Preparation of an ϵ-deficient chloroplast coupling factor 1 having a high ATPase activity

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A modification of the chloroform extraction preparation of CF_1, by elevating the pH during the DEAE–Sephadex purification step to 7.8 yields a 5-subunit CF_1. Different 4-subunit CF_1 preparations deficient in either δ- or ϵ-subunits, as well as pure β-subunits are obtained by fractionation of intact CF_1 complex by anion-exchange high-performance liquid chromatography. Unlike the 5-subunit latent enzyme, ϵ-deficient CF_1 has high Ca-ATPase activity which can be inhibited by purified ϵ-subunits. After trypsin or octylglucoside activations all preparations have identical high ATPase activity. These results suggest that the subunit regulates CF_1-ATPase activity.

Chloroplast coupling factor 1 (CF_1) *ATPase regulation* *ϵ-Subunit* *High-performance liquid chromatography*

1. INTRODUCTION

Chloroplast coupling factor 1 (CF_1) is the extrinsic membrane protein sector of the energy-transducing reversible ATPase. When released from the membranal sector (CF_0) it is a soluble latent ATPase which is composed of 5 different polypeptide subunits, termed α, β, γ, δ and ϵ in decreasing M_r order [1].

A simple and rapid method to prepare CF_1 is by chloroform extraction [2]. The main disadvantage of this technique is that following the final purification step on DEAE–Sephadex column a δ-less CF_1 preparation is obtained, which does not recouple photophosphorylation when added to CF_1-deficient chloroplast particles, in contrast to 5 subunit preparations obtained by other procedures [3,4].

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Abbreviations: CF_1, chloroplast coupling factor 1; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; OG, octyl-D-glucopyranoside (octylglucoside); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis

The latent CF_1 can be activated to hydrolyze ATP in the presence of Ca^{2+} by treatments with trypsin [5], DTT [6] or heat [7] or in the presence of Mg^{2+} by ethanol [8] or OG [9]. However, the mechanism which controls the expression of CF_1 ATPase activity seems to be complex and is not yet fully understood. Protein inhibitory subunits [10,11], thiol modulation [12] and tightly-bound adenine nucleotides [13] have been suggested to be involved in this regulation. The possible role of the ϵ-subunit of CF_1 as an ATPase inhibitor has been controversial. In [10] it was demonstrated that a purified ϵ-subunit, obtained from pyridine-treated CF_1, inhibits Ca-ATPase activity of heat-activated 5-subunit CF_1 and it was suggested that ϵ is the ATPase inhibitor of CF_1. This suggestion was criticized recently since activation of CF_1 ATPase by proteolytic digestion is correlated with a modification of the α-subunit rather than ϵ [14,15].

It is also puzzling that in analogous enzymes from mitochondria and the bacterium *Escherichia coli* the ϵ-subunit seems to play different roles. The functions and the analogy of the ϵ-subunits from these enzymes to that of the CF_1 are reviewed in [1].

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We here report on the preparation of an ϵ-deficient CF_1 by a mild non-denaturing technique. This preparation has high Ca-ATPase activity without any further activation.

2. MATERIALS AND METHODS

Spinach thylakoid membranes were prepared as in [4]. Chloroform extraction was performed as in [2]. The CF_1-containing supernatant was applied to a DEAE–Sephadex column preequilibrated with 20 mM Tris–SO_4, 1 mM EDTA, 0.1 mM ATP, 10 mM $(NH_4)_2SO_4$ and 10% (v/v) glycerol at a final pH of 7.8. The protein was eluted by a linear gradient (10–400 mM) of $(NH_4)_2SO_4$ in the same buffer. The CF_1 was eluted off the column as a broad peak between 0.28 and 0.36 M ammonium sulfate.

HPLC was performed on an Altex Model 330 liquid chromatograph (Beckman Instruments). A Mono-Q HPLC anion exchange column (5 × 50 mm) was equilibrated with 50 mM lithium–borate (pH 8.5), in 20% (v/v) ethylene glycol (buffer A). CF_1 preparation was loaded at a flow rate of 0.5 ml/min. The column was then washed with buffer A for 15 min, followed by a 60-min linear gradient of NaCl (0–500 mM) in buffer A. The protein elution profile was obtained by an automated fluorescamine monitoring system [16].

Purification of ϵ-subunits from spinach CF_1 was performed as in [17].

Protein was determined as in [18] using bovine serum albumin as a standard.

Mg-ATPase with or without 40 mM OG, was assayed as in [9]. Ca-ATPase with or without trypsin was assayed as in [17]. Heat activation of Ca-ATP was performed as in [19].

SDS–PAGE (10%) was performed as in [20].

DEAE–Sephadex and the MONO-Q HPLC column were purchased from Pharmacia, OG was obtained from Calbiochem and the other chemicals were purchased from Sigma.

3. RESULTS

CF_1 was prepared from spinach chloroplasts by the chloroform extraction technique [2] and purified on DEAE–Sephadex A-50 column preequilibrated and eluted at pH 7.8. The high pH causes a shift in the salt concentrations required to elute the enzyme off the column in comparison to [3] and it is eluted as a broad peak between 0.28 and 0.36 M $(NH_4)_2SO_4$. The purified enzyme contains 5 subunits when analyzed by SDS–PAGE (fig.2, lane 5). This preparation is identical in composition to 5-subunit CF_1 preparations prepared by EDTA extraction [3] or by the sucrose ATP extraction procedures [4] (not shown), but differs from the reported preparation obtained by chloroform extraction followed by DEAE–Sephadex purification at pH 7.1 which lacks the δ-subunit [2]. It appears, therefore, that the more alkaline pH prevents the detachment of the δ-subunit during the DEAE–Sephadex purification step.

Similar to other CF_1 preparations, the chloroform-extracted purified enzyme has very low Ca-ATPase and Mg-ATPase activities which can be exposed by trypsin or by octylglucoside, respectively (table 1, control).

In order to test the purity of this CF_1 preparation the enzyme was analyzed by HPLC on an anion exchange column and eluted with a linear gradient of NaCl (0–0.5 M). The elution profile in fig.1 demonstrates 4 main protein peaks, 3 (c–e) very close to each other and one (a) separated from them. The subunit composition of the protein fractions was analyzed by SDS–PAGE (fig.2). The analysis demonstrates that 3 different CF_1 species, composed of different subunits, have been resolved (lanes 2–4). A δ-less CF_1 (fraction c, lane 2), an

Table 1

ATPase activities of the different CF_1 fractions

Fraction	Mg^{2+} ATPase		Ca^{2+} ATPase	
	Untreated	OG activated	Untreated	Trypsin activated
Control	0.3	21.9	1.2	12.3
c	0.04	23.5	0.6	11.6
d	0.09	22.8	0.8	10.8
e	1.5	23.1	8.6	12.3

Ca^{2+}- or Mg^{2+}-dependent ATPase activities of the different CF_1 preparations eluted from the HPLC (fig.1) were assayed with or without activation as described in section 2. Control is CF_1 before fractionation by HPLC. Rates are expressed in μmol ATP hydrolyzed · mg protein^{-1} · min^{-1}

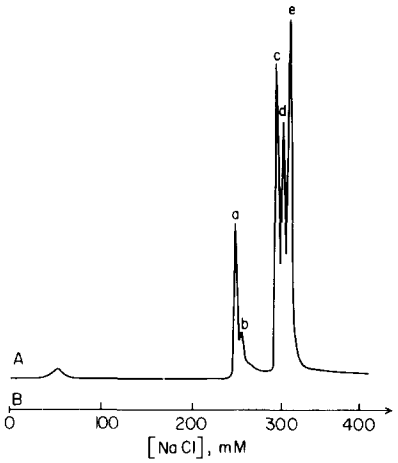

Fig.1. Fractionation of CF_1 by anion-exchange HPLC. CF_1 (3 mg) was resolved on a Mono Q HPLC column at pH 8.5 by an NaCl gradient. The protein elution profile (A) was obtained by an automated fluorescamine method [16]. NaCl concentration (B) is indicated. For further details see section 2.

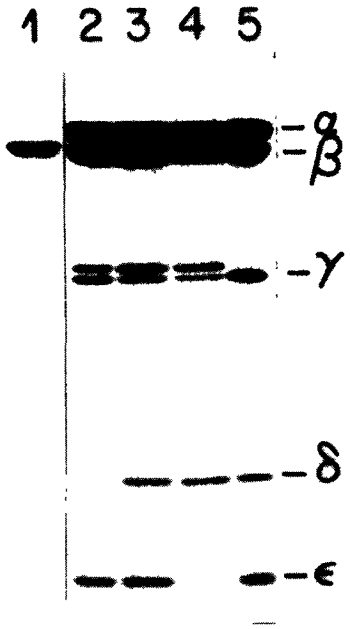

Fig.2. SDS–PAGE (10%) of fractions from HPLC of CF_1. Lanes 1–5 represent peaks a, c, d, e (fig.1) and unfractionated CF_1, respectively. 10 (lane 1) or 25 (lanes 2–5) μg protein were applied.

intact CF_1 (fraction d, lane 3), and an ϵ-less CF_1 (fraction e, lane 4) are sequentially eluted off the column by increasing salt concentrations.

ATPase activities of the different CF_1 fractions were tested with or without further activation (table 1). The results clearly demonstrate that the unactivated ϵ-deficient preparation (fraction e) has much higher Ca-ATPase and Mg-ATPase activities than the δ-less or the intact 5-subunit CF_1. However, following a Mg-ATPase activation by octylglucoside or Ca-ATPase activation by trypsin, the activities of all 3 preparations become essentially identical. Ca-ATPase activity of the ϵ-deficient CF_1 is strongly inhibited by purified spinach ϵ-subunits (table 2). These results strongly suggest that the ϵ-subunit does play a role in suppressing ATPase activity of CF_1.

Analysis of fraction a (fig.1) demonstrates that it contains pure β-subunit (fig.2, lane 1). The identification of this protein was confirmed by immunoprecipitation with rabbit anti-β antiserum and *Staphylococcus aureus* protein A as in [21] (not shown).

The minor peak adjacent to the β-subunit (peak b) contains a polypeptide which may be a contamination in the CF_1 preparation. This protein was identified by labeling with fluorescein isothiocyanate at pH 7 and it migrates as a narrow band close to the β-subunit band of CF_1 on SDS–PAGE (submitted).

Table 2

Inhibition of Ca-ATPase activity of ϵ-deficient CF_1 by purified ϵ

Preparation	Ca-ATPase	
	Control	+ Purified ϵ
Control heat-activated CF_1	12.3	4.2
ϵ-Deficient CF_1	7.8	1.4
ϵ-Deficient heat-activated CF_1	11.2	3.8

CF_1 preparations (2 μg) were preincubated for 5 min at 23°C with or without purified ϵ (3 μg) in 0.5 ml containing 40 mM Na–tricine (pH 8). Heat-activation and measurement of Ca-ATPase activity was performed as described in section 2. Rates are expressed in μmol ATP hydrolyzed · mg protein^{-1} · min^{-1}

4. DISCUSSION

This work demonstrates that the two smallest CF_1 subunits (δ and ϵ) may be dissociated from the enzyme by anion-exchange chromatography under specified conditions. Thus, careful control of the pH during the DEAE–Sephadex purification step following chloroform extraction of spinach CF_1 will determine whether or not δ dissociates from the enzyme. Furthermore, the resolution of CF_1 by anion-exchange HPLC into 3 distinct enzyme populations may be due either to a preexisting heterogenous population of CF_1 or, alternatively, to a partial dissociation of δ or ϵ from CF_1 during chromatography. The latter interpretation seems more likely since there is a large increase in the overall Ca-ATPase activity (without further activation) following HPLC which cannot be accounted for by a preexisting ϵ-deficient CF_1. Since the δ- and ϵ-subunits are the first to be detached from CF_1 by treatment with trypsin [14,15] pyridine or digitonin + heat [10], and since δ binds strongly to DEAE–Sephadex [2] it is not surprising that these subunits will be detached from CF_1 after HPLC. It is not clear at present whether it is the high pressure or the high charge density of the HPLC anion exchanger which lead to the detachment of δ or ϵ. It is also not clear at present whether the presence of pure β-subunits is due to a complete dissociation of part of the enzyme on the column or to preexisting β-subunits which have been copurified with CF_1. Another puzzling observation for which we have no explanation is the appearance of a split γ on SDS–PAGE following the HPLC purification (fig.2).

The role of the ϵ-subunit as an ATPase protein inhibitor in CF_1 has been controversial. Authors in [10] demonstrated that the purified ϵ-subunit isolated from pyridine-treated CF_1 inhibits Ca-ATPase activity of heat-activated CF_1, and suggested that ϵ may be the ATPase inhibitor of CF_1. However, the physiological significance of these results may be questioned because the heat-activated CF_1 preparation used in these experiments was not depleted of its native ϵ-subunit. In the work described here an ϵ-deficient CF_1 preparation was purified by a relatively mild procedure. The clear correlation between the presence of the ϵ-subunit and ATPase inhibition (table 1) and the sensitivity of the ϵ-deficient preparation to purified subunits (table 2) strongly suggest that ϵ is indeed the native ATPase inhibitor of CF_1.

The role of the ϵ-subunit in the regulation of CF_1 ATPase has been previously criticized since it was demonstrated that activation of CF_1 Ca-ATPase by proteolytic digestion is correlated with a modification of the α-subunit but with no apparent effect on the ϵ [14]. Similarly, we previously observed that CF_1-Mg-ATPase activation by OG micelles was independent of the presence or absence of the ϵ-subunit [15]. The demonstration that both trypsin and OG lead to an additional activation of ϵ-depleted CF_1 (table 1) is also consistent with these results. This apparent discrepancy concerning the role of the ϵ-subunit may be reconciled by assuming that the derepression of CF_1-ATPase activity involves a sequential mechanism in which the dislocation of ϵ leads to the activation of the catalytical subunits (α and β). ATPase activation by trypsin or by OG may thus bypass the ϵ subunit by directly affecting the catalytical subunits of CF_1.

It should be mentioned that the latency of CF_1 ATPase has broader implications on the activation of ATP synthesis in illuminated thylakoids. It has been demonstrated that in chloroplasts there is a correlation between the level of energization and the rate of ATP synthesis, activation of ATP hydrolysis and the exchange of tightly bound nucleotides, which suggests that energization induces a conformational change in CF_1 which triggers its catalytical activity (review [22]). It is suggested, therefore, that energization of chloroplast thylakoid membranes by ΔpH^+ induces a sequence of conformational changes in CF_1. Dislocation of the ϵ-subunit is a primary event which in turn leads to changes in the catalytical subunits, possibly in α, leading to the derepression of the catalytic activity.

The isolation of a pure, water-soluble, β-subunit by HPLC may also be of help in future studies for assessing its specific role in ATP synthesis and hydrolysis and in studies of reconstitution of CF_1 from its individual subunits.

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