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Subunit structure of ATP synthase from *Chloroflexus aurantiacus*

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An ATP synthase has been isolated from green nonsulfur photosynthetic bacterium *Chloroflexus aurantiacus*, a representative of a lower branch of eubacteria. The enzyme, reconstituted with the bacterial lipids into proteoliposomes, is shown to catalyze [^{32}P]P_i–ATP exchange (at a rate of 180 nmol [^{32}P]ATP/min/mg). The ATP synthase is composed of nine polypeptide species (60, 50, 33, 19, 16.5, 15.5, 14.5, 13, and 8 kDa as determined by urea-SDS-PAGE). The catalytic part of the ATP synthase (which is detached by chloroform treatment) contains the first four polypeptides. In the intact ATP synthase the 14.5 and 13 kDa polypeptides are connected by disulfide bonds to form a heterodimer of 25 kDa.

ATP synthase; Subunit structure; Evolution; *Chloroflexus aurantiacus*

1. INTRODUCTION

Recent studies on eukaryotic vacuolar ATPases and arhaebacterial ATP synthases have attracted attention to the evolution of the ATP synthase (for recent review see [1]). In spite of the broadening studies, ATP synthase subunit composition has been determined only for representatives of three upper eubacterial branches [2]: gram-positive bacteria (PS3 [3], *Streptococcus faecalis* [4], *Bacillus firmus* [5], *B. alcalophilus* [6]; purple bacteria and their relatives (*Rhodospirillum rubrum* [7,8], *Rhodobacter capsulatus* [9], *Escherichia coli* [10], *Vibrio alginalyticus* [11]); and cyanobacteria-chloroplasts [12,13].

All these ATP synthases are composed of a catalytic part, F_1, and a transmembrane proton channel complex, F_0. The F_1 part detached from the F_0 is a soluble ATPase, which is composed of five different subunits in the stoichiometry $\alpha_3 \beta_3 \gamma_1 \delta_1 \varepsilon_1$. The F_0 part consists of either three subunit species in the stoichiometry a_1 b_2 c_{9-11} or four species, where two related subunits b and b' are present instead of two copies of subunit b.

This study shows that the ATP synthase of a representative of a lower eubacterial branch differs in subunit composition from the standard ATP synthases of upper eubacterial branches.

2. EXPERIMENTAL

2.1. *Cell growth and membrane preparation*

Chloroflexus aurantiacus strain B3 (an isolate from a hot spring of Lake Baikal) was obtained from the collection of Moscow State University. Cells were grown as described earlier [14]. Washed cells were suspended in medium containing 50 mM Tris-HCl (pH 8.0), 2 mM $MgSO_4$, 0.1 mg/ml DNAase, 1 mM phenylmethylsulfonylfluoride and sonicated for 2 × 20 s at 0–4°C. Unbroken cells and debris were removed by centrifugation (3,000 × *g*, 10 min). Membranes were sedimented from the supernatant solution at 70,000 × *g* for 30 min and washed in 10 mM Tris-HCl (pH 8.0).

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2.2. *Isolation of the ATPase part*

The membrane pellet was suspended to a final protein concentration 3 mg/ml in a medium containing 10 mM Tris, 1 mM EDTA (pH 7.9), 10% glycerol. A quarter volume of chloroform was added and the mixture was shaken for 30 s at room temperature and centrifuged at 20,000 × *g* for 5 min. The upper phase was centrifuged for another 20 min at the same speed. The supernatant was brought to 50 mM Tris-HCl (pH 8.0) and 50 mM $(NH_4)_2SO_4$ and loaded on a DEAE-Sephacel column (5 × 1 cm) equilibrated with a buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM $(NH_4)_2SO_4$. The column was washed by 10 ml of the same buffer. The ATPase was then eluted with a 20 ml linear gradient of 50–200 mM $(NH_4)_2SO_4$ in 50 mM Tris-HCl (pH 8.0). Collected active fractions were concentrated on a small Phenyl-Sepharose column (1 × 0.8 cm) and passed through a Sephacryl S-300 column (67 × 0.9 cm) equilibrated with 20 mM Tris-HCl (pH 8.0).

2.3. *Isolation of the ATP synthase*

The membranes were additionally washed with a buffer containing 0.8% Na-cholate, 2 mM $MgSO_4$, 100 mM $(NH_4)_2SO_4$, 40 mM Tris-HCl (pH 8.0), and then stirred for 1 h on ice at a final protein concentration 5 mg/ml in a buffer containing 0.23% Triton X-100, 2 mM $MgSO_4$, 50 mM $(NH_4)_2SO_4$, 40 mM Tris-HCl (pH 8.0). Unsolubilized material was sedimented at 250,000 × *g* for 1 h. Subsequent steps of purification were the same as in the case of the ATPase except for the addition of Triton X-100 to 0.2% at the stage of the DEAE-Sephacel column and to 0.05% at the stage of the Sephacryl S-300 column. Before the last step the protein fractions were concentrated by swelling of Sephadex G-25.

2.4. *Preparation of proteoliposomes*

C. aurantiacus lipids were used. Extraction and fractionation of the lipids have been described previously [15]. The suspension of the swollen lipids in 10 mM Tricine-NaOH (pH 7.8 at 50°C), octylglucoside solution in the same buffer, and ATP synthase preparation were mixed at 52°C to give the final concentration of 2% lipids, 2% octylglucoside and ca. 0.8 mg/ml protein. 5 min later the mixture was diluted ten-fold at the same temperature by gradual addition of a buffer

containing 100 mM Tricine-NaOH (pH 7.8 at 50°C), 2 mM $MgSO_4$, 5 mM K_2SO_4, and 2 mg/ml bovine serum albumin.

2.5. *Analytical procedures*

[^{32}P]P_i–ATP exchange, ATPase activity and protein concentration were assayed as in [15]. Electrophoresis was carried out according to the methods of Laemmli [16] (SDS-PAGE), or Schagger and von Jagow, in the presence of 6 M urea [17] (urea-SDS-PAGE) as indicated in legends. Samples were prepared in 100 mM Tris-HCl (pH 6.8), 1% SDS, 1% mercaptoethanol (omitted when indicated), 10% glycerol. Background-free saturation staining in colloidal Coomassie G-250 [18] was employed.

3. RESULTS

The ATP synthase reconstituted with *C. aurantiacus* lipids into proteoliposomes catalyzed [^{32}P]P_i–ATP exchange at the rate of 180 nmol P_i/min/mg, confirming its integrity. ATP hydrolysis rate (330 nmol/min/mg) was stimulated 2.8-fold by 1 μM gramicidin.

SDS-PAGE revealed eight bands whose Coomassie staining intensities along gel filtration fractions paralleled ATPase activity (Figs. 1 and 2A). In urea-SDS-PAGE the ATP synthase displayed nine polypeptides (Fig. 2B). The four upper bands in urea-SDS-PAGE coincided with the subunits of the ATPase part and were designated as α, β, γ, and δ. The additional bands were preliminarily designated as 1, 2, 3, 4 and 5 according to decreasing molecular mass in urea-SDS-PAGE. Two-dimensional PAGE (Fig. 3) showed that the relative positions of bands δ, 1, 2, 3, and 4 were different in the two systems and that in SDS-PAGE subunits 1 and 3 moved together. The silver staining of the gel revealed no additional subunits, but slight spots of the dimers of subunits 3, 4, and 5 are visible over the corresponding spots.

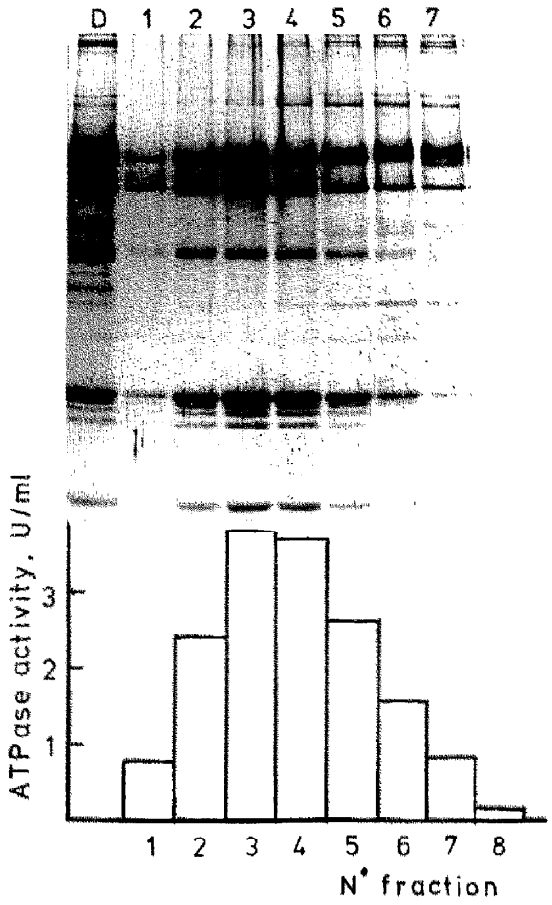

Fig. 1. Last step of purification of *Chloroflexus aurantiacus* ATP synthase (Sephacryl S-300 column). Top, SDS-PAGE (13–18% acrylamide gradient) of fractions. The equal volume (4 μl) of each fraction was applied. Lane D, collected fractions from DEAE-Sephacel column. Bottom, ATPase activity in fractions.

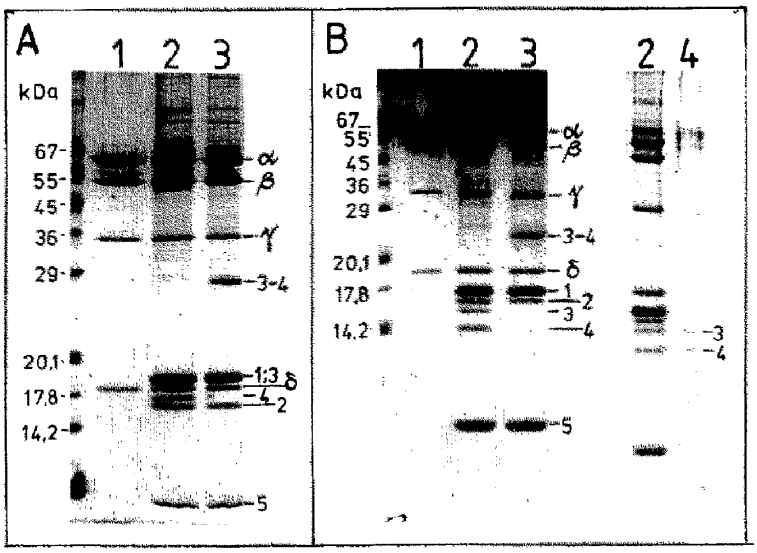

Fig. 2. Electrophoretic analysis of the ATP synthase subunit structure. (A) SDS-PAGE, 15% acrylamide. (B) urea-SDS-PAGE, 12% acrylamide. Lanes: 1, ATPase, 2 μg. 2. ATP synthase, 4 μg; 3, as 2, but without mercaptoethanol in sampler buffer; 4, the band 3–4 of lane B3 excised and soaked in sampler buffer containing mercaptoethanol.

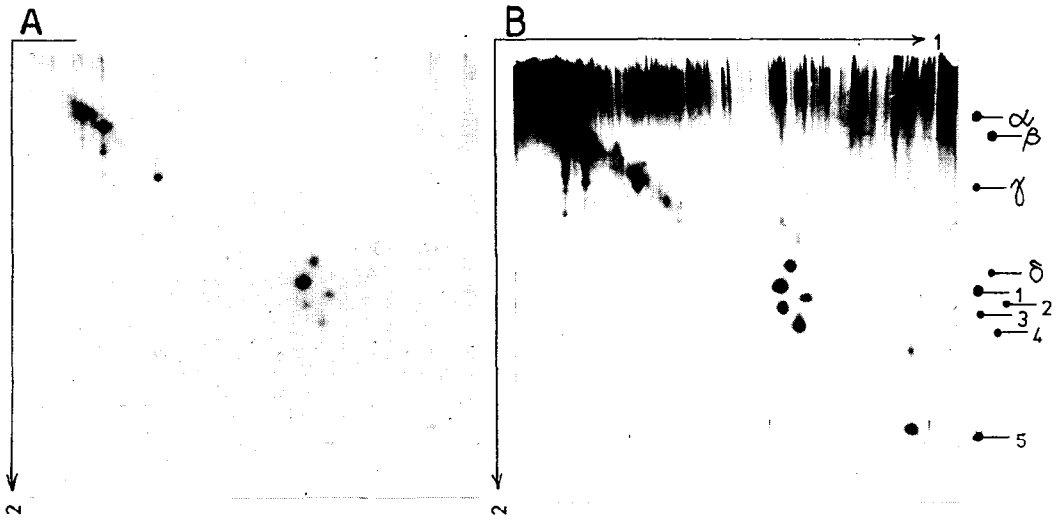

Fig. 3. Two-dimensional SDS-PAGE of the ATP synthase. First dimension – SDS-PAGE (the strip represented in Fig. 2A, lane 2 was used), second dimension – urea-SDS-PAGE. Panel B, the gel overstained by silver [19].

Table I represents the molecular masses and the relative Coomassie staining of the subunits, that may serve as a rough estimation of their stoichiometry. The relative staining ratio for subunits α, β, and γ was very close to the molar ratio of the corresponding subunits of standard ATP synthases. That for subunits γ, δ, 2, 3, and 4, which were assumed to be equimolar, was close to unity. Subunit 1 stained unexpectedly intensely. On the basis of molecular mass it may correspond to double-copy subunit *b*, but its number of copies apparently exceeds two. Subunit 5 evidently corresponds to dicyclohexylcarbodiimide-binding protein due to the large number of copies and its molecular mass.

If mercaptoethanol was omitted in the samples of the ATP synthase, bands 3 and 4 disappeared and another band of approximately their combined molecular mass appeared in their place (Fig. 2, lanes A3 and B3). When excised and soaked in sample buffer containing mercaptoethanol, the latter band dissociated into the bands of subunits 3 and 4 (Fig. 2B, lane 4). One may conclude that polypeptides 3 and 4 are connected as a heterodimer by S–S bond(s) in the intact enzyme.

Table I

The molecular masses and relative Coomassie staining of *C. aurantiacus* ATP synthase subunits.

Subunit	Molecular mass (kDa)		Relative staining
	SDS-PAGE	urea-SDS-PAGE	
α	63	60	2.9
β	54	50	3.0
γ	36	33	1.0
δ	17	19	1.2
1	17.5	16.5	4.4
2	15.5	15.5	1.3
3	17.5	14.5	0.95
4	16.5	13	1.2
5	10	8	6.4
heterodimer 3–4	28	25	1.1

For the latter column, lane 1 of Fig. 2B was scanned at 590 nm and the areas under peaks were divided by corresponding molecular mass determined by urea-SDS-PAGE.

4. DISCUSSION

The results presented show that the ATP synthase of a lower branch of eubacteria differs in subunit structure from the standard ATP synthases of upper eubacterial branches. Subunits α, β, γ, and 5 obviously have their counterparts in standard ATP synthases, while each of the residual subunits displays some unusual properties.

The F_1 part of standard ATP synthases during some isolation procedures loses the δ subunit [4,20,21]. The residual four-subunit complex retains ATPase activity but is unable to bind to the membrane part. Thus the δ subunit is assumed to be involved in functional binding of the F_1 and F_0. Being detached by sonication [14] or chloroform treatment, the F_1 part of the *C. aurantiacus* ATP synthase contained only four subunits. The possibility cannot be excluded that *C. aurantiacus* F_1 actually consists of five subunits. Then, in contrast to standard F_1-ATPases, it loses its smallest subunit (cf. lanes 1 and 2 in Fig. 2B) during its detachment or purification.

The F_0 of standard ATP synthases is composed of three or four subunit species depending on whether it contains two-copied subunit *b* or two related subunits *b* and *b'*: configurations $a_1b_2c_n$ and $a_1b_1b'_nc_n$, respectively. If the F_1 of *C. aurantiacus* consists of four subunit species as the purified catalytic part shows, the F_0 sector must contain an excess of subunit species as compared to standard ATP synthases. If we assume that *C. aurantiacus* F_1 is actually composed of five subunit species it must still contain the excess of polypeptides, since

polypeptide 1 is represented in more than one copy. However, if we treat the heterodimer as a single subunit, the ATP synthase will acquire pattern of eight subunits, where the heterodimer and subunit 1 (16.5 kDa) could take, respectively, the places of the *a* and *b* subunits of standard eubacterial ATP synthases. If the F_1 comprises five subunit species, one would assume subunit 2 (15.5 kDa) to be its smallest subunit. The task of further studies is to determine functional role of *C. aurantiacus* ATP synthase subunits with unusual features and possible homology with their putative counterparts.

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