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The dithiothreitol-stimulated dissociation of the chloroplast coupling factor 1 ϵ-subunit is reversible

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The chloroplast coupling factor 1 complex (CF_1) contains an ϵ-subunit which inhibits the CF_1 ATPase activity. Chloroform treatment of *Chlamydomonas reinhardtii* thylakoid membranes solubilizes only forms of the enzyme which apparently lack the δ-subunit. Four interrelated observations are described in this paper. (1) The dithiothreitol- (DTT) induced ATPase activation of $CF_1(-\delta)$ and the DTT-induced formation of a physically resolvable $CF_1(-\delta,\epsilon)$ from the $CF_1(-\delta)$ precursor are compared. The similar time-courses of these two phenomena suggest that the dissociation of the ϵ-subunit is an obligatory process in the DTT-induced ATPase activation of soluble CF_1. (2) The reversible dissociation of the ϵ-subunit of the CF_1 is demonstrated by the exchange of subunits between distinguishable oligomers. ^{35}S-labelled chloroplast coupling factor 1 lacking the δ and ϵ subunits [$CF_1(-\delta,\epsilon)$] was added to a solution of non-radioactive coupling factor 1 lacking only the δ subunit [$CF_1(-\delta)$]. After separation of the two enzyme forms, via high resolution anion-exchange chromatography, radioactivity was detected in the chromatographic fractions containing $CF_1(-\delta)$. (3) ϵ-deficient CF_1 can be resolved from DTT pretreated ϵ-containing CF_1 for several days after the removal of DTT. On the other hand, brief incubation of the DTT pretreated ϵ-containing CF_1 with low concentrations of *o*-iodosobenzoate results in chromatographs containing only the peak of ϵ-containing CF_1. A simple explanation for this phenomenon is that reduction of CF_1 with DTT increases the apparent dissociation constant for the ϵ-subunit to an estimated $3.5 \cdot 10^{-8}$ M ($\pm 1.0 \cdot 10^{-8}$ M) from a value of $\leqslant 5 \cdot 10^{-11}$ M for the oxidized enzyme. (4) ATPase activity data show that oxidation of the ϵ-deficient enzyme does not completely inhibit its manifest activity, but oxidation of DTT pre-treated CF_1 which contains the ϵ-subunit completely inhibits manifest activity. A simple model is proposed for the influence of the oxidation state of the soluble enzyme on the distribution of ATPase-inactive and ATPase-active subunit configurations.

Introduction

The chloroplast coupling factor 1 (CF_1) is an oligomeric protein containing the catalytic domain of the proton-translocating ATP synthase. This enzyme can be readily solubilized from the thylakoid membrane-embedded proton channel, CF_0. The estimated molecular weight of the CF_1 holoenzyme is 420000 and the most probable stoichiometry of non-identical subunits is $\alpha_3\beta_3\gamma_1\delta_{1-2}\epsilon_1$, in order of descending mass (see Refs. 1–3 for review). The enzyme derived from the freshwater alga *Chlamydomonas reinhardtii* via the chloroform extraction technique lacks an apparent δ-subunit [4,5].

The ϵ-subunit inhibits the ATPase activity of the soluble CF_1 [6,7]. The dissociation of the ϵ-subunit has been correlated with ATPase activation via detergents [8] or heat (under certain conditions; Refs. 9, 10). However, it has been suggested [7,11,12] that the room-temperature DTT ATPase activation process does not involve dissociation of the ϵ-subunit. Recent experiments [11] relating DTT activation and ϵ-subunit-dependent ATPase inhibition were interpreted as evidence

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Abbreviations: PMSF, phenylmethylsulfonyl fluoride; TLCK, *N*(α)-*p*-tosyl-L-lysine chloromethyl ketone; DTT, dithiothreitol; Tricine, *N*-tris(hydroxymethyl)methylglycine; Mops, 3-(*N*-morpholino)propanesulfonic acid; Chl, chlorophyll; FPLC, fast protein liquid chromatography; DEAE-Sephadex A-50, diethylaminoethyl-Sephadex A-50 anion-exchange resin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CF_1, chloroplast coupling factor 1; $CF_1(-\delta)$, chloroplast coupling factor 1 lacking the δ subunit; $CF_1(-\delta,\epsilon)$, chloroplast coupling factor 1 lacking the δ and ϵ subunits.

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for the creation of three additional, low-affinity binding sites, and a concurrent masking of inhibition at the 'intrinsic' high-affinity binding site, for the ϵ-subunit in the presence of DTT. We have recently observed that preincubation of ϵ-containing CF_1 with increasing amounts of DTT lead to an increased population of ϵ-deficient CF_1, as quantitated via high-resolution anion-exchange chromatography [5].

In this paper, we test the possibility that the apparent resolution of ϵ-deficient CF_1 from ϵ-containing DTT-activated precursors is an artifact induced via removal of the ϵ-subunit by the chromatographic column. If this were the case, then the addition of radioactively labelled $CF_1(-\delta,\epsilon)$ to a solution of DTT-treated non-radioactive $CF_1(-\delta)$ followed by chromatography of the mixture would result in the elution of radioactivity only with the ϵ-deficient enzyme form. On the other hand, if reversible dissociation of the ϵ-subunit from the non-radioactive enzyme occurs, then the free ϵ-subunit could bind to radioactive $CF_1(-\delta,\epsilon)$. This would result in the elution of radioactivity in the $CF_1(-\delta)$ fraction. Our experiments demonstrate that reversible dissociation, and not simply aberrant chromatography, is indeed occurring.

The stability of the reduced forms of the enzyme and the role of oxidation in the ϵ-subunit dissociation/rebinding cycle are also addressed.

Materials and Methods

Preparation of CF_1. The modified chloroform extraction purification procedure, a composite of previously published methods [4,13,14–16,35], was described in ref. 5.

For certain experiments, the CF_1 was first purified from either spinach or *C. reinhardtii* via EDTA extraction and DEAE-Sephadex A-50 chromatography [17]. The CF_1 forms were then repurified via anion-exchange chromatography on a Mono Q HR 5/5 column in the absence of DTT as described in the section below, 'Experiment to test for subunit exchange'.

Preparation of ^{35}S-labelled $CF_1(-\delta,\epsilon)$. A 300 ml culture of *C. reinhardtii* strain 137+ was grown in TAP medium [4]. Part of this culture was washed with low-sulfate TAP medium (in which the $MgSO_4$ was replaced with $MgCl_2$), then was used to inoculate 2 liters of low-sulfate TAP medium containing 10 mCi of $Na_2{}^{35}SO_4$. ^{35}S-labelled $CF_1(-\delta)$ was purified as above with the exceptions that cells were broken via sonication and that only three pyrophosphate washes of the membranes were employed. The ^{35}S-labelled $CF_1(-\delta)$ fraction was diluted into a buffer with final concentrations of 150 mM DTT/20 mM Tris-HCl (pH 7.5). It was incubated for 4 h at 25 °C, followed by removal of any insoluble material via centrifugation at $30\,000 \times g$ (4 °C). The sample was applied to a Mono Q HR 5/5 column 5 h after addition to the DTT solution and chromatographed as above. ^{35}S-labelled $CF_1(-\delta,\epsilon)$ eluted as the second of two peaks in the gradient (as confirmed by SDS-PAGE and fluorography; see Fig. 3, lane D) and was used without further treatment. The approximate specific activity was 13 800 cpm/μg.

Experiment to test for subunit exchange. Non-radioactive $CF_1(-\delta)$ prepared by the chloroform extraction technique (or an equal volume of low-salt FPLC buffer) was diluted to an approximate final concentration of 20 μg/ml into a solution with final concentrations of 70 mM DTT, 10% (v/v) glycerol, 20 mM Tris-HCl (pH 7.5) and incubated 4 h at 25 °C. Approx. 150 000 cpm of purified ^{35}S-labelled $CF_1(-\delta,\epsilon)$ (or an equal volume of low-salt FPLC buffer for the control) was added to the solution; this represents a protein increase of about 5%. Following sample clarification ($30\,000 \times g$; 4 °C), a 10 ml aliquot was applied to the Mono Q HR 5/5 column 5 h after the initial dilution into the DTT solution. The column was isocratically washed with low-salt FPLC buffer for a total of 30 ml, then protein was eluted with a continuous 60 ml gradient of low to high salt buffer (120 to 400 mM NaCl in 10% glycerol/1 mM EDTA/20 mM Tris-HCl (pH 7.5)). Fractions of 1 ml were collected; 50-μl aliquots were assayed for protein content and for radioactivity.

Time-course of ATPase activation. $CF_1(-\delta)$ and $CF_1(-\delta,\epsilon)$ were purified from *C. reinhardtii* via the modified chloroform extraction procedure as described above. On the day of the assay, these two enzyme forms were repurified via chromatography in the absence of dithiothreitol, then promptly diluted to 16 μg/ml in a buffer of 20 mM Mops–NaOH (pH 7.5) with or without 50 mM DTT and incubated at 25 °C. Triplicate aliquots (0.48 μg) were assayed for ATPase activity at timed intervals following addition of DTT. Enzyme was assayed at 25 °C, 4 mM [γ-^{32}P]ATP, 2 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.5) for 7.5 min before assay termination [18].

Time-course of $CF_1(-\delta,\epsilon)$ formation. $CF_1(-\delta)$ was purified from *C. reinhardtii* via the modified chloroform extraction procedure as described above, then repurified via chromatography in the absence of dithiothreitol just before the experiment. $CF_1(-\delta)$ was diluted to 16 μg/ml in a buffer of 20 mM Mops–NaOH (pH 7.5) with or without 50 mM DTT and incubated at 25 °C. 10 ml aliquots were then chromatographed at timed intervals after the addition of DTT and the elution of protein from the column was monitored by the absorbance of 280 nm light. The areas of the two resultant peaks were cut out from photocopies of the chromatograph and weighed. Assuming [19] that the extinction coefficients of $CF_1(\pm\epsilon)$ are approximately the same, the $\%CF_1(-\delta,\epsilon)$ was computed as the ratio of the weight of the second peak ($CF_1(-\delta,\epsilon)$) over the sum of the weights of the first ($CF_1(-\delta)$) and second peaks.

o-Iodosobenzoate inhibition of ATPase activity. *C. reinhardtii* $CF_1(-\delta)$ was originally prepared via the EDTA extraction technique [17], then repurified via FPLC (Mono Q 5/5). An aliquot of $CF_1(-\delta)$ was incubated overnight (> 15 h) in 100 mM DTT/20 mM Mops–NaOH (pH 7.5), then chromatographed on Mono Q HR 5/5 column in the absence of DTT as described above. This procedure provided purified fractions of activated $CF_1(-\delta)$* and of $CF_1(-\delta,\epsilon)$. For a latent control, $CF_1(-\delta)$ was handled identically except for the strict absence of DTT. The second chromatograph provided a purified fraction of latent $CF_1(-\delta)$. 0.5 μg of each enzyme form was incubated with varying concentrations of *o*-iodosobenzoate (the acid form was dissolved in water by titration with sodium hydroxide) for 5 min at ambient temperature (approx. 25 °C). At the end of the 5 min pre-incubation, the tube containing the enzyme was put into a 37 °C water-bath and ATPase assay mixture was added to bring the solution concentration to 10 mM [γ^{32}P]ATP/5 mM $MgCl_2$/1 mM EDTA/20 mM Tricine-NaOH (pH 8.0). After 10 min, the assay was terminated and inorganic [γ-^{32}P]phosphate measured as previously described [18].

Miscellaneous techniques. SDS-PAGE analyses used a 4% acrylamide stacking gel and a 15% running gel as described by Laemmli [20]. Radioactive protein in gels was visualized using the fluorographic technique described by Bonner and Laskey [21]. Protein content was estimated by a variation of the Bradford assay [22] in which 50 μl of sample was mixed with 200 μl of Bradford Reagent in a microtiter plate and the resultant absorbance at 590 nm measured with a BIO-TEK model EL-308 EIA Reader. Incorporation of ^{35}S into protein was assayed by scintillation counting of 50 μl of sample in 5 ml of Bio-Safe II™ scintillation cocktail (Research Products International).

Materials. *Chlamydomonas reinhardtii* wild-type strain 137 + was grown under continuous light in 14 liter cultures under conditions previously described [4]. Spinach (Bloomsdale variety) was grown indoors with a 12 h light cycle. Leaves were harvested about 6–8 weeks after germination. Dithiothreitol was obtained from Boehringer Mannheim Biochemicals. $Na_2\ ^{35}SO_4$ was purchased from DuPont. The FPLC, the Mono Q HR 5/5 and 10/10 anion-exchange columns were purchased from Pharmacia. [γ-^{32}P]ATP was prepared essentially as previously described [23]. All other materials were of reagent quality or better.

Results

Comparison of ϵ-deficient CF_1 formation and ATPase activity expression

When the *C. reinhardtii* chloroplast coupling factor 1 complex containing the ϵ-subunit ($CF_1(-\delta)$) was diluted into a solution of 50 mM DTT and then chromatographed, two peaks were resolved. The first peak contained $CF_1(-\delta)$, whereas the second peak lacked the ϵ-subunit ($CF_1(-\delta,\epsilon)$). When aliquots of such a solution were chromatographed at timed intervals after the dilution of protein into the DTT solution, the relative proportion (and absolute amount) of the $CF_1(-\delta,\epsilon)$ recovered rapidly increased within the first few hours of the experiment, then began to approach a plateau of approx. 53% of the recovered protein (Fig. 1A). This contrasted to the behavior of the control $CF_1(-\delta)$ which had been diluted into an equivalent buffer lacking DTT. Without the addition of DTT, no time-dependent formation of ϵ-deficient CF_1 was observed. Less than about 4% of the recovered protein from the DTT-deficient control experiments could be assigned to the $CF_1(-\delta,\epsilon)$ peak and this measurement conservatively included a small uncharacterized contaminating peptide [5].

Dithiothreitol was reported to increase the ATPase activity of *C. reinhardtii* CF_1 approx. 2-fold [4]. However, it was likely that the CF_1 used for these determinations was an unresolved mixture of forms containing and lacking the ϵ-subunit, since these experiments were done prior to the general availability of high-resolution chromatographic columns. It was important to assess the effect of DTT on the ATPase activity of purified $CF_1(-\delta)$ and $CF_1(-\delta,\epsilon)$ from this alga. The ATPase activity of freshly re-purified $CF_1(-\delta)$ was increased from complete latency (the apparent ATPase activity without DTT-treatment was indistinguishable from the non-enzymatic background control) to an apparent specific activity of about 0.7 to 0.8 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ (at 25 °C, pH 7.5, 4 mM ATP, 2 mM $MgCl_2$ and with no ATP regeneration system). This activation process proceeded on a time-scale comparable to that observed in the chromatographic experiments. In contrast, the freshly repurified $CF_1(-\delta,\epsilon)$ had an apparent specific activity of about 2.0 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ without any DTT treatment. Preincubation with DTT only modestly raised the *C. reinhardtii* $CF_1(-\delta,\epsilon)$ ATPase activity, by about 15%, and this enhancement was apparently complete within the first few minutes of preincubation. Fig. 1B is a plot of the time-course of the DTT-activation of the *C. reinhardtii* $CF_1(-\delta)$ ATPase activity. This activity is expressed as a percentage of the $CF_1(-\delta,\epsilon)$ ATPase

* The notation CF_1 or $CF_1(-\delta)$ is being used to designate ϵ-containing forms of the coupling factor that have been previously reduced dithiothreitol. These forms will elute with CF_1 or $CF_1(-\delta)$ on an anion-exchange column, as well as exhibit the same peptide pattern upon SDS-PAGE analysis. However, these forms will display partial manifest activity and will also redistribute into ϵ-deficient enzyme forms upon subsequent rechromatography in the absence of DTT. The notation CF_1 and $CF_1(-\delta)$ is thus used to avoid apparent contradiction of our proposed hypothesis that the ϵ-deficient population of soluble coupling factor is the active ATPase form.

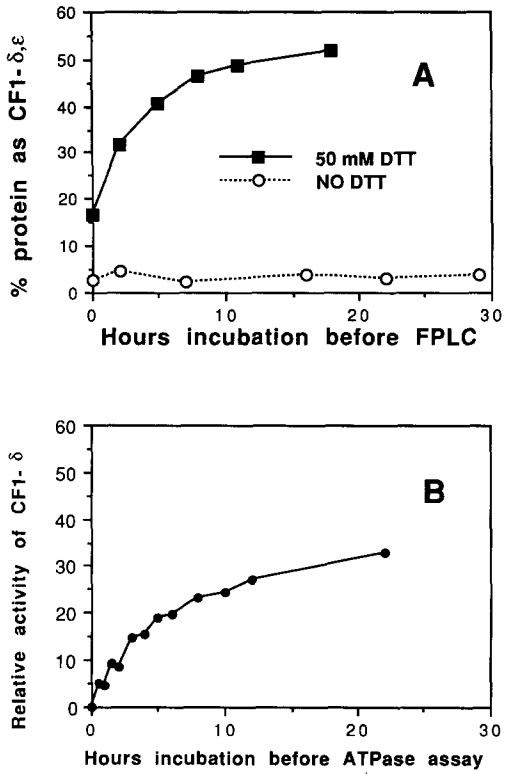

Fig. 1. Comparison of formation of chromatographically resolved $CF_1(-\delta,\epsilon)$ forms with the formation of an active ATPase enzyme. $CF_1(-\delta)$ was diluted into buffer containing 50 mM DTT (solid squares) or no DTT (open circles) as described in Materials and Methods. At various time intervals following dilution, aliquots were chromatographed and the resolved enzymes were quantitated. Results are expressed (panel A) as the percentage of $CF_1(-\delta,\epsilon)$ in the total recovered enzyme. An experimental uncertainty of 10 to 15 min (not shown) exists along the axis of abscissas due to the time required to load protein solution onto the chromatographic column. $CF_1(-\delta)$ was diluted into buffer containing 50 mM DTT (solid circles) and aliquots were assayed for ATPase activity at varying time intervals following dilution, as described in Materials and Methods. $CF_1(-\delta)$ that was not treated with DTT showed no enzymatic activity. The ATPase activity is plotted (panel B) as a percentage of the ATPase activity (2.3 $\mu mol \cdot mg^{-1} \cdot min^{-1}$) of $CF_1(-\delta,\epsilon)$ which was similarly treated with 50 mM DTT.

activity assayed under identical conditions. Note that this curve has a shape similar to the curve obtained from the chromatographic experiments. Also, the relative magnitudes of each phenomenon (ATPase activity and ϵ-deficiency) are consistent with each other. The two curves are not identical, most noticeably near the origin, partly because of the different time resolution of the two assays and possibly also because of necessary differences in solution conditions following the preincubation periods.

The starting material for the above experiments ($CF_1(-\delta)$) was obtained via two successive chromatographic purifications (see Materials and Methods) of the original enzyme preparation (via the modified chloroform extraction procedure), rather than only one. This decreased the population of contaminating $CF_1(-\delta,\epsilon)$ arising from the previously reduced $CF_1(-\delta)$. This point will be elaborated on below in the section The stability of the effect of DTT.

Evidence for the exchange of the ϵ-subunit of CF_1

The incubation of $[^{35}S]CF_1(-\delta,\epsilon)$ with a solution of once-purified, non-radiolabelled, DTT-treated $CF_1(-\delta)$ resulted in the incorporation of radioactivity in the $CF_1(-\delta)$ fraction following chromatographic resolution of the two enzyme forms. The chromatographic profiles obtained from such experiments are presented in Fig. 2. In these experiments, the non-radiolabelled $CF_1(-\delta)$ was treated with 70 mM DTT prior to addition of $[^{35}S]CF_1(-\delta,\epsilon)$. Radiolabelled protein was incorporated into the ϵ-containing fraction. It is clear from the chromatographs shown in Fig. 2 that this observation was

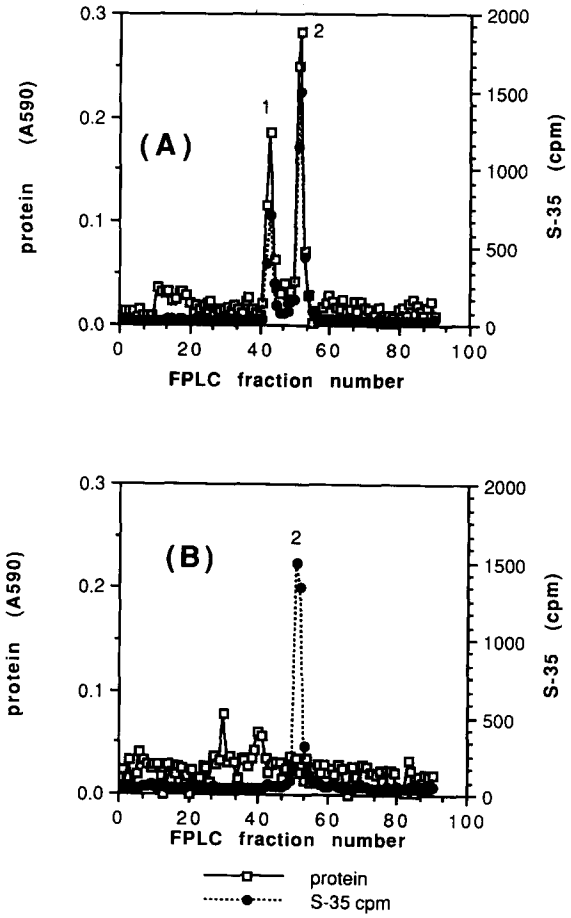

Fig. 2. Chromatographic demonstration of $[^{35}S]CF_1(-\delta,\epsilon)$ subunit exchange into $CF_1(-\delta)$. Non-radioactive $CF_1(-\delta)$ was preincubated with 70 mM DTT, $[^{35}S]CF_1(-\delta,\epsilon)$ was added, and the sample was then chromatographed as described in Materials and Methods. An average of 95% (±S.D. of 9% from six related experiments) of the radioactivity applied to the column was recovered. Panel B shows the chromatograph obtained when only $[^{35}S]CF_1(-\delta,\epsilon)$ was chromatographed. (Note: a larger aliquot was used for panel B vs. panel A to offset the nonspecific loss of protein, which is significant at very low protein concentrations.) The axes represent raw data without corrections for volumes or external standards.

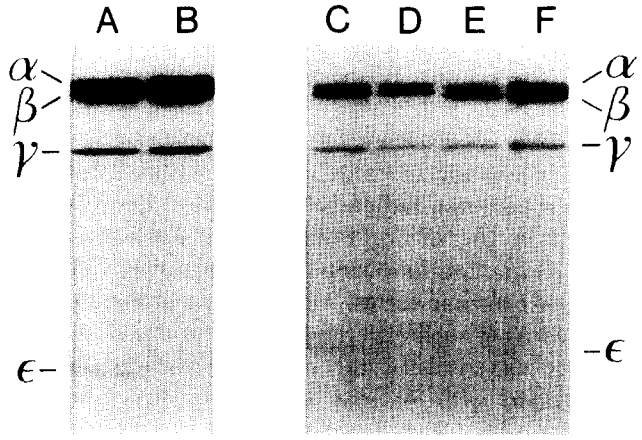

Fig. 3. SDS-PAGE analysis of chromatographically resolved CF_1 forms. Samples were obtained as in Fig. 2 with the exception that the incubation was done at approx. 55 μg/ml. Lanes A and B: Coomassie-stained visualization of the first and second chromatographic peaks, respectively. These two electrophoretic patterns were always diagnostic of the respective peaks in all such chromatographs. Lanes C and D: Fluorographic visualizations of the first and second chromatographic peaks, respectively, obtained during the original purification of $[^{35}S]CF_1(-\delta,\epsilon)$ (Materials and Methods). The ε-peptide from the radioactive precursor enzyme is presented as a positive control. Lanes E and F: Fluorographic visualizations of the first and second chromatographic peaks, respectively, obtained from a mixture of non-radiolabelled $CF_1(-\delta)$ and $[^{35}S]CF_1(-\delta,\epsilon)$ essentially as in Fig. 2 panel A. Similar fluorographs were obtained from other experiments as described in the text.

not due to contamination from unresolved peaks. Other data ensure that this observation was not the result of contaminated radiolabelled material. First, the control chromatograph of $[^{35}S]CF_1(-\delta,\epsilon)$ contained only one peak (Fig. 2B). Second, comparison of the Coomassie-stained (Fig. 3, lanes A and B) vs. fluorographic (Fig. 3, lanes E and F) visualizations of SDS-PAGE analysis of the two chromatographic peaks showed that the first peak contained only non-radiolabelled ε and that the radioactivity was contributed only by the three-subunit enzyme. (The radioactive ε-peptide band in Fig. 3, lane C, was obtained from the precursor to the $[^{35}S]CF_1(-\delta,\epsilon)$ and is presented as a positive control for the fluorographic data.)

Radioactive protein was incorporated in the $CF_1(-\delta)$ peak experiments involving the chromatography of a mixture of non-radioactive $CF_1(-\delta)$, radioactive $CF_1(-\delta,\epsilon)$, but no added DTT (data not shown). This again is evidence of subunit exchange. This observation was consistent with our previous findings [5] and ε-subunit dissociation often occurred to a limited extent in the absence of added DTT.

The stability of the effect of DTT

The evidence demonstrating that the ε-subunit reversibly dissociates even after ε-subunit-containing CF_1 is repurified in the absence of DTT, and the variable extent to which this phenomenon occurs, was at first puzzling. However, the enzymes used in the preceding experiments had originally been purified via the chloroform extraction technique, which includes DTT in the extraction buffer [4,14–16]. Since the enzyme is originally purified under non-equilibrium conditions with respect to DTT reduction, one might expect a variable mixture of reduced and non-reduced CF_1. If the reduced form of the enzyme is stable in the absence of excess reductant, and if the reduced form of the enzyme has a greater dissociation constant for the ε-subunit than does the oxidized (or nonreduced) form, then one might expect that the rechromatography of partially reduced CF_1 would yield $CF_1(-\epsilon)$ even in the absence of additional DTT. Also, successive chromatographic repurifications of *partially* reduced CF_1, as should be obtained from the chloroform extraction procedure, will successively decrease the amount of ε-deficient CF_1 observed.

To test the stability of ε-subunit dissociation after the removal of excess reductant, CF_1 was incubated with 70 mM DTT overnight (15 h) at 25°C. The enzyme mixture was repurified via FPLC in the absence of DTT to remove the reductant and the $CF_1(-\epsilon)$ which was formed. Aliquots of this CF_1 were rechromatographed at intervals up to 7 days) following DTT removal. Both CF_1 and $CF_1(-\epsilon)$ were obtained upon rechromatography; the distribution of enzyme forms was about the same on the 1st day as it was on the 7th. For the sake of clarity, such experiments are schematically illustrated in Fig. 4. These results were observed whether the enzyme was derived from spinach or from *C. reinhardtii*; in the latter case, $CF_1(-\delta)$ rather than CF_1 was used.

One may estimate an apparent dissociation constant (K'_D) describing the affinity of the ε-subunit for DTT-treated CF_1 if certain assumptions are made. The first is that equilibrium has been reached and that the equilibrium is not perturbed by the loss of solubility of the ε-subunit. The second is that one ε-subunit has dissociated for every $CF_1(-\epsilon)$ observed. Since the dissociated ε-subunit has not yet been completely accounted for, these are non-trivial assumptions. Nonetheless, they allow one to approximate K'_D as $[CF_1(-\epsilon)]^2/[CF_1]$ (or as $[CF_1(-\delta,\epsilon)]^2/[CF_1(-\delta)]$ according to the parent form of the enzyme). Thus for the spinach enzyme a K'_D of $7.1 \cdot 10^{-8}$ M was estimated. These data were derived from pre-treatment of 51 μg CF_1/ml with 100 mM DTT for 14 h at 25°C; the average wt% $CF_1(-\epsilon)$ obtained was 51%. This reflected an increase over the control K'_D of $\leqslant 6 \cdot 10^{-11}$ (no DTT pretreatment) by three orders of magnitude if the enzyme was prepared without exposure to exogenous reductant, as is the case with the EDTA extraction procedure [17].

Similarly, an average K'_D value of $3.5 \cdot 10^{-8}$ M ($\pm$S.D. of $1.0 \cdot 10^{-8}$ M; $n = 5$) was estimated when the *C. reinhardtii* $CF_1(-\delta)$ enzyme form was used as the

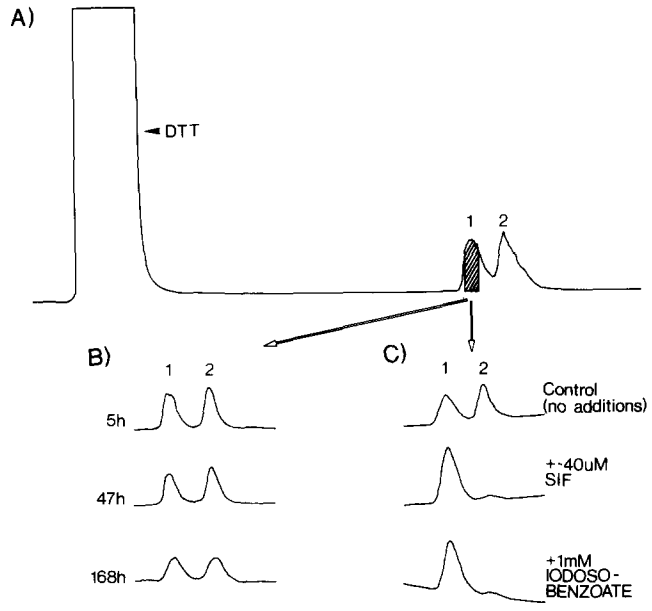

Fig. 4. Rechromatography of DTT pre-treated CF_1 with and without oxidants. Spinach CF_1 was incubated with 100 mM DTT overnight, then rechromatographed (panel A). The shaded region represents the fraction of DTT-activated, ϵ-subunit-containing CF_1 used for the subsequent experiments. Panel B shows the distribution of $CF_1(\pm\epsilon)$ when the enzyme was rechromatographed after dilution into 20 mM Mops–NaOH (pH 7.5) at 5, 47 or 168 h after elution of the DTT-activated, ϵ-subunit-containing enzyme. Panel C shows the distribution of $CF_1(\pm\epsilon)$ when the enzyme was rechromatographed after 1 h incubation in 20 mM Mops–NaOH (pH 7.5) containing either no additions (control), approx. 40 μM SIF or 1 mM *o*-iodosobenzoate. The traces were obtained from ultraviolet absorbance (280 nm) of the FPLC effluent. In all traces, peak 1 contains CF_1, peak 2 contains $CF_1(-\epsilon)$.

starting material. In experiments in which the enzyme concentration was varied above 10 μg/ml, the wt% $CF_1(-\epsilon)$ value diminished, whereas the K'_D estimate (above) was essentially constant. Lower values (about $6 \cdot 10^{-9}$ M) were obtained if the protein concentration was less than 10 μg/ml during preincubation. This may have been due to such factors as increased error of measurement or loss of enzyme at low protein concentrations. In all cases, the relative increase over the control K'_D (no DTT pretreatment) was much greater if the enzyme was originally prepared via the EDTA extraction procedure than by the chloroform extraction procedure due to the lower control K'_D value no greater than $5 \cdot 10^{-11}$ M.

The role of oxidation in ϵ-binding and ATPase inhibition

The discovery that the low ϵ-affinity (high K'_D) form of CF_1 was stable even after the removal of DTT greatly facilitated the study of the effect of oxidation on the ϵ-subunit dissociation. One could simply saturate the enzyme with 100 mM DTT overnight, rechromatograph the reduced CF_1 (from spinach) or reduced $CF_1(-\delta)$ (from *C. reinhardtii*) to remove DTT and residual ϵ-deficient enzyme forms, incubate the DTT-pretreated enzyme with various oxidants, then assay the distribution of $(\pm\epsilon)$enzyme forms via FPLC analysis. A representative experiment is diagrammed in Fig. 4.

FPLC analysis of DTT-pretreated CF_1 that was incubated with *o*-iodosobenzoate for 1 h (at concentrations of either 1 mM or 50 μM) demonstrated that essentially all of the enzyme chromatographs as the ϵ-containing form. Rapidly vortexing the DTT-pretreated CF_1 with 1 mM *o*-iodosobenzoate, followed by immediate FPLC analysis (sample was completely applied to the column in 10 min) resulted in the appearance of only a single peak of the ϵ-containing form. This contrasted to the control experiment lacking any oxidants in which two smaller peaks appeared, which corresponded to both ϵ-containing and ϵ-deficient forms of the enzyme.

Similar experiments suggested that potassium ferricyanide, although it is often used to inactivate the ATPase activity of reduced $CF_1 \cdot CF_0$ [24–26], was not an appropriate oxidant for the study of the dissociation and rebinding of the ϵ-subunit to the reduced, solbule CF_1. The total amount of recovered CF_1 enzyme forms (relative to non-oxidized controls) was always greatly diminished after treatment with potassium ferricyanide. One possible explanation could be that potassium ferricyanide effectively destroyed the recognizable soluble forms of the enzyme. It has already been noted that ferricyanide can oxidize tyrosine and tryptophan, and thus, "one cannot generally assume the simple formation of disulfides from the action of ferricyanide on enzymes" [36]. Alternatively, the potassium ferricyanide may have formed a complex with the enzyme which did not elute from the Mono Q column under the conditions used. The use of potassium ferricyanide as a model oxidant was not pursued further.

On the other hand, incubation of DTT-pretreated CF_1 with low concentrations (estimated to be on the order of 40 μM; Selman-Reimer, S., unpublished data) of the partially purified soluble inactivating factor (SIF) [27] resulted in the formation of a single chromatographic peak of the ϵ-containing CF_1, without the detectable loss of total CF_1. In this respect, *o*-iodosobenzoate was perhaps a better functional analog to SIF than was potassium ferricyanide. *o*-Iodosobenzoate has been used by many laboratories to oxidize vicinal dithiols in CF_1 or $CF_1 \cdot CF_0$ [11,25,28,29,37].

Inhibition of ATPase activity of DTT pretreated $CF_1(\pm\epsilon)$ by o-iodosobenzoate

Treating the reduced ϵ-containing CF_1 with compounds that putatively oxidize vicinal dithiols increased the affinity for the ϵ-subunit, as described above. It has been shown by others that oxidation of ϵ-containing CF_1 or of ϵ-deficient CF_1 plus purified ϵ-subunit inhibits the ATPase activity [11]. The question arose as to

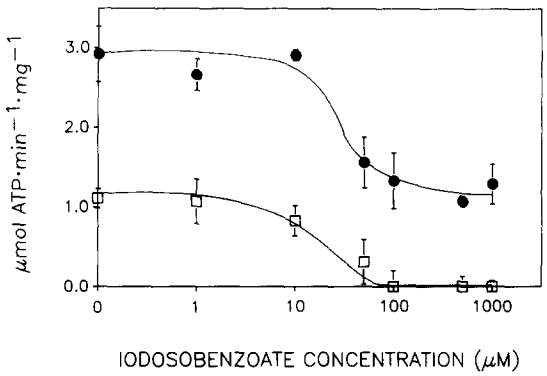

Fig. 5. Inhibition of DTT-enhanced $CF_1(\pm\epsilon)$ by *o*-iodosobenzoate. *C. reinhardtii* $CF_1(-\delta)$ was handled as described in Materials and Methods to yield either latent $CF_1(-\delta)$, DTT-activated $CF_1(-\delta)$ or DTT-activated $CF_1(-\delta,\epsilon)$. The three enzyme forms were incubated with various concentrations of *o*-iodosobenzoate for 5 min at aprox. 25 °C, then ATPase activity assayed as described in Materials and Methods. The latent $CF_1(-\delta)$ possessed no detectable activity above the non-enzymatic control and is not shown. DTT-activated $CF_1(-\delta,\epsilon)$ is represented by solid circles and DTT-activated $CF_1(-\delta)$ is represented by open squares.

whether oxidation of the CF_1 in the absence of the ϵ-subunit is sufficient to inhibit the ATPase activity. This related to the primary mechanism of converting a completely latent soluble CF_1 into a CF_1 with manifest ATPase activity during the room temperature DTT activation process. Was it the reduction step itself, or was it the dissociation of the ϵ-subunit?

For this experiment, *C. reinhardtii* was the source of the enzyme forms. As described in Materials and Methods, activated $CF_1(-\delta)$ and $CF_1(-\delta,\epsilon)$ were prepared by presaturating enzyme with DTT followed by FPLC repurification; latent $CF_1(-\delta)$ was obtained by identical handling of enzyme in the strict absence of DTT. The results of assaying these three enzyme forms for ATPase activity is plotted in Fig. 5. In the absence of additional stimulants, the latent $CF_1(-\delta)$ had no detectable ATPase activity over the non-enzymatic control. As anticipated, the ϵ-deficient enzyme had a greater activity than did the ϵ-containing enzyme in the absence of *o*-iodosobenzoate; these activities were unchanged by incubation with 1 μM *o*-iodosobenzoate. Increasing the *o*-iodosobenzoate concentration above 10 μM inhibited the ATPase activity of both enzyme forms. However, preincubation of the ϵ-deficient CF_1 with 100 μM to 1 mM inhibited the ATPase activity to approx. 40% of the control value, whereas preincubation of the ϵ-containing CF_1 completely inhibited the ATPase activity. Thus oxidation of reduced coupling factor in the absence of the ϵ-subunit was not sufficient to return the enzyme to a completely inactive ATPase. The ϵ-subunit was also required to completely inactivate the ATPase activity in the species we have studied.

Discussion

We have demonstrated the exchange of radiolabelled subunits from $CF_1(-\delta,\epsilon)$ into $CF_1(-\delta)$. A simple explanation for this is that the ϵ-subunit has dissociated from the nonlabelled $CF_1(-\epsilon)$ enzyme and rebound to the radiolabelled $CF_1(-\delta,\epsilon)$ enzyme. Another possibility is that the major CF_1 subunits (α, β, and/or γ) have dissociated and reassembled. This, however, is less likely for at least two reasons. One reason is that we observe no isolated β subunit, which should elute just before $CF_1(-\delta)$ under the chromatographic conditions used (Refs. 35, 14 and R.J.D. unpublished results). The second reason is that techniques for dissociation of the major subunits into reconstitutively active isolated subunits typically require extreme conditions (molar concentrations of chaotropic salts or of urea; see Refs. 30, 31). This contrasts to the dissociation of ϵ-subunit, which occurs during some treatments that activate ATPase activity, such as the addition of detergent [8] or heating in the presence of ATP [9,10]. Due to the observation that $CF_1(-\delta,\epsilon)$ can be formed via DTT incubation of the precursor $CF_1(-\delta)$ [5], we interpret our results as evidence for the reversible dissociation of the ϵ-subunit, rather than merely evidence for a direct transfer of the ϵ-subunit.

From the results presented here, and from our previous results [5], we conclude that an effect of DTT is to enhance the dissociation of the ϵ-subunit, resulting in an increased population of ϵ-deficient CF_1. This is in contrast to previous findings [11] which were based on SDS-PAGE analysis of potentially heterogeneous protein solutions. Our conclusions are not in disagreement with the fundamental suggestion [11,32] that the γ- and ϵ-subunits interact. Moreover, our results do not contradict published observations [11] which show that the 'holoenzyme' and $CF_1(-\epsilon)$ are inhibited in near-identical fashions by addition of the ϵ-subunit. The comparison of protein distributions and relative specific activities of the CF_1 forms during the DTT incubation time course leads one to suggest that the ϵ-deficient population is the active enzyme form in a DTT-activated mixture. Thus one may again raise the issue of whether dissociation of the ϵ-subunit is an obligatory step in the formation of an active ATPase.

The subtle variability in the amount of $CF_1(-\epsilon)$ generated from a CF_1 precursor in the putative absence of DTT appears to be due to the stability of the reduced enzyme. Since most of our work employed enzyme purified via the chloroform extraction technique, which contains subsaturating amounts of DTT, the proportion of $CF_1(-\epsilon)$ generated from CF_1 in the absence of additional DTT has ranged from the limits of detection ($\leqslant 3\%$ of the total recovered CF_1) to as high as approx. 33% of the recovered CF_1. This variability was not random, but was dependent upon the protein con-

centration of the experiment and the number of chromatographic purification cycles preceding the experiment, in addition to the DTT exposure history of the enzyme. The amount of $CF_1(-\epsilon)$ generated from CF_1 repurification is nearly eliminated when the enzyme is originally prepared via EDTA extraction followed by FPLC.

All of the above observations are consistent with dissociative behavior described by a simple K'_D for each of two states. Andralojc and Harris [11] have estimated apparent dissociation constants for the spinach system based on the inhibition of ATPase activity by addition of purified ϵ-subunit. The fact that our estimates, independently derived from the distribution of enzyme forms obtained from biological sources, are similar to the previous estimates supports the accuracy of the constants. However, our techniques clarify the point that DTT activation can indeed free the 'intrinsic' ϵ-subunit from CF_1.

We have not yet identified the specific mechanism by which DTT enhances the dissociation of the ϵ-subunit from CF_1. Earlier work suggested that there were no intersubunit disulfide bonds in CF_1 [33,34]. Chemical modification of the single cysteine residue on the ϵ-subunit did not prevent the modified ϵ-subunit from inhibiting ATPase activity [32]. Therefore, an indirect (i.e. noncovalent) mechanism involving protein dynamics is probably involved. The slow rate of formation of a high-K'_D form via room-temperature reduction and the requirement of extremely high DTT concentrations suggests that there is a significant energy barrier to be overcome and that the high-K'_D form is at a higher energy level. This would be consistent with the much faster and more efficient process of oxidative inactivation.

It will be interesting to compare the ATPase activation of F_1 from certain cyanobacteria with the behavior of the CF_1 forms studied in this paper. Cysteines 198 and 204 of the γ-subunit, which are thought to form the disulfide bridge reduced by DTT [29], are conserved in *C. reinhardtii* [38] and spinach [39], but not in *Synechococcus* 6301 [40] or *Anabaena* 7120 [41]. However, the γ-subunits, from all four photosynthetic species appear to retain the 'light-accessible sulfhydryl' (cysteine 89) originally described in the spinach system [42].

Taken together, the data presented here and in our previous paper [5] allow us to present a simple model for the configurational behavior of the DTT-treated CF_1 enzyme forms. This is summarized in Fig. 6. We propose that the active ATPase forms are the forms deficient in the ϵ-subunit and that the completely latent forms are the ones that contain the ϵ-subunit. Both forms can be further stimulated by a number of techniques. However, several of these techniques, such as detergent treatment [8], heating in the presence of ATP [9] and methanol activation [35] have already been

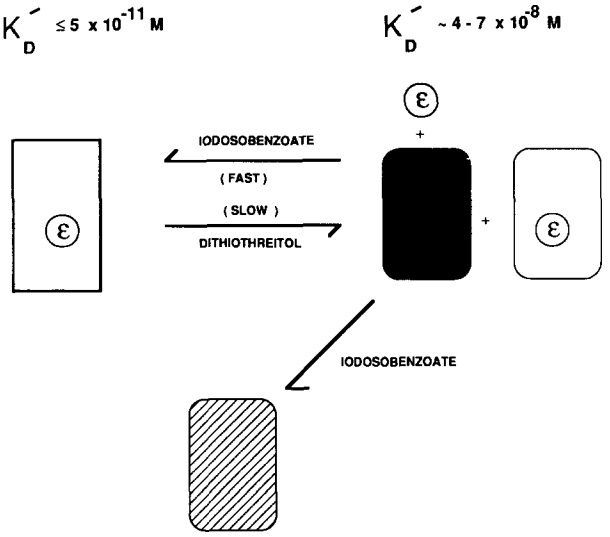

Fig. 6. A simple model for the relationship between ϵ-affinity, oxidation state, and ATPase activity of soluble CF_1. Unshaded enzyme forms have completely latent ATPase activity, fully shaded enzyme forms have manifest ATPase activity, and partially shaded forms have partially inhibited ATPase activity.

shown to overcome the inhibitory effect of the ϵ-subunit. Further investigation is needed to determine whether all known MgATPase activation techniques involve an obligatory transition to an ϵ-deficient configuration. Understanding how (and if) the behavior of the soluble CF_1 oligomer relates to the synthetic activity of the $CF_1 \cdot CF_0$ will be even more challenging.

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