.0
n-pentane	0.0	0.0
n-hexane	0.0	0.0

Results

General properties of the chloroform-released ATPase

Chloroform treatment of *R. rubrum* chromatophores allows the preparation of a highly stable oligomycin-insensitive ATPase complex. Table I indicates that other organic solvents are less specific in the extraction of the enzyme.

The addition of metal salts ($CaCl_2$, $MgCl_2$, $MnCl_2$), chelating agents (EDTA, EGTA), nucleotides (ATP, ADP, AMP), inorganic phosphate or sucrose over a large concentration range had no effect on stability, yield and specific activity of the ATPase. It became almost completely solubilized in 50 mM Tris-HCl, pH 7.5, at a bacteriochlorophyll concentration of 0.2 mM.

The various steps and yields of the present chloroform procedure are summarized in Table II. Purification of the Ca^{2+}-dependent ATPase activity was 200-fold and the yield based on membrane-bound Ca^{2+}-ATPase activity was about 20%. However, the yield of Mg^{2+}-ATPase activity was much lower (<1%) and its purification was less effective (about 6-fold). The dialysis step following ammonium sulfate precipitation yielded no increase in the specific Mg^{2+}-ATPase activity, whereas the specific Ca^{2+}-ATPase was increased.

As shown by analytical disc electrophoresis in 5% polyacrylamide gels (Fig. 1 A) the chloroform-released ATPase appears to be homogeneous after gel filtration on Sepharose CL-6B. Both the Ca^{2+}- and Mg^{2+}-dependent ATPase activities are associated with the single slowly moving protein band

Table II. Purification of chloroform-released *R. rubrum* ATPase. ATPase activity was measured at 37 °C in 10 ml of 50 mM Tris-HCl buffer, pH 8.0, containing 1 mM ATP and 1 mM $CaCl_2$ or 1 mM $MgCl_2$, respectively. Analytical polyacrylamide gel electrophoresis (PAGE) was carried out as described in Materials and Methods.

Purification step	Total protein [mg]	Specific Ca^{2+}-ATPase activity [U/mg]	Total Ca^{2+}-ATPase activity [U]	Specific Mg^{2+}-ATPase activity [U/mg]	Total Mg^{2+}-ATPase activity [U]	Protein bands in PAGE
chromatophores	1500.0	0.11	165.0	0.14	210.0	–
chloroform extract	41.3	3.0	124.0	0.35	14.5	6
30–60% $(NH_4)_2SO_4$ fraction	8.8	3.4	30.0	0.57	5.0	2–3
dialyzed $(NH_4)_2SO_4$ fraction	8.7	8.8	76.6	0.38	3.3	2–3
eluate of Sepharose CL-6B	1.5	21.7	32.6	0.90	1.4	1

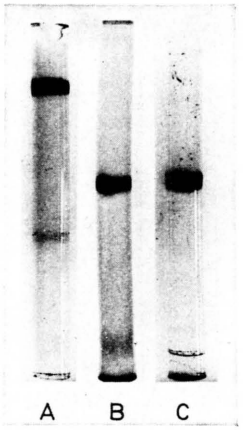

Fig. 1. Analytical disc electrophoresis on 5% polyacrylamide gels of various ATPase forms. (A) Chloroform-released ATPase (50 μg), (B) ATPase from an acetone powder extract (40 μg), and (C) ultrasonic preparation of ATPase (40 μg).

as could be visualized by the method of Wachstein and Meisel [20]. The ATPase forms prepared by the other two methods migrated significantly faster than the chloroform-released enzyme (Fig. 1 A – C).

The purified chloroform-extracted ATPase could be stored at 0 – 4 °C in 45 mM Tris-HCl, pH 7.5, containing 10% glycerol (v/v) for at least 4 weeks without loss in enzymic activity. At minus 20 °C in the presence of 50% glycerol (v/v) the enzyme was stable for months.

The ultraviolet absorption spectrum of the chloroform-released ATPase shows a maximum at $\lambda = 277$ nm and a minimum at $\lambda = 253$ nm as well as distinct shoulders at 270 and 261 nm. The quotient E_{280}/E_{260} of 1.26 ± 0.05 indicates a nucleotide content of about 3 nucleotides per molecule [21]. The $E^{1\%}_{280}$ nm value is 15.6.

The amino acid composition of chloroform-released ATPase is shown in Table III. All values are based on ion-exchange chromatography as described under MATERIALS AND METHODS. In addition to these data the fluorescence emission peak at 305 nm is in good agreement with the published value of 310 ± 5 nm for tyrosine [22, 23] and is close to the fluorescence emission peak of 302 nm for pure CF_1 [24]. The fluorescence emission ratio at 300 nm to that at 350 nm is 14 (see Fig. 2). Such a ratio indicates a high degree of purity for *R. rubrum* ATPase [23].

The molecular weight of the chloroform-released ATPase was determined by gel filtration on a

Table III. Amino acid composition of chloroform-released ATPase from *R. rubrum FR1*. Values are expressed in mol % (mol/100 mol amino acid) and have been obtained after acidic and basic hydrolysis as described in Materials and Methods.

Amino acid	Amount (mol %)
Asx	9.2 ± 0.60
Thr	7.2 ± 0.04
Ser	5.4 ± 0.05
Glx	11.5 ± 0.35
Pro	2.6 ± 0.50
Gly	10.5 ± 0.25
Ala	11.4 ± 0.06
Val	9.3 ± 0.14
Cys	0.4 ± 0.50
Met	0.8 ± 0.30
Ile	5.4 ± 0.03
Leu	9.4 ± 0.10
Tyr	1.1 ± 0.10
Phe	3.4 ± 0.20
Lys	5.5 ± 0.20
His	1.3 ± 0.21
Arg	5.6 ± 0.20
Trp *	0

* The absence of tryptophan was further indicated by the fluorescence emission spectrum (see Fig. 2).

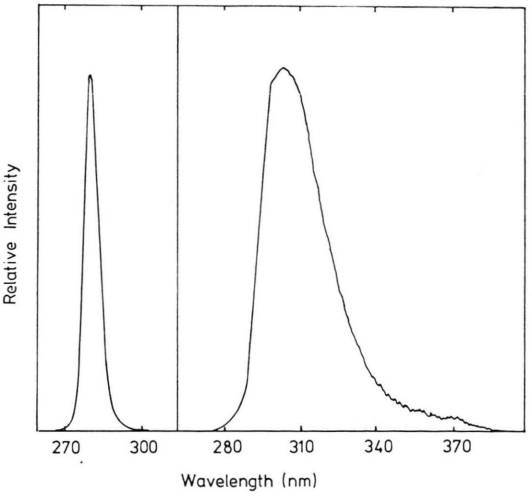

Fig. 2. Fluorescence excitation and emission spectra of chloroform-released ATPase. Left curve: Fluorescence excitation spectrum (emission at 305 nm), right curve: Fluorescence emission spectrum (excitation 280 nm).

Sepharose CL-6B column (1×100 cm) calibrated with ferritin, catalase, and hemoglobin. In Fig. 3 log molecular weights of the marker proteins are plotted versus the K_{av} values. They result in a straight line. The midpoint of the eluted ATPase peak corresponds with a molecular weight of

400,000 ±30,000 for the chloroform-released enzyme. Under identical conditions the molecular weight of the sonicated ATPase was found to be 370,000 ±30,000.

Subunit composition of various ATPase forms

The polypeptide components of the chloroform-released ATPase were analyzed by sodium dodecyl sulfate gel electrophoresis and compared with those of the ATPase complexes prepared by acetone powder extraction and ultrasonication (Fig. 4 A – D). The chloroform-released ATPase (Fig. 4 A) is composed of five different classes of subunits which are designed according to ref. 25 as α, β, γ, δ, and ε. Two more low molecular weight polypeptides in small amounts were detected in some chloroform preparations. They could not be identified. It should be noted that the protein band designed as γ shows a higher staining intensity in the case of the chloroform-released enzyme.

The ATPase extracted from an acetone powder and purified to homogeneity by affinity chromatography (Fig. 4 B) is composed of four classes of subunits (α, β, γ, and δ, but no ε). Replacing affinity chromatography by preparative disc gel electrophoresis as final purification step yields an active ATPase complex composed of only three different subunits α, β, and γ (Fig. 4 C).

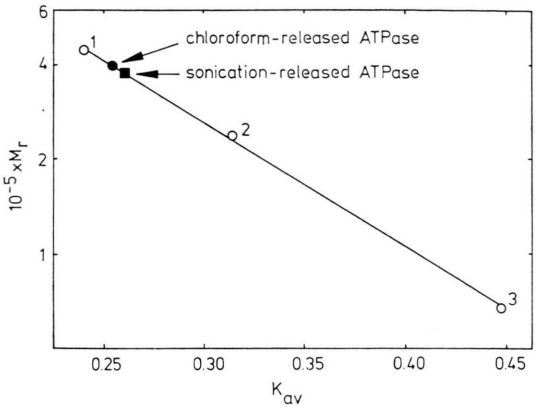

Fig. 3. Molecular weight of chloroform-released ATPase (●) and sonicated ATPase (■) determined by gel filtration. (1) Ferritin, (2) catalase, (3) hemoglobin.

Ultrasonication of chromatophores yields an ATPase complex that is mainly composed of the three major subunits α, β, γ, and minute amounts of δ (Fig. 4 D). The molecular weights of the subunit proteins of the various ATPase complexes are summarized in Table IV.

Some kinetic properties of the ATPase complexes

The chloroform-released ATPase shows a maximum activity of 21.7 U/mg with Ca-ATP. The other Me^{2+}-ATP complexes tested were less potent

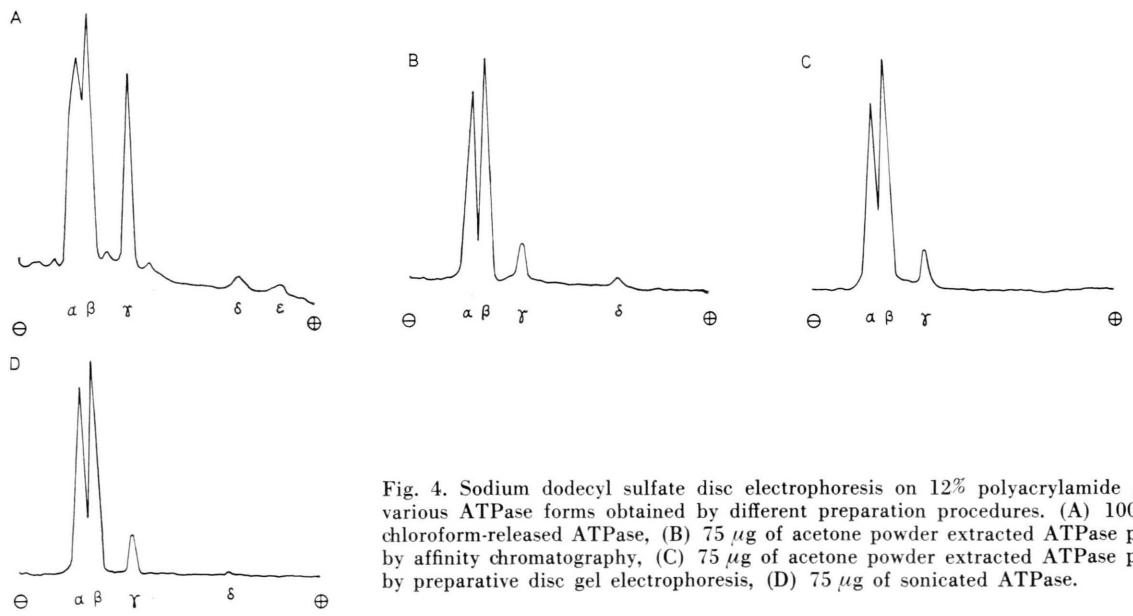

Fig. 4. Sodium dodecyl sulfate disc electrophoresis on 12% polyacrylamide gels of various ATPase forms obtained by different preparation procedures. (A) 100 μg of chloroform-released ATPase, (B) 75 μg of acetone powder extracted ATPase purified by affinity chromatography, (C) 75 μg of acetone powder extracted ATPase purified by preparative disc gel electrophoresis, (D) 75 μg of sonicated ATPase.

Table IV. Subunit structure of various ATPase forms from *R. rubrum* chromatophores.

Preparation method	Composition and molecular weights of subunits				
	α	β	γ	δ	ε
(A) chloroform procedure	58,000	53,500	39,000	18,500	14,000
(B) acetone powder extraction	56,000	51,000	38,000	21,000	
(C) preparative gel electrophoresis of (B)	56,000	51,000	38,000		
(D) ultrasonication	56,000	51,000	38,000	(21,000)	

substrates ($Cd^{2+} > Mg^{2+} > Mn^{2+}$). Some kinetic parameters of the chloroform-released ATPase are summarized in Table V and compared with related data found with membrane-bound ATPase and other soluble ATPase forms. In contrast to the chloroform-released ATPase, the enzymes obtained by acetone powder extraction and ultrasonication of chromatophores are strictly Ca^{2+}-dependent ATPase complexes (no ATP hydrolysis in absence of Ca^{2+} and in presence of Mg^{2+}, Cd^{2+}, or Mn^{2+}).

The K_m values (Ca-ATP) obtained for all soluble ATPase forms are strikingly similar, but they are considerably higher than the values for the membrane-bound state. The K_m values for Mg-ATP and Cd-ATP obtained for the membrane-bound ATPase are not changed during solubilization of the enzyme by chloroform treatment.

Discussion

Low electrophoretic mobility in 5% polyacrylamide gels and cold stability are common properties of chloroform-released ATPases from *R. rubrum* and beef heart mitochondria [16]. The molecular weight of about 400,000 agrees well with the molecular weight for chloroform-released mitochondrial ATPase from *yeast* [26] and would be consistent with a suggested subunit stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$. The molecular weight of the chloroform-released enzyme is slightly higher than that of the sonicated *R. rubrum* ATPase form (370,000). This difference is likely due to the lack of polypeptides δ (18,500) and ε (14,000) in the sonicated enzyme.

The amino acid composition of *R. rubrum* ATPase shows close similarities with the coupling factor ATPases from mitochondria [2], chloroplasts [27], and other bacteria [28–30]. Both the amino acid analysis data and the fluorescent emission measurements suggest that tryptophan is missing in the chloroform-released preparation of *R. rubrum* ATPase. Our results are in accordance with the findings of Farron [27] and Penefsky and Warner [31] who demonstrated that CF_1 from spinach chloroplasts and F_1 from beef heart mitochondria, respectively, also lack tryptophan. However, amino acid analysis data of coupling factor ATPases from the oxidative bacteria *Micrococcus spec.* [30], *Micrococcus lysodeicticus* [32], and *Bacillus megaterium* [28] reveal the presence of tryptophan in the ATPase molecules.

Table V. Kinetic parameters of various *R. rubrum* ATPase forms.

Substrate	ATPase preparation									
	Chromatophores		Chloroform method		Acetone powder extraction		Preparative gel electrophoresis		Sonication	
	spec. act. [a] [U/mg]	K_m [mM]	spec. act. [U/mg]	K_m [mM]	spec. act. [U/mg]	K_m [mM]	spec. act. [U/mg]	K_m [mM]	spec. act. [U/mg]	K_m [mM]
$(Ca\text{-}ATP)^{2-}$	0.09	0.036	21.7	0.170	11.0	0.150	2.6	0.200	12.5	0.150
$(Mg\text{-}ATP)^{2-}$	0.14 [b]	0.028	0.9 [c]	0.029	—	—	—	—	—	—
$(Cd\text{-}ATP)^{2-}$	0.32	0.020	4.6	0.014	—	—	—	—	—	—
$(Mn\text{-}ATP)^{2-}$	0.11	n.d.	0.9	n.d.	—	—	—	—	—	—

[a] Specific activity was measured in the presence of 1 mM ATP and 1 mM $MeCl_2$ as described in the legend of Table II.

[b, c] At optimal molar ratios of $Mg^{2+}/ATP = 0.25$ the specific activity could be increased about 2.5 times up to 0.34 U/mg and 2.2 U/mg of protein, respectively.

n.d., not determined.

The ATPase complex bound to chromatophore membranes of *R. rubrum* can be gradually disintegrated by application of different preparation methods. Recently, Philosoph *et. al.* [33] have shown that LiCl treatment of chromatophores in the presence of ATP releases only the inactive β-subunit(s) from the membrane-bound ATPase.

The ATPase complex released from the chromatophores by ultrasonication and purified under the conditions described here contains only the three major subunits α, β, γ. By purification of an acetone powder extract by affinity chromatography, however, we have obtained an ATPase complex consisting of four different subunits (α, β, γ, δ). Application of preparative disc gel electrophoresis during purification of ATPase from acetone powder yields, on the other hand, an enzyme exclusively composed of α-, β-, and γ-subunits.

The densitometric pattern of the acetone powder-extracted ATPase shown in Fig. 4 B differs from the acetone powder-extracted enzyme described earlier by Johansson and Baltscheffsky [25]. Our preparation is lacking the ε-subunit. However, the molecular weight of the δ-subunit (21,000) presented here is exactly the sum of the molecular weights of the δ- and ε-subunits (13,000 and 7,500, respectively) reported by Johansson and Baltscheffsky [24]. These differences in the subunit structure of the acetone powder-extracted ATPase forms are likely related to the distinct purification procedures used.

The various preparation procedures also affect the kinetic parameters of the ATPase complexes. The affinity to Ca-ATP of the chloroform-released ATPase appears to become decreased during solubilization (increase of K_m value). In contrast, the K_m values obtained with Mg-ATP and Cd-ATP remain unchanged. This deviation in kinetic behaviour may suggest the presence of two different types of Me^{2+}-ATP binding sites: one highly specific for Ca-ATP, the other (also) specific for Mg-ATP and Cd-ATP. Thus the sonicated and acetone powder-extracted ATPase forms would only carry Ca-ATP specific sites. An alternative explanation of the differences in kinetic behaviour is the assumption that Me^{2+}-specific regulatory sites may become affected during sonication or acetone powder extraction. Related effects are not uncommon [3–6].

The chloroform-released ATPase appears to be more closely related to the membrane-bound enzyme than the other ATPase forms inasmuch as the kinetic data suggest only minor conformational changes of the enzyme protein.

Very recently Webster and Jackson [34] have described the preparation of a Ca^{2+}- and Mg^{2+}-dependent ATPase from wild-type *Rhodospirillum rubrum* by the same chloroform extraction method and subsequent affinity chromatography. These authors have obtained an enzyme which shows only three bands (α, β, and γ) upon sodium dodecyl sulfate disc electrophoresis and a kinetic behaviour similar to that of the ATPase isolated from acetone powder of *R. rubrum* chromatophores.

The discrepancies to the chloroform-released ATPase complex described in this paper may be related to the use of different *R. rubrum* strains (FR1 (DSM-No. 1068) versus wild-type) and/or to artifacts occurring during preparation, perhaps due to proteolytic degradation [30].

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