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Purification of the H^+-ATPase from *Rhodobacter capsulatus*, identification of the F_1F_0 components and reconstitution of the active enzyme

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A simple method has been adopted for the isolation of the H^+-ATPase from cell membranes of *Rhodobacter capsulatus* that is based on octylglucoside solubilization. The highly purified F_0F_1 enzyme catalyzes ATP hydrolysis and DCCD-sensitive ATP-P_i exchange. The isolated complex consists of nine different polypeptides with apparent molecular masses of 50, 45, 34, 29, 23, 20, 18.5, 11 and 7.5 kDa. The N-terminal sequence of the eight largest subunits were determined by automated Edman degradation. The five F_1 polypeptides could be readily identified by their homology to the F_1 subunits α, β, γ, δ and ε from other Rhodospirillaceae. In contrast to prokaryotic F_0 complexes known today, the membrane portion of H^+-ATPase from *R. capsulatus* was found to be composed of four polypeptides. Three of these correspond to subunit *a, b and c of the Escherichia coli* enzyme (29, 18.5 and 7.5 kDa). A fourth subunit of 23 kDa may be also related to subunit *b*. The ATP-synthase of this photosynthetic bacterium is in this respect similar to that of chloroplasts.

Introduction

The membrane-bound ATP-synthases of the F_1F_0 type catalyze the terminal reaction of oxidative and photo-phosphorylation. This enzyme, which functions as a proton-dependent reversible ATPase (H^+-ATPase), is composed of a hydrophilic portion F_1 which contains the nucleotide-binding sites [1] and a hydrophobic portion F_0 which is supposed to act as a proton channel [2]. The hydrophilic F_1 portion, which has ATPase activity, is generally composed of five subunits (α, β, γ, δ, ε) in a stoichiometry of 3 : 3 : 1 : 1 : 1. The structure of this enzyme has been remarkably conserved among prokaryotes, mitochondria and chloroplasts [3].

The hydrophobic F_0 portion of the enzyme was first characterized in prokaryotic systems like *Escherichia coli* and the thermophilic bacterium PS3 [4,5]. In *E. coli* the complex includes three subunits (*a*, *b* and *c*) present in various copy numbers: 1 : 2 : 9–12. The primary structure of the three polypeptides derived from the DNA sequence of the *unc* operon from *E. coli* [6] was

Abbreviations: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, carbonylcyanide *p*-(trifluoromethoxy)-phenylhydrazone; octylglucoside, *n*-octyl-β-D-glucopyranoside; Triton X-100, octylphenoxypoly(ethoxyethanol); Tricine, *N*-[2-hydroxy-1,1-(bis(methyl)ethyl]glycine; DTT, dithiothreitol; EDTA, ethylene dinitrile tetra acetic acid; HPLC, high performance liquid chromatography; P_i, inorganic phosphate.

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found to be homologous to that of F_0 subunits from mitochondria and chloroplasts [7]. The eukaryotic systems, however, included one to four additional proteins. Thus, the eight polypeptides of the *E. coli* H^+-ATPase seem to compose a minimal catalytic structure that has been strictly conserved during evolution.

The F_1 portion of the H^+-ATPase from photosynthetic bacteria has been previously biochemically characterized [8] and the amino acid sequences of the F_1 polypeptides from *Rhodopseudomonas blastica* and *Rhodospirillum rubrum* were derived from DNA sequence analysis of the ATP operon [9,10]. The genes coding for the F_0 polypeptides were not found to be part of the same transcriptional unit as in the case of *E. coli* and, only recently, the DNA sequence of the F_0 genes from *R. rubrum* has been determined [11]. In these photosynthetic organisms, as in the case of the cyanobacterium *Synechococcus* [12], four structural F_0 genes have been identified. Besides subunit *a* and *c* they encode two proteins which are both homologous with subunit b of *E. coli* and have been termed *b* and *b'*.

A procedure to isolate the ATP-synthase from photosynthetic bacteria was developed only for *R. rubrum* [13,14], but the polypeptide composition of the F_0 part was not reported. Previous investigation indicated that the H^+-ATPase from *R. capsulatus* can be light-activated and is, in this respect similar to that of chloroplasts [15].

In this study we describe the purification of the H^+-ATPase from *Rhodobacter capsulatus* and the identification of nine polypeptide components. Thus all three membrane complexes: cytochrome b/c_1 complex, photochemical reaction center and ATP-synthase have been biochemically characterized in one species of photosynthetic purple bacteria [16,17], and can be used in the future for in vitro reconstitution of the complete photophosphorylating system.

Materials and Methods

Preparation of H^+-ATPase. Rhodobacter capsulatus strain 'GA' [16] was grown photosynthetically in a fermenter. Chromatophores were prepared from 26 g (wet weight) of cells, as described in Ref. 18. The photosynthetic membranes were resuspended to a protein concentration of 7 mg/ml in a buffer containing 50 mM glycylglycine and 5 mM $MgCl_2$ (pH 7.4) (buffer A). Membranes were selectively solubilized by mixing the suspension with an equal volume of buffer A containing 2% octylglucoside. The mixture was stirred for 20 min on ice and centrifuged at $300\,000 \times g$ for 1 h at 4° C. Saturated (20° C) ammonium sulfate solution was added to the supernatant up to 47% saturation and the mixture stirred for 30 min on ice before being centrifuged 10 min at 12 000 rpm. The pellet was discarded and the supernatant brought to 58% saturation, stirred and centrifuged as before. The precipitate, enriched in H^+-ATPase, was collected and resuspended at a protein concentration of 10 mg/ml in buffer A in which 3% octylglucoside and 30 mg/ml phosphatidylcholine from soybean (Sigma) were previously suspended by sonication. The suspension was loaded on a 20–40% (w/v) linear source density gradient in buffer A plus 1% octylglucoside and centrifuged at 49 000 rpm for 19 h. The H^+-ATPase activity banded as a colorless zone at 30% sucrose. The gradient was collected in 0.3 ml fractions from the bottom, proteins were analyzed by SDS-PAGE and fractions containing pure H^+-ATPase were combined, frozen in liquid nitrogen and stored at −20° C without significant loss of activity over at least one month. The protein content was determined according to Bradford [19].

ATPase activity. The ATPase activity was tested in a buffer containing 50 mM Tris-Cl (pH 7.4), 5 mM $MgCl_2$ and 5 mM ATP (ATP buffer). A suspension of 50 mg asolectine in 1 ml of ATP buffer was prepared by sonication. Routinely 20 µl of lipid suspension and 20 µg of protein were added to 600 µl of ATP buffer. This mixture was incubated at 37° C for 10 min before the reaction was stopped by adding 2% perchloric acid. The P_i concentration was determined as described in Ref. 20. The ATPase activity of chromatophores was tested in the presence of 1 µM FCCP.

ATP-$^{32}P_i$-exchange activity. Proteoliposomes were prepared by diluting 50 µl of protein solution (1 mg/ml) with 625 µl of a buffer containing 80 mM Tricine-NaOH (pH 8) and 8 mM DTT. After 10 min at 37° C, 125 µl of a liposomal suspension containing 50 mg/ml asolectin, 50 mM Tricine-NaOH and 0.1 mM EDTA (pH 8), was

added. This mixture was incubated another 10 min at 20 °C before addition of 200 µl of 50 mM ATP (pH 8) and 50 mM $MgCl_2$. After another 10 min at 20 °C the reaction was started by addition of 20 µl of 0.1 M $Na_2H^{32}PO_4$ (10^5–10^6 cpm). The reaction was stopped after 10 min incubation at 37 °C with 100 µl of 30% trichloroacetic acid. After centrifugation, aliquots of the supernatant were taken and the ATP-$^{32}P_i$-exchange rate analyzed according to Ref. 21. Alternatively proteoliposomes were prepared by detergent dialysis. The protein solution (1 mg) was added to 1 ml buffer containing 50 mg asolectine, 1.6% cholate, 0.8% desoxycholate, 50 mM tricine, 0.1 mM EDTA (pH 8), and 0.5 mM DTT. This solution was dialyzed against 1000 volumes of 10 mM Tricine (pH 8), 0.1 mM EDTA and 0.2 mM DTT for 24 h with three changes of buffer. Then the ATP-$^{32}P_i$-exchange activity was measured as described above. Inhibition by DCCD was tested after a 30 min incubation period at 20 °C with different concentrations of DCCD dissolved in 5 µl of methanol.

DCCD labelling. 16 nmol ^{14}C-DCCD (Amersham, 57 Ci/mol) in toluene were dried by a stream of nitrogen and then dissolved in 10 µl of MeOH/pyridine (100 : 1). The H^+-ATPase (0.16 nmol in 100 µl of buffer A plus 1% octylglucoside) was added and the samples incubated for 2 h at 4 °C. The unreacted DCCD was separated by centrifuging the samples through a 1 ml Sephadex-G-50 column [22], pre-equilibrated with the same buffer. The samples were then concentrated (Centricon-30 tube, Amicon) and loaded onto a 15% acrylamide-SDS gel. Autoradiography of dried gels was performed using Hyperfilm-βmax (Amersham).

Determination of N-terminal sequences. SDS-PAGE was performed according to Laemmli [23]. N-terminal sequences were determined according to Eckerskorn et al. [24] as follows: the separated subunits were electroblotted in a semi-dry apparatus (Sartorius, Göttingen) onto siliconized glass fiber sheets (Glassybond, Biometra). Electrotransfer was performed in 50 mM boric acid, 10% methanol (pH 9) with constant current (1 mA/cm^2) for 3 h (high-molecular-weight subunits, 12% acrylamide gel) or for 6 h (low-molecular-weight subunits, 15% acrylamide gel). The transferred proteins were stained with 0.1% (w/v) Coomassie blue R 250, 30% (v/v) methanol, 10% (v/v) acetic acid for 2 min. The blot was destained in water/methanol/acetic acid (60 : 30 : 10, v/v/v), washed with bidistilled water and dried. The detected protein bands were excised and placed directly in a gas phase sequencer (type 470A, Applied Biosystems). The phenylthiohydantoin amino acids were analyzed using an isocratic HPLC system [25].

Results

Purification of H^+-ATPase

The purification procedure of an active H^+-ATPase from chromatophores of *R. capsulatus* is described here. It is similar to that used for the isolation of the H^+-ATPase [26] and the cytochrome b_6/f from spinach chloroplasts or the cytochrome b/c_1 complex from photosynthetic bacteria [27]. However, instead of a mixture of 0.9% octylglucoside and 0.5% cholate, the purification of H^+-ATPase from *R. capsulatus* was performed using only octylglucoside. The omission of cholate was found to be advantageous for selective solubilization of chromatophores and improved purity of the preparation. Length of incubation with detergent, temperature and the protein/detergent ratio strongly influenced the efficiency of solubilization. Optimal conditions (Fig. 1, lane A) are a protein concentration of 3.5 mg/ml at 1% octylglucoside and incubation for 20 min under stirring at 0 °C. After centrifugation most of the H^+-ATPase was found in the supernatant (Fig. 1, lane C) while the reaction center and the light-harvesting complexes were only partially solubilized (Fig. 1, lane B).

The enzyme was further purified by ammonium sulfate fractionation. The H^+-ATPase was precipitated between 47% and 58% saturation, very little of reaction center and light-harvesting complexes were co-precipitated (Fig. 1, lane D).

Addition of lipids to the buffer used to resuspend the ammonium sulfate precipitate improved the yield and the purity of the preparation in the following and last step, which consisted of a sucrose density gradient centrifugation. SDS-PAGE of the purified enzyme showed nine polypeptide components with relative molecular masses

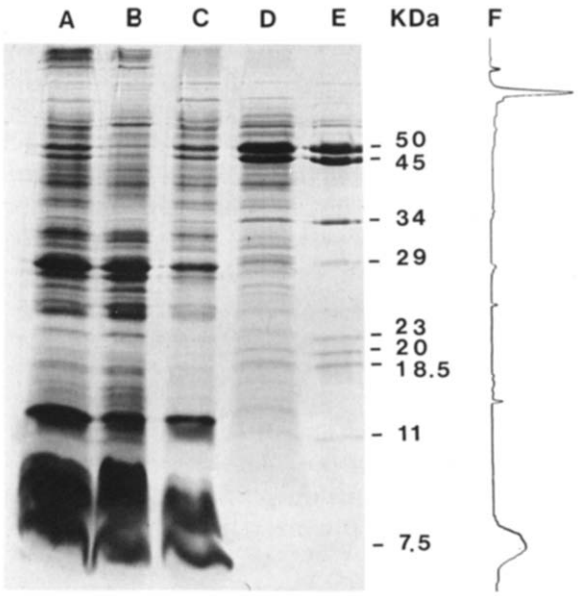

Fig. 1. Coomassie blue staining of a 15% acrylamide SDS-gel, showing the purification of the H^+-ATPase from *R. capsulatus* and the labelling by [^{14}C]DCCD. (A) 36 μg of chromatophores; (B) 25 μg 1% octylglucoside-unsolubilized pellet; (C) 18 μg 1% octylglucoside-solubilized supernatant (80% of the total H^+-ATPase); (D) 15 μg 47–58% ammonium sulfate precipitate; (E) 5 μg purified H^+-ATPase; (F) labelling by [^{14}C]DCCD of subunit *c*. The labelled protein is visualized by densitometric scanning of the autoradiogramm. Before electrophoresis proteins were incubated for 10 min at 65°C in 5% SDS and 2% 2-mercaptoethanol.

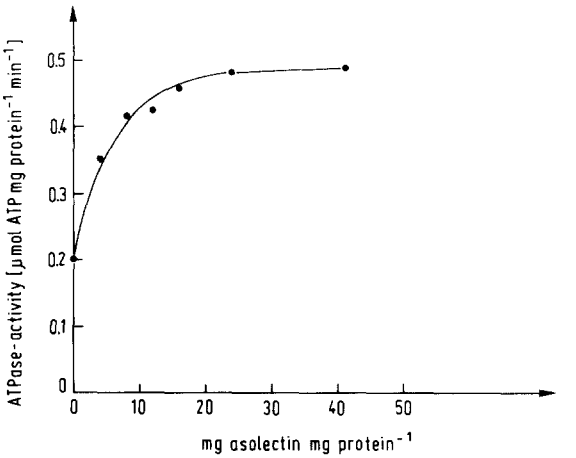

Fig. 2. Stimulation of the ATPase activity by phospholipids. For experimental details, see Materials and Methods.

of 50, 45, 34, 29, 23, 20, 18.5, 11 and 7.5 kDa (Fig. 1, lane E). The major contaminant was a 62 kDa polypeptide. Table I summarizes the purification procedure.

Characterization of the H^+-ATPase activity

Total ATPase activity increased during the initial step of solubilization four-fold (Table I). Such stimulation of the ATPase activity upon solubilization has been reported for other ATPases before [8]. In the following purification step involving precipitation with ammonium sulfate, the specific ATPase activity decreased; this was probably due to partial inactivation of the enzyme by this treatment. After the sucrose density gradient centrifugation, the pure enzyme in detergent micelles catalyzed the hydrolysis of 0.5 μmol ATP/min per mg H^+-ATPase. The enzyme exhibited maximal activity at pH values around pH 7.4 and could be stimulated up to 2.3-fold by addition of lipids (Fig. 2). The highest activity was measured in the presence of 50 mg asolectin per mg protein.

The ATP-$^{32}P_i$-exchange activity of the preparation was determined as described under Materials and Methods. When the enzyme was incorporated into liposomes by means of detergent dialysis, a specific activity of 1.3 nmol ^{32}P/min per mg protein was measured. A higher activity of 3.8 nmol ^{32}P/min per mg protein was found when the

TABLE I

ATPASE ACTIVITY AND ATP-P_i EXCHANGE AT EACH STEP OF PURIFICATION

	Total protein (mg)	Total activity (μmol ATP/min)	Specific activity (μmol ATP/min per mg protein)	ATP-P_i exchange (nmol AT^{32}P/min per mg protein)
Chromatophores	360	11.2	0.031	0.2
1% Octylglucoside	171	48	0.28	n.d.
Ammonium sulfate	49.9	7.5	0.15	n.d.
Sucrose gradient	4.8	2.4	0.52	3.8

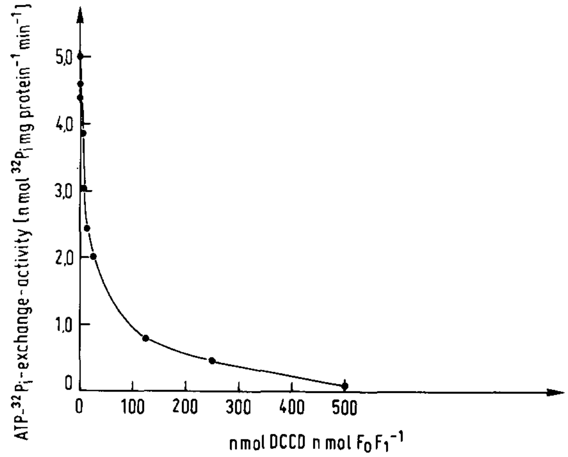

Fig. 3. Inhibition of the ATP-P_i exchange activity by DCCD. For experimental details, see Materials and Methods.

enzyme was incorporated into liposomes by detergent dilution.

The sensitivity of the H^+-ATPase towards DCCD, a specific inhibitor of the proton conductive portion F_0, is shown in Fig. 3. The ATP-$^{32}P_i$-exchange could be strongly inhibited by DCCD. Complete inhibition of the activity was found at a DCCD/enzyme ratio of 500. A 50% inhibition was obtained at a ratio of 12.

Polypeptide composition

The water soluble F_1 portion of the H^+-ATPase from *R. capsulatus* has been previously characterized [8]. Similarly to the F_1 from mitochondria, chloroplasts and other prokaryotes [3], the coupling factor from photosynthetic bacteria was shown to be composed of five polypeptides with a relative molecular mass of 54 kDa (α), 50 kDa (β), 32 kDa (γ), 13 kDa (δ) and 10 kDa (ε) [8]. The subunits of the H^+-ATPase from *R. capsulatus* are shown in Fig. 1 together with their relative molecular weights. Small differences are observed depending on the gel system used. Five of the components form the water-soluble F_1 portion and the four remaining components are assumed to be part of the membrane-bound F_0 portion.

The primary structure of the F_1 polypeptides was derived from the DNA sequence analysis of the ATP operon from two species of Rhodospirillaceae [9,10]; we expected to be able to identify the *R. capsulatus* F_1 polypeptides by sequence homology. The polypeptides were separated by preparative SDS-PAGE and blotted onto glass fibers, as described in Materials and Methods. Proteins detectable by Coomassie blue staining were excised and subjected to automated Edman degradation. The N-terminal sequence of the eight largest polypeptides could be determined. All proteins lacked the N-formylmethionine residue and had free N-termini. The small polypeptide of 7.5 kDa was not detected with the system used.

The comparison of the N-terminal sequences obtained from the H^+-ATPase polypeptides of *R. capsulatus* with known sequences of the F_1 components from two related Rhodospirillaceae provided unambiguous identification (Fig. 4).

The N-terminal sequence of the 50 kDa subunit was determined for the first 19 amino acid residues. The sequence was identical to that of *R.*

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50 kDa R.c.       G I Q A A E I S A I L K E Q I K N F G
                  * * * * * * * * * * * * * * * * * * *
     α R.b.     M G I Q A A E I S A I L K E Q I K N F G

45 kDa R.c.                 L S K G K V T Q V I G A V V D V Q F
                              * * * * * * * * * * * * * * * * *
     β R.b.     M T M A T T V S K G K V T Q V I G A V V D V Q F

34 kDa R.c.       P(S)L K D L K N
                  * * * * * * * *
     γ R.b.     M P S L K D L K N

29 kDa R.c.       V X F T L P K N S K
                        * * *
     a Sy.      M G S A T L P S D L M

23 kDa R.c.      (G)N E T H A V E A A A(A)V A G X(A A)E
                          *     * * *             *
     b R.r.     A E T A E H G G E A A S H G G L F A D P

20 kDa R.c.      (A)E P A(S)I S A A I A G R Y A T A I F(D)L(A Q)E
                  * *   * * * *     * *   * * * * *   *   *     *
     δ R.b      M A E A A S I S Q G I A E R Y A T A L F E L S K E

18.5 kDa R.c.  (A)E G P F V S L R N A H - F V I L V A F L I F I
                              *     *     * *     *   *
     b E.c.     M N L N A T I L G Q A I A F V L F V L F C M K Y

11 kDa R.c.      (A)D T M Q F D L V S P E R X L
                  *   *   * * * * * * * * *   *
     ε R.b.     M A A T L Q F D L V S P E R R L
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Fig. 4. Identification of the F_1F_0 components of *R. capsulatus* H^+-ATPase by amino acid sequence homology. The N-terminal sequence of the *R. capsulatus* F_1 subunits: R.c., (α, β, γ, δ, ε) was compared with that of *R. blastica*: R.s. [9]. The homologies in the N-terminal sequences of the 29 kDa subunit *b*. [9] with subunit *a* of *Synechococcus*: Sy [12], of the 23 kDa subunits with subunit *b* of *R. rubrum*: R.r. [11] and of the 18.5 kDa with subunit *b* of *E. coli*: E.c. [6] are shown. Identical amino acid residues (single-letter code) are marked by an asterix. Amino acids in brackets were identified with a low degree of confidence.

blastica subunit α [9]. The identity with the corresponding polypeptide from *R. rubrum* was 84%.

The sequence of the first 18 amino acid residues from the β subunit (45 kDa) and that of the first eight residues from the γ subunit (34 kDa) from *R. capsulatus* H^+-ATPase matched the corresponding sequences from *R. blastica* also very well. The homology with the respective polypeptides from *R. rubrum* was again lower with 60% homology.

Less homology between *R. capsulatus* and *R. blastica* was found in the N-terminal sequence of subunits δ and ϵ. A stretch of 24 amino acid residues of the δ subunit (20 kDa) showed 71% identity with *R. blastica* and only 37.5% with *R. rubrum*. Subunit ϵ (11 kDa) had 80% identity in the determined N-terminal sequence with that of *blastica* and 67% with that of *rubrum*.

The sequences of the F_0 polypeptides from *E. coli, Synechococcus* and *R. rubrum* were used to identify the corresponding components in the *R. capsulatus* complex [6,11,12] (Fig. 4). The N-terminal sequences of the 23 kDa and 18.5 kDa polypeptides exhibited significant homology with the N-terminal region of *R. rubrum* subunit *b* (26%) and with *E. coli* subunit *b* (29%), respectively (Fig. 4). In agreement with genetic evidence reported for other photosynthetic prokaryotes, the two proteins seems to be both related with subunit *b*, accordingly they have been respectively identified as subunit *b* and *b'* [11,12].

Only 10 amino acid residues of the 29 kDa subunit could be sequenced and this was too short to reveal significant homology with the corresponding *E. coli* or other known F_0 sequences. However, some identities with the N-terminal sequence of subunit *a* from *Synecococcus* [12] was detected. This evidence and the observation that this protein is poorly stained by Coomassie blue, suggested that it could correspond to subunit *a* [2,7,28].

No sequencing data were obtained on the 7.5 kDa protein; however, labelling with ^{14}C-DCCD indicated that this protein corresponds to subunit *c* of the F_0 portion. Fig. 1 (lane F) shows a scanning of the autoradiogramm indicating the specific labelling by DCCD of the 7.5 kDa subunit. The peak in the 100 kDa region most probably is an aggregate of subunit *c* [29].

Discussion

A general method for the purification of H^+-ATPase and b/c_1 or b_6/f complexes from photosynthetic membranes [26,27] has been adopted for the purification of the H^+-ATPase from *R. capsulatus*. The new procedure could also be used to isolate the b/c_1 complex of the same organism if, prior to detergent solubilization, the chromatophore membranes were washed in 2 M NaBr to remove the F_1 ATPase (data not shown). The solubilization of both membrane complexes from chromatophores was found to be more selective by octylglucoside than by a mixture of this detergent and cholate, which had been previously used to isolate the b/c_1 complex [30]. A short incubation with octylglucoside can preferentially extract the ATP synthase. This complex is less hydrophobic than other major membrane complexes of chromatophores (light harvesting, reaction center and b/c_1 complex).

The method described was simple to perform and reproducible. It could possibly be applied to other prokaryotic organisms. Methods to isolate the same enzyme from Rhodospirillaceae were previously developed only for *R. rubrum* ATP synthase and were based on Triton X-100 solubilization [13,14]. Small detergents with small polar head groups like octylglucoside, however, seem to be more suitable solubilizing agents of membrane proteins for the purpose of crystallization. Bacteriorhodopsin, porin and a photochemical reaction center are examples of membrane proteins which have been crystallized in the presence of octylglucoside [31,32,33].

The ATPase activity of the preparation could be stimulated by phospholipids. A maximal ATP-$^{32}P_i$-exchange activity was obtained when the enzyme was reconstituted into liposomes by detergent dilution. The activity was similar to that measured in dark chromatophores under the same conditions, assuming a 4% ATP-synthase content based upon coomassie blue staining intensity. Slightly higher values were previously obtained with the reconstituted preparation from *R. rubrum* [13] and elevated ATP-$^{32}P_i$-exchange activity was measured in *R. capsulatus* chromatophores in the presence of 0.2 mM succinate [18]. The significance of these differences is at present uncertain.

It was further demonstrated that this H^+-ATPase and by continuous illumination [35]. Energization of the membranes upon illumination established a more strict coupling between the electron-transport reactions and the rate of photophosphorylation [36]. This activation, which requires active cyclic electron flow, cannot be expected for the isolated enzyme, unless the ATP synthase is co-reconstituted with the electron-transport complexes. This experimental approach is presently under investigation.

The analysis of the polypeptide composition of the H^+-ATPase from *R. capsulatus* revealed nine subunits. A similar composition was reported for the chloroplast enzyme [26,28,37]. Previously characterized prokaryotic F_1F_0 preparations contained only eight subunits [38–40]. The mitochondrial enzyme includes several additional F_0 subunits besides the five polypeptide components of the F_1 [41]. A comparison of the N-terminal sequences of the five F_1 polypeptides of *R. capsulatus* ATP synthase revealed a striking homology with the corresponding sequences from *R. blastica* [9]. The homology in the N-terminal regions was in agreement with the taxonomic position of the species of purple bacteria examined [42]. The degree of conservation among the F_1 polypeptides was found to be variable as previously observed [3].

The membrane portion of this ATP-synthase includes four different polypeptide chains. The 7.5 kDa subunit was identified by DCCD labelling as subunit *c*. This protein which is also termed proteolipid because of its peculiar property of being extractable by organic solvents, is known to bind DCCD covalently [2]. The hydrophobic subunit *a* was found to be mostly conserved between *E. coli*, mitochondria and chloroplast (subunit IV) [2,6,40,41] in the C-terminal region. As expected the N-terminal sequence of the 29 kDa subunit of *R. capsulatus* F_0 showed only a weak homology with known sequences of subunit *a*. Both the 23 and 18.5 kDa polypeptides showed homology with subunit *b* of other prokaryotes [11,6] in their N-terminal regions. One copy of each polypeptide may be present in the F_0 of photosynthetic prokaryotes rather than two identical *b* subunits as in the case of *E. coli* [11]. This difference may be related to regulatory phenomenon of light activation exhibited by the photosynthetic ATP-synthases.

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