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Two distinct types of ε-binding site exist in chloroplast coupling factor (CF_1)

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Isolated chloroplast coupling factor (CF_1), when depleted of its ε-subunit, has a high ATPase activity which can be inhibited by binding ε to a single high-affinity ($K_d = 1.4 \times 10^{-10}$ M) site. In CF_1 reduced by dithiothreitol (DTT), however, ε at this binding site is no longer inhibitory. Instead, 3 equivalent, lower affinity ($K_d = 6 \times 10^{-8}$ M), inhibitory binding sites for ε are observed, whether or not the high-affinity site contains a bound ε-subunit. Binding of ε 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 α, β: for a review see [1]). However, two other subunits of CF_1, the γ- and ϵ-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 γ-subunit [5] or by cleavage of this subunit by trypsin [6]. Reduction of the γ-subunit of isolated CF_1 also increases its ATPase activity [7]. However, the ϵ-subunit has also been implicated in the activation of isolated CF_1. Thus, removal of the ϵ-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 ϵ removal, has, however, remained unclear.

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

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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

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°C or kept at 4°C as a precipitate in 2 M ammonium sulphate, pH 7.5. ϵ and ϵ-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 ϵ-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-

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

DTT activation of CF_1 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_1, 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, ϵ was added from a 0.1 mg/ml solution to 0.12 ml aliquots containing 20 μg/ml CF_1, 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_1 was modified from [8]. To 0.75 ml of a solution containing 40 μg/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 ϵ, 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 μl of 0.1 mg/ml TPCK-trypsin in 2 mM HCl. 80 μl aliquots were withdrawn and quenched at the indicated times by addition to 40 μl of 0.1 mg/ml soybean trypsin inhibitor, and Ca-ATPase activity determined.

The concentration of ϵ was determined using fluorescamine [11] with bovine serum albumin as protein standard. Unbound ϵ was effectively removed from solution by centrifugation through a Sephadex G-75 column (0.75 cm × 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 ϵ [15], of 400 kDa for CF_1 [16] and of 385300 Da for ϵ-depleted CF_1. DTT, TPCK-trypsin and soybean trypsin inhibitor were supplied by Sigma (England), and all reagents were of analytical grade.

3. RESULTS

Both ϵ depletion of CF_1 and DTT treatment increase the ATPase activity of our CF_1 preparation (fig.1). However, DTT treatment does not result in the loss of the ϵ-subunit from CF_1. DTT activation, followed by centrifugation through a column of Sephadex G-75, results in a CF_1 preparation containing as much ϵ-subunit as native CF_1 (fig.1, tracks 3 and 4). Tracks 1 and 2 show that free ϵ itself is adsorbed in the column. Control experiments (not shown) indicate that ϵ is also retained by the column in the presence of either albumin or beef heart F_1-proteins which show no specific interaction with ϵ. Thus, DTT activation cannot involve freeing the ϵ-subunit from CF_1.

In spite of the endogenous ϵ in DTT activated

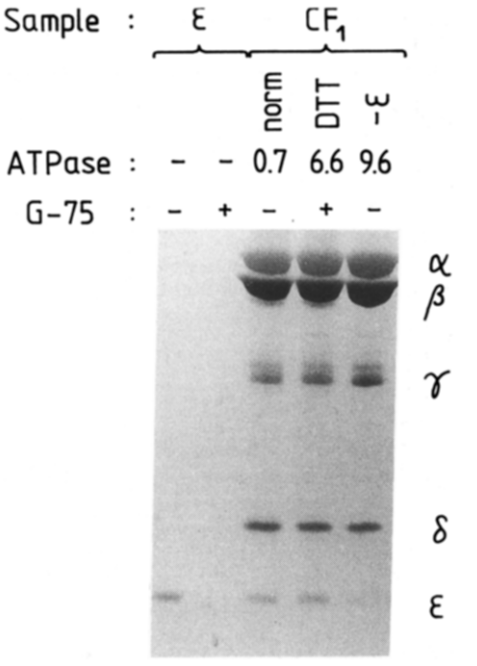

Fig.1. ϵ content of activated CF_1 preparations. 4 μg of CF_1, following: no pretreatment (norm), DTT treatment (DTT), or ϵ removal ($-\epsilon$) or 0.2 μg of ϵ were subjected to SDS-PAGE with (+) or without (−) prior passage through Sephadex G-75. Corresponding ATPase activities are expressed in μmol P_i released/min per mg protein.

CF_1, the preparation is still inhibited by added ϵ. This is demonstrated in fig.2, where ϵ binding to various CF_1 preparations is measured. This figure also shows that the affinity of DTT activated CF_1 for ϵ is considerably lower than that of ϵ-depleted CF_1. 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 ϵ in the preincubation, E_0 the concentration of ϵ binding sites and α the fractional saturation of CF_1 with ϵ (calculated on the assumption that ϵ bound is directly proportional to the fall in ATPase activity). Such treatment shows that the dissociation constant K_d (slope of line) increases from 1.4×10^{-10} M (for ϵ-depleted CF_1) to 6×10^{-8} M (for DTT activated CF_1). In addition the number of binding sites (intercept) increases, from 1.1 to 3.2 mol ϵ/mol CF_1.

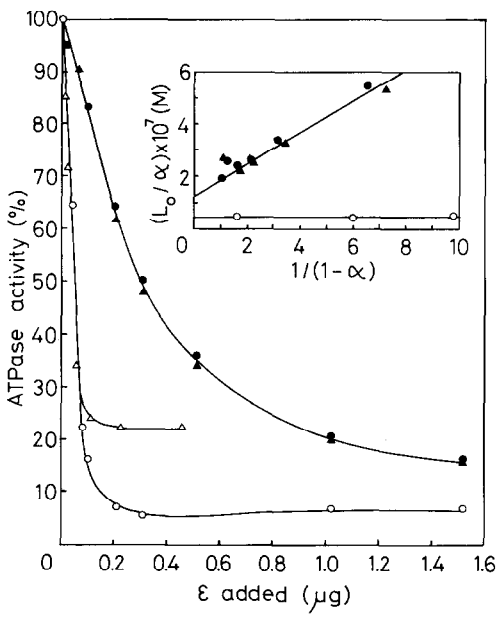

Fig.2. Inhibition of ATPase activity of CF_1 preparations by purified ϵ-subunit. (△) Native CF_1, (○) ϵ-depleted CF_1. Open symbols before, closed symbols after, DTT treatment. ATPase activities at 100% were (△) 0.88; (○) 4.3; (▲) 8.8; and (●) 13.3 μmol/min per mg. (Inset) Linearisation of the same data, as explained in text.

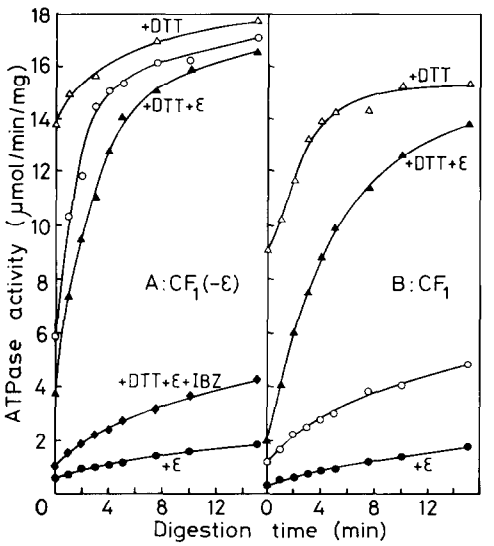

Fig.3. Trypsin activation of CF_1 with ϵ bound at either the high- or low-affinity binding sites. (A) ϵ-depleted CF_1; (B) native CF_1. Prior to trypsin treatment: (○) no further pretreatment; (●) addition of ϵ; (△) DTT treatment; (▲) DTT treatment followed by addition of ϵ; and (♦) DTT treatment, ϵ addition, then reoxidation using iodosobenzoate (IBZ). For experimental details, see section 2.

This experiment was repeated with DTT activated CF_1 which had been ϵ-depleted prior to DTT treatment. Surprisingly, ϵ 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 ϵ on CF_1, DTT treatment also prevents access of added ϵ to the preexisting ϵ binding site even when this is empty. These effects of DTT are reversible: reoxidation of ϵ-depleted, DTT-activated CF_1 with iodosobenzoate yields a preparation with a single, high-affinity, binding site for ϵ as seen in CF_1 which has been simply depleted of its ϵ-subunit (not shown). The additional observation (fig.2) that the relatively low ATPase activity of native CF_1 is also reduced by ϵ addition, could possibly be attributed to a small proportion of the CF_1 sample which lacks ϵ at its high-affinity site.

There thus appear to be 2 classes of ϵ binding site on CF_1 with very different affinities, which are non-overlapping (since ϵ can bind to both simultaneously, at least on DTT-activated CF_1). Further evidence that these sites are discrete come from studies on activation of CF_1 by trypsin, which activates by cleavage at a site other than on the ϵ-subunit [18,19]. Despite having a high ATPase activity as compared to native CF_1, both DTT-activated and ϵ-depleted CF_1 can be further activated, about 2-fold, by a brief treatment with trypsin (fig.3). ϵ can be bound to both these preparations but, as shown above, after DTT treatment only the low-affinity sites are filled. Rebinding of ϵ to the high-affinity binding site considerably decreases the rate of activation of CF_1 by trypsin (fig.3A, cf. ○, ●). However, ϵ 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. △, ▲). It is concluded that ϵ bound in native CF_1 protects a site susceptible to tryptic cleavage which affects CF_1 activity (as previously shown by Richter et al. [8]), while in DTT-treated CF_1 neither ϵ 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, ◆) and isolated, untreated CF_1 shows some indication that a small proportion of it lacks ϵ at the high-affinity site (fig.3B, cf. ○, ●) as both the initial rate of ATP hydrolysis and its subsequent increase are diminished by ϵ addition.

4. DISCUSSION

Trypsin treatment, reduction with DTT and removal of the ϵ-subunit are all procedures which lead to an increased ATPase activity of isolated CF_1. Their ultimate effects are presumably manifest on the catalytic (β) subunit of this enzyme, but their immediate effects are disparate. Trypsin activation seems to correlate with partial digestion of the α- and γ-subunits of soluble CF_1 [18], DTT activation with reduction of thiols on the γ-subunit [7], and the ϵ-subunit may interact with either the γ-subunit [8] or directly with the β-subunit of CF_1 [20].

CF_1 appears to have one tight and 3 weaker binding sites for ϵ, 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 ϵ certainly suggests that these reside on the α- or β-subunits, the only subunits present in triplicate [16,20]. Indeed, ϵ or its equivalent has been shown to interact with the β-subunit of F_1 from *E. coli* [21] or ox heart [22].

DTT appears to mask the effect of the intrinsic ϵ subunit of CF_1: after treatment, ϵ bound at the high-affinity site no longer inhibits nor protects CF_1 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 β-subunits to function independently.

To extrapolate these data to the situation in vivo is difficult. As the presence of 2 ϵ per CF_1 has been commonly reported [23–25] both the high- and low-affinity binding sites may be at least partially occupied in native CF_1. Whatever the stoichiometry in vivo, a means of regulation involving the alternation of ϵ 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 ϵ. Of possible relevance is the increase in the accessibility of the ϵ-subunit of thylakoid bound CF_1 to anti-ϵ antibody during illumination, recently reported by Richter and McCarty [26]. Their further observation that the antibody actually removed ϵ from CF_1 under such conditions, might be explained by a concomitant reduction in affinity of CF_1 for ϵ (i.e. a rise in K_d). It is therefore feasible that ϵ displacement from a high- to a low-affinity binding site is involved in the activation process in vivo.

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