

# Two distinct types of $\epsilon$ -binding site exist in chloroplast coupling factor ( $CF_1$ )

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Isolated chloroplast coupling factor ( $CF_1$ ), when depleted of its  $\epsilon$ -subunit, has a high ATPase activity which can be inhibited by binding  $\epsilon$  to a single high-affinity ( $K_d=1.4 \times 10^{-10}$  M) site. In  $CF_1$  reduced by dithiothreitol (DTT), however,  $\epsilon$  at this binding site is no longer inhibitory. Instead, 3 equivalent, lower affinity ( $K_d=6 \times 10^{-8}$  M), inhibitory binding sites for  $\epsilon$  are observed, whether or not the high-affinity site contains a bound  $\epsilon$ -subunit. Binding of  $\epsilon$  to the high-affinity site protects  $CF_1$  against trypsin activation, while binding to the low-affinity site does not, showing that occupation of each class of site has a different effect on  $CF_1$  structure. The effects of DTT can be interpreted in terms of a reduction in intersubunit cooperativity in  $CF_1$ .

Chloroplast; ATP synthase;  $F_1$ -ATPase; ATPase inhibitor protein; Subunit interaction

## 1. INTRODUCTION

The ATP synthase of chloroplasts ( $CF_1$ - $CF_0$ ) couples the dissipation of a light-induced pH gradient to the phosphorylation of ADP. It also possesses a latent ATPase activity, which is revealed after treatment with dithiols or trypsin. The nucleotide binding and catalytic sites of this complex reside on the two largest subunits of  $CF_1$  (designated  $\alpha$ ,  $\beta$ : for a review see [1]). However, two other subunits of  $CF_1$ , the  $\gamma$ - and  $\epsilon$ -subunits, are implicated in regulation of its ATPase activity [2–4].

In membrane-bound  $CF_1$ , ATPase activation can be correlated with the reduction of a disulphide bridge (using DTT) within the  $\gamma$ -subunit [5] or by cleavage of this subunit by trypsin [6].

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*Abbreviations:*  $CF_1$ , chloroplast coupling factor 1;  $F_1$ , coupling factor 1; DTT, DL-dithiothreitol; IBZ, *o*-iodosobenzoate; TEA, triethanolamine; tricine, *N*-tris(hydroxymethyl)methylglycine; Tris, tris(hydroxymethyl)aminomethane; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

Reduction of the  $\gamma$ -subunit of isolated  $CF_1$  also increases its ATPase activity [7]. However, the  $\epsilon$ -subunit has also been implicated in the activation of isolated  $CF_1$ . Thus, removal of the  $\epsilon$ -subunit (by ion-exchange chromatography) increases ATPase activity, while rebinding leads to inhibition [3,4], which is complete at a 1:1 molar ratio [8]. The relationship between DTT activation and  $\epsilon$  removal, has, however, remained unclear.

We show here that DTT activation of  $CF_1$  does not remove its intrinsic  $\epsilon$ -subunit, but masks its inhibitory properties. Further, DTT activated  $CF_1$  can be inhibited by added  $\epsilon$ ; this binds not to the binding site of the intrinsic  $\epsilon$  but to 3 lower affinity binding sites on  $CF_1$ , which may well lie on the three (catalytic)  $\beta$ -subunits.

## 2. MATERIALS AND METHODS

$CF_1$  was prepared from fresh market spinach according to the method of Lien and Racker [9] and was frozen in liquid nitrogen and stored at  $-70^\circ\text{C}$  or kept at  $4^\circ\text{C}$  as a precipitate in 2 M ammonium sulphate, pH 7.5.  $\epsilon$  and  $\epsilon$ -depleted  $CF_1$  were prepared by the method of Richter et al. [8], except that DEAE-Sephacel was used in the place of DEAE-cellulose, and the  $\epsilon$ -elution buffer was 25 mM TEA-NaOH, pH 7.9, 2 mM ATP, 20 mM NaCl, 20% ethanol (v/v) and 30% glycerol (v/v) (buf-

fer A). Eluted  $\epsilon$  was concentrated by ultrafiltration using an Amicon model 8010 with a YM5 membrane.

DTT activation of CF<sub>1</sub> was by a modification of a published procedure [10]. 0.4 ml of 20 mM tricine-NaOH, pH 8.0, 1 mM EDTA, 1 mM ATP and 50 mM DTT containing 1–2 mg/ml CF<sub>1</sub>, was incubated for 3 h at room temperature. The samples were then diluted 5-fold with 20 mM tricine-NaOH, pH 8.0, 1 mM EDTA and used immediately. (Further dilution of these samples in the various incubation media resulted in a final DTT concentration of less than 1 mM.) For the titrations,  $\epsilon$  was added from a 0.1 mg/ml solution to 0.12 ml aliquots containing 20  $\mu$ g/ml CF<sub>1</sub>, 20 mM tricine-NaOH, pH 8.0, and 1 mM EDTA, and were mixed immediately. After 5 min at room temperature Ca-ATPase activity was assayed, by a published procedure [9].

Trypsin activation of CF<sub>1</sub> was modified from [8]. To 0.75 ml of a solution containing 40  $\mu$ g/ml coupling factor, 20 mM tricine-NaOH, pH 8.0, and 1 mM EDTA, was added 0.24 ml of either 0.102 mg/ml purified  $\epsilon$ , or buffer A alone. Where indicated, samples were then reoxidised by a 10 min incubation with 2 mM *o*-iodosobenzoate (IBZ). Digestion was initiated using 10  $\mu$ l of 0.1 mg/ml TPCK-trypsin in 2 mM HCl. 80  $\mu$ l aliquots were withdrawn and quenched at the indicated times by addition to 40  $\mu$ l of 0.1 mg/ml soybean trypsin inhibitor, and Ca-ATPase activity determined.

The concentration of  $\epsilon$  was determined using fluorescamine [11] with bovine serum albumin as protein standard. Unbound  $\epsilon$  was effectively removed from solution by centrifugation through a Sephadex G-75 column (0.75 cm  $\times$  6 cm) equilibrated with 25 mM TEA-NaOH, pH 7.9, 1 mM ATP, 1 mM EDTA, as described by Penefsky [12]. Coupling factor subunits were identified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using an 8–12% linear acrylamide gradient and the discontinuous buffer system of Laemmli [13], and were stained by the method of Morrissey [14]. A molecular mass of 14700 Da was used for  $\epsilon$  [15], of 400 kDa for CF<sub>1</sub> [16] and of 385300 Da for  $\epsilon$ -depleted CF<sub>1</sub>. DTT, TPCK-trypsin and soybean trypsin inhibitor were supplied by Sigma (England), and all reagents were of analytical grade.

### 3. RESULTS

Both  $\epsilon$  depletion of CF<sub>1</sub> and DTT treatment increase the ATPase activity of our CF<sub>1</sub> preparation (fig.1). However, DTT treatment does not result in the loss of the  $\epsilon$ -subunit from CF<sub>1</sub>. DTT activation, followed by centrifugation through a column of Sephadex G-75, results in a CF<sub>1</sub> preparation containing as much  $\epsilon$ -subunit as native CF<sub>1</sub> (fig.1, tracks 3 and 4). Tracks 1 and 2 show that free  $\epsilon$  itself is adsorbed in the column. Control experiments (not shown) indicate that  $\epsilon$  is also retained by the column in the presence of either albumin or beef heart F<sub>1</sub>-proteins which show no specific interaction with  $\epsilon$ . Thus, DTT activation cannot involve freeing the  $\epsilon$ -subunit from CF<sub>1</sub>.

In spite of the endogenous  $\epsilon$  in DTT activated

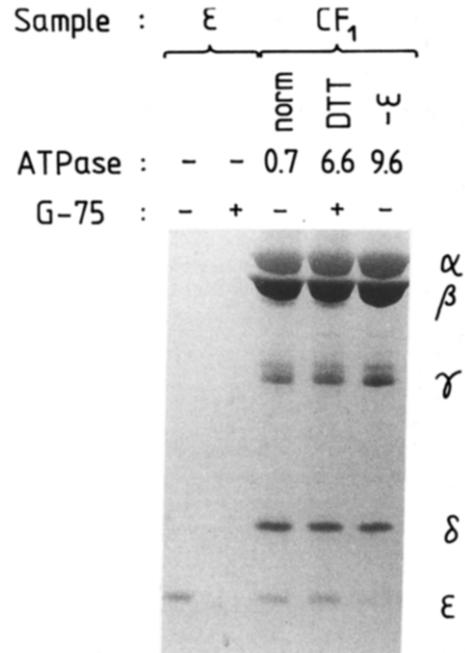


Fig.1.  $\epsilon$  content of activated CF<sub>1</sub> preparations. 4  $\mu$ g of CF<sub>1</sub>, following: no pretreatment (norm), DTT treatment (DTT), or  $\epsilon$  removal (- $\epsilon$ ) or 0.2  $\mu$ g of  $\epsilon$  were subjected to SDS-PAGE with (+) or without (-) prior passage through Sephadex G-75. Corresponding ATPase activities are expressed in  $\mu$ mol P<sub>i</sub> released/min per mg protein.

CF<sub>1</sub>, the preparation is still inhibited by added  $\epsilon$ . This is demonstrated in fig.2, where  $\epsilon$  binding to various CF<sub>1</sub> preparations is measured. This figure also shows that the affinity of DTT activated CF<sub>1</sub> for  $\epsilon$  is considerably lower than that of  $\epsilon$ -depleted CF<sub>1</sub>. These data were analysed as in [15] (fig.2, inset) using the linearisation,

$$\frac{L_0}{\alpha} = \frac{K_d}{1 - \alpha} + E_0$$

where  $L_0$  is the concentration of added  $\epsilon$  in the preincubation,  $E_0$  the concentration of  $\epsilon$  binding sites and  $\alpha$  the fractional saturation of CF<sub>1</sub> with  $\epsilon$  (calculated on the assumption that  $\epsilon$  bound is directly proportional to the fall in ATPase activity). Such treatment shows that the dissociation constant  $K_d$  (slope of line) increases from  $1.4 \times 10^{-10}$  M (for  $\epsilon$ -depleted CF<sub>1</sub>) to  $6 \times 10^{-8}$  M (for DTT activated CF<sub>1</sub>). In addition the number of binding sites (intercept) increases, from 1.1 to 3.2 mol  $\epsilon$ /mol CF<sub>1</sub>.

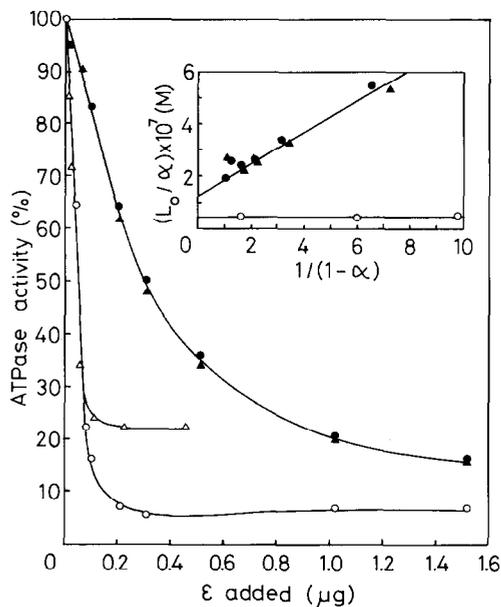


Fig. 2. Inhibition of ATPase activity of CF<sub>1</sub> preparations by purified  $\epsilon$ -subunit. ( $\Delta$ ) Native CF<sub>1</sub>, ( $\circ$ )  $\epsilon$ -depleted CF<sub>1</sub>. Open symbols before, closed symbols after, DTT treatment. ATPase activities at 100% were ( $\Delta$ ) 0.88; ( $\circ$ ) 4.3; ( $\blacktriangle$ ) 8.8; and ( $\bullet$ ) 13.3  $\mu\text{mol}/\text{min}$  per mg. (Inset) Linearisation of the same data, as explained in text.

This experiment was repeated with DTT activated CF<sub>1</sub> which had been  $\epsilon$ -depleted prior to DTT treatment. Surprisingly,  $\epsilon$  binding to this preparation followed a curve indistinguishable from that observed with the undepleted preparation (fig. 2, upper curves). Thus, besides revealing 3 inhibitory, low-affinity binding sites for  $\epsilon$  on CF<sub>1</sub>, DTT treatment also prevents access of added  $\epsilon$  to the preexisting  $\epsilon$  binding site even when this is empty. These effects of DTT are reversible: reoxidation of  $\epsilon$ -depleted, DTT-activated CF<sub>1</sub> with iodosobenzoate yields a preparation with a single, high-affinity, binding site for  $\epsilon$  as seen in CF<sub>1</sub> which has been simply depleted of its  $\epsilon$ -subunit (not shown). The additional observation (fig. 2) that the relatively low ATPase activity of native CF<sub>1</sub> is also reduced by  $\epsilon$  addition, could possibly be attributed to a small proportion of the CF<sub>1</sub> sample which lacks  $\epsilon$  at its high-affinity site.

There thus appear to be 2 classes of  $\epsilon$  binding site on CF<sub>1</sub> with very different affinities, which are non-overlapping (since  $\epsilon$  can bind to both simultaneously, at least on DTT-activated CF<sub>1</sub>).

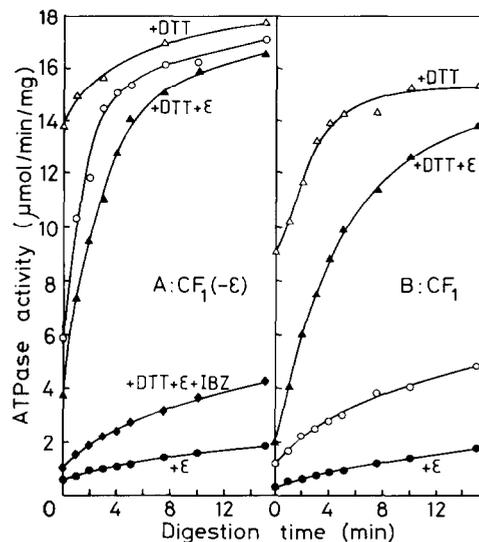


Fig. 3. Trypsin activation of CF<sub>1</sub> with  $\epsilon$  bound at either the high- or low-affinity binding sites. (A)  $\epsilon$ -depleted CF<sub>1</sub>; (B) native CF<sub>1</sub>. Prior to trypsin treatment: ( $\circ$ ) no further pretreatment; ( $\bullet$ ) addition of  $\epsilon$ ; ( $\Delta$ ) DTT treatment; ( $\blacktriangle$ ) DTT treatment followed by addition of  $\epsilon$ ; and ( $\blacklozenge$ ) DTT treatment,  $\epsilon$  addition, then reoxidation using iodosobenzoate (IBZ). For experimental details, see section 2.

Further evidence that these sites are discrete come from studies on activation of CF<sub>1</sub> by trypsin, which activates by cleavage at a site other than on the  $\epsilon$ -subunit [18,19]. Despite having a high ATPase activity as compared to native CF<sub>1</sub>, both DTT-activated and  $\epsilon$ -depleted CF<sub>1</sub> can be further activated, about 2-fold, by a brief treatment with trypsin (fig. 3).  $\epsilon$  can be bound to both these preparations but, as shown above, after DTT treatment only the low-affinity sites are filled. Rebinding of  $\epsilon$  to the high-affinity binding site considerably decreases the rate of activation of CF<sub>1</sub> by trypsin (fig. 3A, cf.  $\circ$ ,  $\bullet$ ). However,  $\epsilon$  binding to the lower affinity sites in the DTT-treated preparations, while inhibiting ATP hydrolysis, yields no such protection and trypsin activation remains rapid (fig. 3, cf.  $\Delta$ ,  $\blacktriangle$ ). It is concluded that  $\epsilon$  bound in native CF<sub>1</sub> protects a site susceptible to tryptic cleavage which affects CF<sub>1</sub> activity (as previously shown by Richter et al. [8]), while in DTT-treated CF<sub>1</sub> neither  $\epsilon$  at the induced low-affinity sites, nor at the modified preexisting site, protect from trypsin digestion. Again,

iodosobenzoate reverses the effect of DTT (fig.3A,  $\blacklozenge$ ) and isolated, untreated CF<sub>1</sub> shows some indication that a small proportion of it lacks  $\epsilon$  at the high-affinity site (fig.3B, cf.  $\circ$ ,  $\bullet$ ) as both the initial rate of ATP hydrolysis and its subsequent increase are diminished by  $\epsilon$  addition.

#### 4. DISCUSSION

Trypsin treatment, reduction with DTT and removal of the  $\epsilon$ -subunit are all procedures which lead to an increased ATPase activity of isolated CF<sub>1</sub>. Their ultimate effects are presumably manifest on the catalytic ( $\beta$ ) subunit of this enzyme, but their immediate effects are disparate. Trypsin activation seems to correlate with partial digestion of the  $\alpha$ - and  $\gamma$ -subunits of soluble CF<sub>1</sub> [18], DTT activation with reduction of thiols on the  $\gamma$ -subunit [7], and the  $\epsilon$ -subunit may interact with either the  $\gamma$ -subunit [8] or directly with the  $\beta$ -subunit of CF<sub>1</sub> [20].

CF<sub>1</sub> appears to have one tight and 3 weaker binding sites for  $\epsilon$ , all of which are inhibitory to ATPase activity by this enzyme (fig.2). The tight binding site has previously been reported by Richter et al. [8]. The weaker sites can be seen only after activation by DTT, though whether they are exposed by DTT, or always present (with their inhibitory effect masked by the similar effect of the higher affinity site) is not clear from these data.

The existence of 3 identical, low-affinity binding sites for  $\epsilon$  certainly suggests that these reside on the  $\alpha$ - or  $\beta$ -subunits, the only subunits present in triplicate [16,20]. Indeed,  $\epsilon$  or its equivalent has been shown to interact with the  $\beta$ -subunit of F<sub>1</sub> from *E. coli* [21] or ox heart [22].

DTT appears to mask the effect of the intrinsic  $\epsilon$  subunit of CF<sub>1</sub>: after treatment,  $\epsilon$  bound at the high-affinity site no longer inhibits nor protects CF<sub>1</sub> from trypsin (fig.3). Further, the high-affinity site itself is masked: if emptied before DTT-treatment, the site cannot be filled unless the reduction is reversed. Such isolation of the high-affinity site may be accomplished by eliminating intersubunit cooperativity, allowing the 3  $\beta$ -subunits to function independently.

To extrapolate these data to the situation in vivo is difficult. As the presence of 2  $\epsilon$  per CF<sub>1</sub> has been commonly reported [23–25] both the high- and low-affinity binding sites may be at least partially

occupied in native CF<sub>1</sub>. Whatever the stoichiometry in vivo, a means of regulation involving the alternation of  $\epsilon$  between the low- and high-affinity sites may be envisaged. Such an exchange would require a change in the relative affinities of the two types of binding site for  $\epsilon$ . Of possible relevance is the increase in the accessibility of the  $\epsilon$ -subunit of thylakoid bound CF<sub>1</sub> to anti- $\epsilon$  antibody during illumination, recently reported by Richter and McCarty [26]. Their further observation that the antibody actually removed  $\epsilon$  from CF<sub>1</sub> under such conditions, might be explained by a concomitant reduction in affinity of CF<sub>1</sub> for  $\epsilon$  (i.e. a rise in  $K_d$ ). It is therefore feasible that  $\epsilon$  displacement from a high- to a low-affinity binding site is involved in the activation process in vivo.

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