

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

Moshe Finel, Menachem Rubinstein⁺ and Uri Pick*

Biochemistry Department and ⁺Virology Department, The Weizman Institute of Science, Rehovot 76100, Israel

Received 21 October 1983

A modification of the chloroform extraction preparation of CF₁, by elevating the pH during the DEAE–Sephadex purification step to 7.8 yields a 5-subunit CF₁. Different 4-subunit CF₁ preparations deficient in either δ - or ϵ -subunits, as well as pure β -subunits are obtained by fractionation of intact CF₁ complex by anion-exchange high-performance liquid chromatography. Unlike the 5-subunit latent enzyme, ϵ -deficient CF₁ 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 CF₁-ATPase activity.

Chloroplast coupling factor 1 (CF₁) ATPase regulation ϵ -Subunit
High-performance liquid chromatography

1. INTRODUCTION

Chloroplast coupling factor 1 (CF₁) is the extrinsic membrane protein sector of the energy-transducing reversible ATPase. When released from the membranal sector (CF₀) 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 CF₁ 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 CF₁ preparation is obtained, which does not recouple photophosphorylation when added to CF₁-deficient chloroplast particles, in contrast to 5 subunit preparations obtained by other procedures [3,4].

* To whom correspondence should be addressed

Abbreviations: CF₁, 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 CF₁ can be activated to hydrolyze ATP in the presence of Ca²⁺ by treatments with trypsin [5], DTT [6] or heat [7] or in the presence of Mg²⁺ by ethanol [8] or OG [9]. However, the mechanism which controls the expression of CF₁ 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 CF₁ as an ATPase inhibitor has been controversial. In [10] it was demonstrated that a purified ϵ -subunit, obtained from pyridine-treated CF₁, inhibits Ca-ATPase activity of heat-activated 5-subunit CF₁ and it was suggested that ϵ is the ATPase inhibitor of CF₁. This suggestion was criticized recently since activation of CF₁ 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 CF₁ are reviewed in [1].

We here report on the preparation of an ϵ -deficient CF₁ 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 CF₁-containing supernatant was applied to a DEAE-Sephadex column preequilibrated with 20 mM Tris-SO₄, 1 mM EDTA, 0.1 mM ATP, 10 mM (NH₄)₂SO₄ and 10% (v/v) glycerol at a final pH of 7.8. The protein was eluted by a linear gradient (10–400 mM) of (NH₄)₂SO₄ in the same buffer. The CF₁ 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). CF₁ 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 CF₁ 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-ATPase 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

CF₁ 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 (NH₄)₂SO₄. The purified enzyme contains 5 subunits when analyzed by SDS-PAGE (fig.2, lane 5). This preparation is identical in composition to 5-subunit CF₁ 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 CF₁ 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 CF₁ 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 CF₁ species, composed of different subunits, have been resolved (lanes 2–4). A δ -less CF₁ (fraction c, lane 2), an

Table 1
ATPase activities of the different CF₁ fractions

Fraction	Mg ²⁺ ATPase		Ca ²⁺ ATPase	
	Un-treated	OG activated	Un-treated	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

Ca²⁺- or Mg²⁺-dependent ATPase activities of the different CF₁ preparations eluted from the HPLC (fig.1) were assayed with or without activation as described in section 2. Control is CF₁ before fractionation by HPLC. Rates are expressed in $\mu\text{mol ATP hydrolyzed} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$

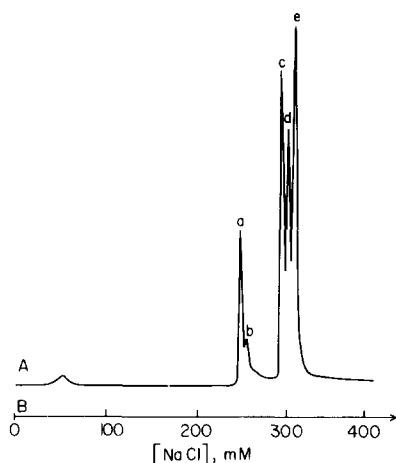


Fig.1. Fractionation of CF₁ by anion-exchange HPLC. CF₁ (3 mg) was resolved on a Mono Q HPLC column at pH 8.5 by an NaCl gradient. The protein elution profile (A) was obtained by an automated fluorescamine method [16]. NaCl concentration (B) is indicated. For further details see section 2.

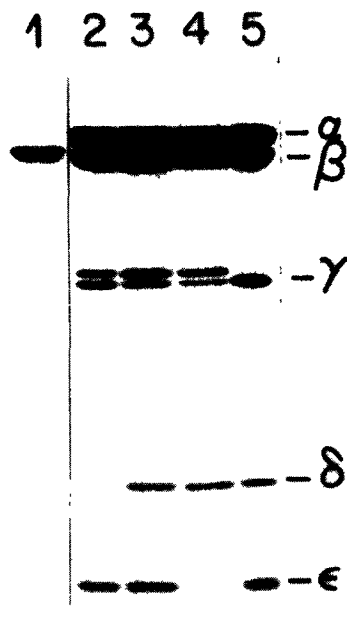


Fig.2. SDS-PAGE (10%) of fractions from HPLC of CF₁. Lanes 1-5 represent peaks a, c, d, e (fig.1) and unfractionated CF₁, respectively. 10 (lane 1) or 25 (lanes 2-5) μ g protein were applied.

intact CF₁ (fraction d, lane 3), and an ϵ -less CF₁ (fraction e, lane 4) are sequentially eluted off the column by increasing salt concentrations.

ATPase activities of the different CF₁ 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 CF₁. 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 CF₁ 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 CF₁.

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 CF₁ 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 CF₁ on SDS-PAGE (submitted).

Table 2

Inhibition of Ca-ATPase activity of ϵ -deficient CF₁ 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

CF₁ preparations (2 μ g) were preincubated for 5 min at 23°C with or without purified ϵ (3 μ g) 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 μ mol ATP hydrolyzed $\cdot$ mg protein⁻¹ $\cdot$ min⁻¹

4. DISCUSSION

This work demonstrates that the two smallest CF₁ 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 CF₁ will determine whether or not δ dissociates from the enzyme. Furthermore, the resolution of CF₁ by anion-exchange HPLC into 3 distinct enzyme populations may be due either to a preexisting heterogeneous population of CF₁ or, alternatively, to a partial dissociation of δ or ϵ from CF₁ 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 CF₁. Since the δ - and ϵ -subunits are the first to be detached from CF₁ 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 CF₁ 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 CF₁. 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 CF₁ has been controversial. Authors in [10] demonstrated that the purified ϵ -subunit isolated from pyridine-treated CF₁ inhibits Ca-ATPase activity of heat-activated CF₁, and suggested that ϵ may be the ATPase inhibitor of CF₁. However, the physiological significance of these results may be questioned because the heat-activated CF₁ preparation used in these experiments was not depleted of its native ϵ -subunit. In the work described here an ϵ -deficient CF₁ 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 CF₁.

The role of the ϵ -subunit in the regulation of CF₁ ATPase has been previously criticized since it was demonstrated that activation of CF₁ 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 CF₁-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 CF₁ (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 CF₁-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 CF₁.

It should be mentioned that the latency of CF₁ 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 CF₁ 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 CF₁. 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 CF₁ from its individual subunits.

ACKNOWLEDGEMENTS

The authors are grateful to Professor Mordhay Avron and to Dr Yosepha Shahak for helpful discussions and comments. This research was sup-

ported by the US-Israel Binational Science Foundation (UP, BSF 2891/82), and by the Ionel Saniei (M.R.) and the W.T. Hogan (U.P.) Career Development Chairs.

REFERENCES

- [1] Nelson, N. (1981) *Curr. Top. Bioenerg.* 11, 1–33.
- [2] Younis, H.M., Winget, G.D. and Racker, E. (1977) *J. Biol. Chem.* 252, 1814–1818.
- [3] Lien, S. and Racker, E. (1971) *Methods Enzymol.* 23, 547–555.
- [4] Strotmann, H., Hesse, H. and Edelmann, K. (1973) *Biochim. Biophys. Acta* 314, 202–210.
- [5] Vambutas, V.K. and Racker, E. (1965) *J. Biol. Chem.* 240, 2660–2667.
- [6] McCarty, R.E. and Racker, E. (1968) *J. Biol. Chem.* 243, 129–137.
- [7] Farron, F. and Racker, E. (1970) *Biochemistry* 9, 3829–3836.
- [8] Sakurai, H., Shinohara, K., Hisabori, T. and Shinohara, K. (1981) *J. Biochem.* 90, 95–102.
- [9] Pick, U. and Bassilian, S. (1982) *Biochemistry* 24, 6144–6152.
- [10] Nelson, N., Nelson, H. and Racker, E. (1972) *J. Biol. Chem.* 247, 7657–7662.
- [11] Younis, H.M. and Morjana, N.A. (1982) *FEBS Lett.* 140, 317–319.
- [12] Mills, T.D., Mitchell, P. and Schurmann, P. (1980) *FEBS Lett.* 112, 173–177.
- [13] Strotmann, H., Bickel, S. and Huchzermeyer (1976) *FEBS Lett.* 61, 194–198.
- [14] Moroney, J.V. and McCarty, R.E. (1981) in: *Energy Coupling in Photosynthesis* (Selman, B.R. and Selman-Reimer, S. eds) pp.169–179, Elsevier, Amsterdam, New York.
- [15] Pick, U. and Bassilian, S. (1981) in: *Energy Coupling in Photosynthesis* (Selman, B.R. and Selman-Reimer, S. eds) pp.251–260, Elsevier, Amsterdam, New York.
- [16] Bohlen, P., Stein, S., Stone, J. and Udenfriend, S. (1977) *Anal. Biochem.* 67, 438–445.
- [17] Nelson, N. (1980) *Methods Enzymol.* 69, 301–326.
- [18] Bradford, M.M. (1976) *Anal. Biochem.* 72, 248–254.
- [19] Lien, S. and Racker, E. (1971) *Methods Enzymol.* 23, 547–555.
- [20] Laemmli, A.K. (1970) *Nature* 227, 680–685.
- [21] Fluhr, R., Fromm, H. and Edelman, M. (1984) *Gene*, in press.
- [22] Schlodder, E., Graber, P. and Witt, H.T. (1982) in: *Topics in Photosynthesis* (Barber, J. ed) vol.4, pp.105–175, Elsevier, Amsterdam, New York.