RESOLUTION OF THE CHLOROFORM-RELEASED CF_1 INTO ATPase COMPLEXES DIFFERING IN THEIR ATPase ACTIVITY

Identification of one subunit as a putative natural inhibitor of the ATPase

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1. Introduction

The thylakoid membrane of chloroplasts, the plasma membrane of bacteria, and the inner membrane of mitochondria contain a H^+-translocating ATPase which is common for energy transduction in such membranes [1]. The enzyme is composed of two sectors, an intrinsic membrane part (F_0) and a part extrinsic to the membrane (F_1, ATPase). Several methods have been advanced for isolation and study of the subunit structure and function of this enzyme [2,3]. Among these procedures, the chloroform method [4,5] is distinguished by its simplicity, suitability for both large and small scale preparations, and ability to extract other membrane peptides which could be associated with the enzyme function [6].

In [5] we described the chloroform-released CF_1 ATPase as containing 7 subunits. At that time, 2 of these were considered as contaminants. Here we show that at least 1 of these 2 subunits has a biological function and should be considered as a true subunit of the enzyme.

2. Materials and methods

Spinach was purchased from a local market and chloroplasts were prepared as in [5]. Preparations 1–10 in table 1 were made as follows: prep. (1) as in [5] to release CF_1 from swollen thylakoid membranes, except the final conc. of 1–2 mg chl/ml prior to treatment with chloroform; prep. (3), (5), (6) were made from prep. (1) when passed through a Sephadex G-200 column (1.7 cm × 100 cm) and eluted at a flow rate of 3–4 ml/h with a solution of 20 mM Tris–HCl, 2 mM EDTA, 1 mM ATP and 1 mM DTT; prep. (6) was eluted first followed by the 5 subunit version of the enzyme and then prep. (5); prep. (7)–(10), the 7 subunit enzyme was treated with pyridine and the protein in the supernatant was precipitated by ethanol [5]. The ethanol precipitate, (3 mg protein) was dissolved in a buffer containing 7 M urea and 20 mM Tris–HCl and applied to a column (1 cm × 6 cm) of DEAE-cellulose. A stepwise gradient of NaCl was used to elute the protein; 4 ml Tris–urea buffer, 10 ml buffer containing 25 mM NaCl, this eluted a fraction containing α- and β-subunits, 15 ml buffer containing 0.1 M NaCl (prep. (8) and (10) were eluted here) and finally 15 ml containing 0.2 M NaCl which eluted prep. (9). Preparations (2) and (4) were made by applying 8 mg purified CF_1 (prep. (3)) dissolved in 20 mM Tris–HCl (pH 8) and 7 M urea to a DEAE-cellulose column (1 cm × 7 cm) equilibrated with the same buffer at a flow rate of 30 ml/h, and was eluted with the same buffer containing 0.2 M NaCl. Fractions of CF_1 lacking either the ϵ-subunit or the ϵ- and δ-subunits were collected.

Chlorophyll was determined as in [7] and protein as in [8] scaled down to 1 ml. Polyacrylamide gel electrophoresis analysis was done as in [9] with 10% polyacrylamide in the presence of 0.05% SDS. Ca^{2+}-dependent ATPase activity of the different proteins was activated and assayed as in [10] and as in table 1. The coupling activity of CF_1, CF_1 in complex with C_2 and CF_1 in complex with C_1 and C_2 was assayed as in [11].

Abbreviations: CF_1, chloroplast coupling factor 1; chl, chlorophyll; EDTA, ethylene diamine tetraacetic acid; Tris, *N*-[tris-(hydroxymethyl)] amino methane; DTT, dithiothreitol; SDS, sodium dodecylsulphate; PMS, *N*-methyl-phenazonium methosulphate

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3. Results and discussion

3.1. *Polyacrylamide gel electrophoretic analysis of CF_1 preparations with modified subunit composition*

Electrophoretic analysis in non-dissociating gels of the CF_1 (table 1) (1–10) showed that all of these were essentially single protein complexes. Typical scans of the SDS gel electrophoretic analysis of some of the preparations listed in table 1 are shown in fig.1. In fig.1(a) the 7 subunits of preparation 1 and their M_r-values are illustrated. Fig.1(b) shows the 5 subunits typical of CF_1 (prep. (3)) [12,13]. Fig.1(c,d) show prep. (5) and (6) which have the 5 subunits typical of CF_1 plus C_1 or, C_2, respectively. In fig.1(e) and 1(f) traces of prep. (4) and (2) (table 1), containing the subunits $\alpha,\beta,\gamma,\delta$ and α,β,γ, respectively, are shown. Preparation (7) containing only α-, β-subunits has been described [5]. A further modified CF_1 which contains α-, β-, δ- and ϵ-subunits was also described in [5]. This preparation was not used here. These results demonstrate that many combinations of the constituent subunits of CF_1 can be obtained. This presents a unique opportunity to study the roles of the subunits in both the hydrolysis of ATP and the capacity of CF_1 to reconstitute ATP synthetic activity.

Table 1
The variation of heat-activated Ca^{2+}-ATPase activity with the subunit composition of CF_1 preparations

Prep. no.	Subunit composition	Ca^{2+}-ATPase activity (μmol . min^{-1} . mg protein^{-1})
1	$\alpha\beta\gamma C_1 \delta \epsilon C_2$	12
2	$\alpha\beta\gamma$	16
3	$\alpha\beta\gamma \quad \delta\epsilon$	17
4	$\alpha\beta\gamma \quad \delta$	16.4
5	$\alpha\beta\gamma C_1 \delta \epsilon$	16.5
6	$\alpha\beta\gamma \quad \delta \epsilon C_2$	13
7	$\alpha\beta$	15.4
8	$\alpha\beta \quad C_1$	15.0
9	$\alpha\beta \quad C_1 \quad C_2$	2.5
10	$\alpha\beta \quad\quad C_2$	3.0

Ammonium sulphate suspensions of the various preparations were centrifuged and the pellets were dissolved in 40 mM Tris–HCl (pH 8) and recentrifuged again at 14 000 × *g* for 10 min. Protein concentrations in the supernatants were adjusted to 2 mg/ml with buffer and were mixed 1:1 (v/v) with a solution of 40 mM Tris–HCl (pH 8), 40 mM ATP and 10 mM DTT before heat activation as described in the text. Ca^{2+}-ATPase activity was determined after addition of 20 μl heat-activated mixture to 0.9 ml assay medium

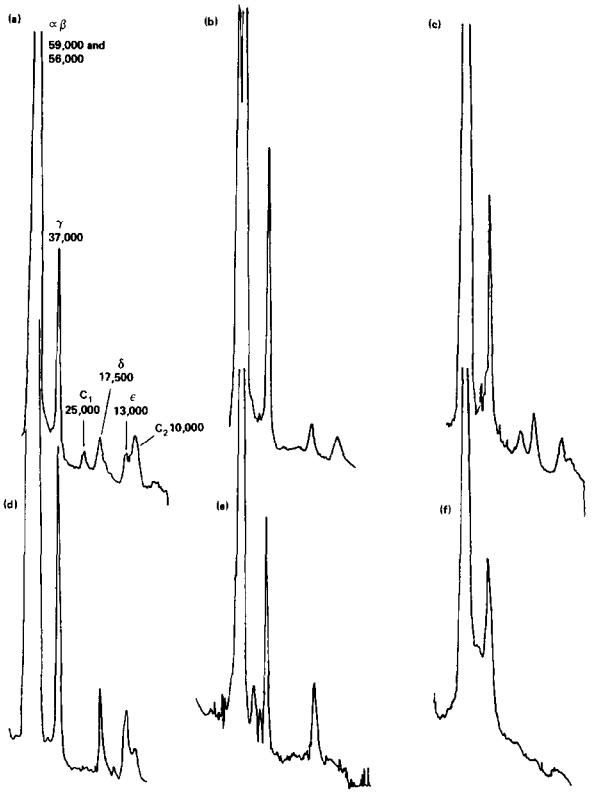

Fig.1. Densitometric traces showing the subunit structure of the different preparations: (a) the 7 subunit enzyme; (b) the 5 subunit enzyme; (c) the 5 subunit enzyme + C_1; (d) the 5 subunit enzyme + C_2; (e) the $\alpha\beta\gamma\delta$ preparation; (f) the $\alpha\beta\gamma$ preparation; Conditions were as in section 2.

3.2. *ATPase activity of various combinations of CF_1-subunits*

From the data presented in table 1 it can seen that the ATPase activity of CF_1 (prep. (3)) was not affected significantly by:

(i) The presence of subunit C_1 (cf. prep. (3) and (5)).
(ii) The absence of the ϵ subunit (cf. prep. (3) and (4)). This is a surprising observation since the ϵ subunit has been shown to inhibit CF_1-ATPase activity [14].
(iii) The removal of the δ- and ϵ-subunits (cf. prep. (2) and (3)).
(iv) The removal of the γ-, δ- and ϵ-subunits (cf. prep. (3) and (7)). This observation suggests that the ATP-hydrolytic site is located on one or both of the major subunits, similar to the conclusion in [15].

Those preparations of CF_1 which contained subunits $C_1 + C_2$ (prep. (1)) or subunit LC_2 (prep. (6))

Table 2
Effect of subunits C_1 and C_2 on the ability of CF_1 to reconstitute photophosphorylation

Preparation	Rate of cyclic photophosphorylation (μmol ATP . h^{-1} . mg chl^{-1})
Broken chloroplasts	290
EDTA particles	5
+ 100 μg CF_1 (prep. (3))	74
+ 100 μg CF_1–C_2 (prep. (6))	72
+ 100 μg CF_1–C_1–C_2 (prep. (1))	73

The preparation of depleted EDTA particles and their reconstitution with different CF_1 preparations, as well as the assay of the PMS cyclic photophosphorylation were done as in [11]

had an ATPase activity which was significantly lower than of CF_1 (prep. (3)). The most dramatic effect on ATPase activity is seen when subunit C_2 is in combination with either the α-, β-subunits (prep. (10)) or the α-, β-, C_1-subunits (prep. (9)). The hydrolytic activity is at least 80% inhibited. We conclude that C_2 has a profound inhibitory effect on the ATPase activity. This inhibitory effect of subunit C_2 is ameliorated when subunit C_2 is in the presence of the γ-, δ- and ϵ-subunits (cf. prep. (1) and (6)). The highest ATPase activity is seen when the subunit C_2 is absent (cf. prep. (1,5) and (3,6)).

The presence or absence of C_1 in any combination of subunits has little effect on ATPase activity (cf. prep. (9,10), (1,6) and (3,5)). However, this subunit has M_r and properties which are similar to those of the 26 500 M_r subunit of the rat liver mitochondrial ATPase in [6]. The presence of this subunit of the rat liver ATPase did not affect the app. K_m of the enzyme for MgATP hydrolysis, but it was essential for the association of the ATPase to silicotungstate-treated rat liver mitochondrial membranes, and for the protection of the enzyme activity against low temperatures. Subunit C_1 may have a similar function and therefore, prep. (5) may be the form of the chloroplast ATPase that corresponds to the type 1 ATPase in [6]. Our observation showed that either C_1 or C_2 can protect CF_1 ATPase activity against inactivation at low temperature.

3.3. *The effect of subunits C_1 and C_2 on the ability of CF_1 to enhance ATP-synthesis*

The data presented in table 2 show that CF_1 (prep. (3)) can enhance significantly the ability of EDTA-particles to perform cyclic photophosphorylation and that $CF_1 + C_2$ (prep. (6)) and $CF_1 + C_1 + C_2$ (prep. (1)) have the same ability as CF_1 to potentiate cyclic photophosphorylation. Thus, although the ATPase activity of CF_1 was inhibited in the presence of C_2 its coupling activity was not affected by either this protein or by C_1.

We conclude that subunit C_2 present in some of these preparations of the chloroplast CF_1-ATPase has functional properties, which are similar to those of the mitochondrial inhibitor protein.

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