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F_0 and F_1 parts of ATP synthases from *Clostridium thermoautotrophicum* and *Escherichia coli* are not functionally compatible

Amaresh Das and Lars G. Ljungdahl

Center for Biological Resource Recovery and Department of Biochemistry, University of Georgia, Athens, GA 30602, USA

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F_1-stripped membrane vesicles from *Clostridium thermoautotrophicum* and *Escherichia coli* were reconstituted with F_1-ATPases from both bacteria. Reconstituted F_1F_0-ATPase complexes were catalytically active, i.e. capable of hydrolyzing ATP. Homologous-type ATPase complexes having F_0 and F_1 parts of ATP synthases from the same origin were DCCD sensitive and supported ATP-driven enhancement of anilinonaphthalene sulfonate (ANS) fluorescence. Hybrid-type ATPase complexes having F_0 and F_1 parts of ATP synthases from different origins were neither DCCD sensitive nor did they support ATP-driven enhancement of ANS fluorescence. Analyzing these results it has been demonstrated that the F_0 and F_1 parts of ATP synthases of these two bacteria are not functionally compatible.

Clostridium thermoautotrophicum; *Escherichia coli*; F_1F_0-ATP synthase; In vitro reconstitution; Functional compatibility

1. INTRODUCTION

The membrane-bound proton-translocating F_1F_0 ATP synthase has been characterized in bacteria, mitochondria and chloroplasts [1]. It catalyzes both synthesis and hydrolysis of ATP [2]. Structurally it consists of two different components, a membrane-bound sector, F_0, which functions as a proton-translocating channel, and an extramembrane sector, F_1, which has catalytic activity [2]. The subunit compositions of both F_1 and F_0 parts of ATP synthases have been known from different sources. A minimum subunit composition has been reported for the ATP synthase of *Clostridium pasteurianum* which has 4 different subunits (3 in F_1 and 1 in F_0) [3], while a maximum subunit composition has been reported from the mitochondrial ATP synthase of bovine heart, which has at least 13 different subunits (5 in F_1 and 8 in F_0) [4]. An intermediate subunit composition has been reported for the ATP synthases from most other sources, including bacteria, mitochondria and chloroplasts, where an average of 8–10 subunits (5 in F_1 and 3–5 in F_0) are present [4]. It is important to know how ATP synthases from various sources are related with each other. Several reports are available on the functional compatibility of ATP synthases from various sources having similar subunit composition [5–7], however, there is no report on similar studies using ATP synthases from different sources having dissimilar subunit compositions.

Clostridium thermoautotrophicum is an anaerobic, thermophilic, acetogenic bacteria capable of both autotrophic and heterotrophic growth [8]. Under both growth conditions it derives energy from a functional electron transport chain using CO as an electron donor [9]. We have previously reported the presence of an F_1-ATPase in *C. thermoaceticum* which is composed of 4 different subunits, viz. α, β, γ and δ [10]. F_1-ATPase of identical subunit composition has been characterized also in *C. thermoautotrophicum* [11]. The whole enzyme (F_1F_0) has been isolated and purified from the latter bacterium in which the F_0 part is found to be comprised of only 2 subunits ([11], Ivey, Das and Ljungdahl, unpublished data). In this report the functional compatibility of the ATP synthase of *C. thermoautotrophicum* has been compared with that of *Escherichia coli* by in vitro reconstitution between F_0 and F_1 parts of their ATP synthases. By these studies is has been shown that the ATP synthases from these 2 bacteria are not functionally compatible.

2. MATERIALS AND METHODS

2.1. *Bacteria and growth*

Clostridium thermoautotrophicum strain 701/5 [12] was grown on 1% glucose at 58°C under 100% CO_2 in a medium whose composition has been described elsewhere [13]. *Escherichia coli* strain TG1 was grown in a minimal medium as described [14] with 0.2% ammonium succinate as the carbon and energy source. Cells from both cultures were harvested at mid log-phase and stored at −20°C until used.

2.2. *Preparation of membranes*

Everted membrane vesicles were prepared after breaking cells in a French press as described [10]. F_1-stripped membrane vesicles of *C.*

Correspondence address: A. Das, Membrane Biology Division, Central Drug Research Institute, Lucknow 226001, India.

Abbreviations: SDS, sodium dodecyl sulfate; DCCD, *N-N'*-dicyclohexylcarbodimide; PMSF, phenylmethylsulfonyl fluoride; ANS, anilinonaphthalene sulfonate.

thermoautotrophicum [10] and *E. coli* [6] were prepared from crude, washed membranes by further washing them in low ionic strength buffer in the presence of 0.5 mM EDTA until the ATPase-specific activities were less than 5% of the original value. The stripped membrane vesicles were finally suspended in buffer A (50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 1 mM DTT, 10% glycerol and 0.1 mM PMSF) to a final concentration of 5 mg protein/ml.

2.3. *Preparation of F_1-ATPases*

F_1-ATPase from *C. thermoautotrophicum* [10] and *E coli* [15] were prepared as described. The purity of the enzyme preparations were checked by SDS-PAGE (see below). The final preparation of F_1-ATPase from *C. thermoautotrophicum* (CTF_1) had a specific activity around 12 U per mg protein, and that of *E coli* (EF_1) of around 60 U per mg protein.

2.4. *Reconstitution experiments*

The procedure described by Steffens et al. [6] was followed with these modifications: F_1-stripped vesicles (2 mg protein), prepared as above, were treated with F_1-ATPase in buffer A to a final volume of 2 ml. The reaction mixture was incubated at 37°C or 60°C (details in the text) for 15 min and then centrifuged at 100,000×*g* for 30 min. The pellet containing the reconstituted membranes was collected and washed twice in buffer A before finally being suspended in the same buffer to a final protein concentration of 1 mg/ml. The ATPase activity of reconstituted membranes was determined as described below.

2.5. *Measurement of ATP-driven fluorescence of anilinonaphthalene sulfonate (ANS) by reconstituted membranes*

ANS fluorescence was measured in an Hitachi spectrofluorometer, model 204, using an excitation wavelength of 365 nm and measuring emission at 480 nm as described [16]. The reconstituted vesicles (0.3–0.5 mg) were suspended in a reaction mixture containing 50 mM Tris-H_2SO_4, pH 7.6, 1 mM $MgSO_4$ and 20 mM ANS, and incubated at a desired temperature (details in the text) for 20 min. The reaction was started by adding ATP (2 mM), and the enhanced fluorescence was measured for 5 min. To determine the effect of DCCD on ATP-driven enhancement of ANS fluorescence, reconstituted membranes in the above reaction mixture were incubated with DCCD (50 μM final concentration) for 30 min before the start of the reaction.

2.6. *Analytical procedure*

ATPase activity was determined according to Ivey and Ljungdahl [10] and the release of inorganic phosphate (P_i) was measured following the method of Baginsky et al. [17]. 1 unit of activity is defined as the release of 1 μmol of P_i per min, and the specific activity is the number of U per mg of protein. SDS-PAGE was done in the presence of 8 M urea following a modified method of Weber and Osborn, as described by Swank and Munkers [18]. Membrane and soluble proteins were determined by a modified Lowry method, as described by Markwell [19].

3. RESULTS

In order to establish a condition which supported the maximum binding of F_1 to stripped membranes, a fixed amount of membrane sample (usually 2 mg of protein) was treated with increasing amounts of F_1 (0.5–10.0 U) in a reconstitution reaction mixture (details in section 2). The reconstituted membranes were isolated and the ATPase activities of bound F_1 were determined. When the ATPase activities of isolated reconstituted membranes were plotted against the units of F_1 added, a ratio of 3 U per mg of membrane protein was established, which supported the optimum activity of bound F_1 in reconstituted membranes. This value was the same for both EF_1 and CTF_1 bound to either homologous or heterologous stripped membranes. Therefore, we always maintained this ratio (i.e. 3 U of F_1 per mg of membrane protein) in all of our reconstitution experiments reported here. The results (Table I) show that the stripped membrane vesicles of *E. coli* and *C. thermoautotrophicum* were capable of re-binding both homologous and heterologous F_1 parts. Higher ATPase activities of bound F_1 were recorded in homologous F_1F_0-ATPase complexes (e.g. EF_0+EF_1 or CTF_0+CTF_1) than in hybrid F_1F_0-ATPase complexes (e.g. EF_0+CTF_1 or CTF_0+EF_1). It was found that the efficiency of binding between F_0 and F_1 was higher when they were from homologous origins than when they were from heterologous origins (unpublished data). In order to see the effect of temperature on the binding of F_1 to stripped membranes, reconstitution reactions were carried out at 2 different temperatures, 37 and 60°C (Table II). It was found that bound EF_1 and CTF_1 showed their optimum activities in both homologous and hybrid F_1F_0-ATPase

Table I

ATPase activities of bound F_1 from *E. coli* (Ec) and *C. thermoautotrophicum* (Ct) after reconstitution with homologous and heterologous stripped (F_1-depleted) membrane vesicles[a]

No.	Source of stripped membrane	Source of F_1-ATP	Reconst. temp. (°C)	ATPase activity of reconstituted vesicles[b]	
				37°C	60°C
1	Ct	Ct	37	0.20	0.85
2	Ct	Ec	37	1.25	0.78
3	Ct	Ct	60	0.28	1.95
4	Ct	Ec	60	0.92	0.64
5	Ec	Ct	37	0.19	0.70
6	Ec	Ec	37	2.00	0.95
7	Ec	Ct	60	0.22	0.84
8	Ec	Ec	60	1.32	0.68

[a] Reconstitutions were done as described in section 2. The results are the average of 3 separate experiments.
[b] ATPase activities are expressed as μmol P_i released per min per mg of protein.

complexes when they were reconstituted and assayed at 37°C and 60°C, respectively. The best results, however, were obtained for homologous F_1F_0-ATPase complexes. The temperature of reconstitution had no significant effect on the activities of bound EF_1 or CTF_1 in hybrid F_1F_0-ATPase complexes provided that they were assayed at their respective optimum temperatures, i.e. 37°C for EF_1 and 60°C for CTF_1. The bound EF_1 became partially inactive at 60°C while bound CTF_1 was completely inactive at 37°C, indicating that CTF_1 is more selective towards its optimum temperature (i.e. 60°C) for activity than EF_1.

DCCD is known to block proton translocation through F_0 during energy transfer reactions catalyzed by F_1F_0-type ATP synthases [20]. Therefore by DCCD inhibition studies it may be possible to find out whether or not the catalytic activity of reconstituted F_1F_0-ATPase complexes is coupled to proton translocation. The results (Fig. 1) show that DCCD effectively inhibited the ATPase activity of bound F_1 in homologous F_1F_0-ATPase complexes but that it had no effect on the ATPase activity of hybrid F_1F_0-ATPase complexes. These results indicate that functional activity was restored in homologous F_1F_0-ATPase complexes but was lost in hybrid F_1F_0-ATPase complexes. The effective concentrations of DCCD which inhibited 50% of ATPase activities of bound F_1 in reconstituted (EF_0+EF_1) and (CTF_0+CTF_1) complexes were found to be 0.5 and 6.0 μM, respectively, indicating that reconstituted homologous complexes from *E. coli* are more sensitive to DCCD than those of *C. thermoautotrophicum*.

ATP hydrolysis by a functionally active F_1F_0-ATPase complex in either intact or reconstituted membrane vesicles develops a membrane potential (positive inside) which attracts negatively charged ANS, and this results in increased fluorescence [16]. Here we found that the ATP-driven enhancement of ANS fluorescence was observed only in homologous F_1F_0-ATPase complexes and was inhibited by DCCD. No ATP-driven enhancement of ANS fluorescence was observed in hybrid F_1F_0-ATPase complexes. These results confirm that the F_1 and F_0 parts of ATP synthases from *C. thermoautotrophicum* and *E. coli* fail to reconstitute functionally active hybrid F_1F_0-ATPase complexes. The functional activity of F_1F_0-ATPase complex is, however, restored when F_1 and F_0 parts of ATP synthases are reconstituted from the same source.

Table II

ATP-driven ANS fluorescence by reconstituted membranes[a]

Type of reconstitution	Assay temp. (°C)	Enhancement of ATP-driven ANS fluorescence (%)	
		-DCCD	+DCCD
EF_0+EF_1	37	68.0	–
EF_0+EF_1	60	41.0	–
EF_0+CTF_1	37	7.0	–
EF_0+CTF_1	60	7.6	–
CTF_0+EF_1	37	10.5	–
CTF_0+EF_1	60	9.8	–
CTF_0+CTF_1	37	36.4	–
CTF_0+CTF_1	60	58.5	–

[a] Details of the fluorescence study are described in section 2. Reconstituted membranes of all types were prepared at 37°C and the fluorescence was measured at 37 and 60°C.

[b] ATP-driven ANS fluorescence by intact membranes of *E. coli* and *C. thermoautotrophicum* were taken as 100%. The % enhancement of fluorescence by reconstituted membranes of *E. coli* and *C. thermoautotrophicum* given in the table are relative to that of their intact membranes, respectively.

4. DISCUSSION

Reconstitution studies using F_1 and F_0 parts of ATP synthases from different sources can provide important information on their relationships at the functional level [21]. In this study we have shown evidence that F_1 and F_0 parts of ATP synthases from *E. coli* and *C. thermoautotrophicum* can bind to each other but fail to reconstitute functionally active hybrid F_1F_0-ATPase complexes. This is probably the first study to report that F_1 and F_0 parts of ATP synthases from two different bacteria are

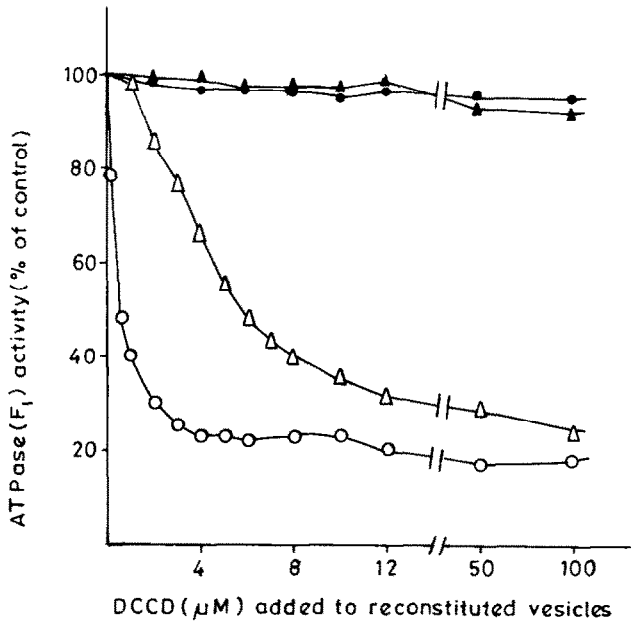

Fig. 1. Effect of DCCD on the ATPase activity of bound F_1 in reconstituted F_1F_0-ATPase complexes. F_1-stripped membrane vesicles of *E. coli* were reconstituted with EF_1 (●) and CTF_1 (▲) at 37°C as described in section 2. F_1-stripped membrane vesicles of *C. thermoautotrophicum* were reconstituted similarly with EF1 (○) and CTF_1 (△) as above. The activities of bound EF_1 and CTF_1 were measured at 37°C and 60°C, respectively. Prior to ATPase assay the reconstituted vesicles (500 μl) were diluted twice with reconstitution buffer (composition given in section 2) and treated with the indicated amount of DCCD in methanol (5 μl) for 30 min. The controls were treated with identical amounts (5 μl) of methanol.

not functionally compatible. By DCCD inhibition and fluorescence studies it has been shown that only homologous F_1F_0-ATPase complexes are active, both functionally and catalytically, while the hybrid F_1F_0-ATPase complexes are active only catalytically. These results indicate that a mere binding between F_1 and F_0 does not always yield a functionally active F_1F_0-ATPase complex, rather a specific binding between the two is required for functional activity. Such a specific binding might have taken place in homologous F_1F_0-ATPase complexes but not in hybrid F_1F_0-ATPase complexes. By in vitro reconstitution studies, either between F_1 and F_0 parts of ATP synthases or by using different combinations of subunits of ATP synthase from different bacteria, it has been demonstrated that ATP synthases from *Salmonella typhimurium*, *Klebsiella pneumoniae* and the thermophilic bacilli, PS3, are functionally compatible with that of *E. coli* [5–7]. These differences in results may be due to several reasons. Firstly, the subunit compositions of both F_1 and F_0 parts of ATP synthases are similar in *S. typhimurium*, *E. coli*, *K. pneumoniae* and PS3 [4,5,7,22] but differ from *C. thermoautotrophicum* as mentioned earlier. Secondly, some of the important subunits of ATP synthases are quite homologous among these species, as evidenced from their primary structure analysis, as well as by in vitro reconstitution, complementation and immunological studies [5–7,23]. Although we do not have sufficient data on comparative analysis of all subunits of F_1 and F_0 parts of ATP synthases from *E. coli* and *C. thermoautotrophicum*, we have at least found that none of the CTF_0 subunits cross-react with antibodies against EF_0 subunits (Das and Ljungdahl, unpublished data). These results indicate that, structurally and immunologically, the ATP synthase from *E. coli* is different from that of *C. thermoautotrophicum*.

Oligomeric structures of F_1 and F_0 parts of ATP synthases play a crucial role in reconstitution reactions, as suggested by Schneider and Altendorf [24]. By in vitro reconstitution studies these authors have demonstrated that the F_1 part of the ATP synthase from *E. coli* only recognizes an oligomeric structures conferred by all 3 subunits of F_0. Further support to the above statement comes from in vitro reconstitution studies between F_0 and F_1 parts of mitochondrial ATP synthases reported by Scatz et al. [25]. These authors demonstrated that the F_1 part of yeast mitochondrial ATP synthase fails to reconstitute oxidative phosphorylation of bovine heart sub-mitochondrial particles which are fully depleted of F_1. Interestingly, the major difference between mitochondrial ATP synthases from these 2 sources is their subunit composition at the F_0 level. The F_0 part of yeast mitochondrial ATP synthase has 5 different subunits while that of bovine mitochondrial ATP synthase has at least 8 different subunits [4]. The F_1 part of ATP synthases from both species has, however, similar subunit composition, i.e. each has 5 different subunits [4]. In another study, Bar-Zvi et al. [26] reported that the F_1 part of the ATP synthase from the thermophilic bacilli, PS3, can bind F_1-depleted lettuce thylakoid chloroplast particles but failed to reconstitute photophosphorylation activity. Here also the subunit composition of the F_0 part of ATP synthases from these 2 sources are different (F_0 from lettuce chloroplast has 4 different subunits while that from PS3 has 3), although both have similar subunit composition in their respective F_1 parts [4,22].

From the above discussion it appears that the oligomeric structures of F_0 and F_1 parts of ATP synthases play a crucial role in reconstitution reactions. One of the essential criteria towards the formation of a functionally active hybrid F_1F_0-ATPase complex is that the oligomeric structures of interspecies F_1's and F_0's should be similar. However, to confirm this it is necessary to do more reconstitution studies using ATP synthases of dissimilar subunit compositions. It is also important to know the primary structures of the important subunits (e.g. β and γ) of these ATP synthases since it may be possible that they are different from that present in *E. coli* or PS3.

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