Inverse regulation of F_1-ATPase activity by a mutation at the regulatory region on the γ subunit of chloroplast ATP synthase

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Chloroplast ATP synthase is a thiol-modulated enzyme whose $\Delta\mu H^+$-linked activation is strongly influenced by reduction and the formation of a disulphide bridge between Cys^{199} and Cys^{205} on the γ subunit. In solubilized chloroplast coupling factor 1 (CF_1), reduction of the disulphide bond elicits the latent ATP-hydrolysing activity. To assess the regulatory importance of the amino acid residues around these cysteine residues, we focused on the three negatively charged residues Glu^{210}-Asp-Glu^{212} close to the two cysteine residues and also on the following region from Leu^{213} to Ile^{230}, and investigated the modulation of ATPase activity by chloroplast thioredoxins. The mutant γ subunits were reconstituted with the α and β subunits from F_1 of the thermophilic bacterium *Bacillus* PS3; the active ATPase complexes obtained were purified by gel-filtration chromatography. The complex formed with a mutant γ subunit in which Glu^{210} to Glu^{212} had been deleted was inactivated rather than activated by reduction of the disulphide bridge by reduced thioredoxin, indicating inverse regulation. This complex was insensitive to the inhibitory CF_1-ε subunit when the mutant γ subunit was oxidized. In contrast, the deletion of Glu^{212} to Ile^{230} converted the complex from a modulated state into a highly active state.

Key words: activation, chloroplast coupling factor 1, thiol modulation, thioredoxin.

INTRODUCTION

F_0F_1-ATP synthase synthesizes ATP from ADP and P_i driven by a proton-motive force [1–3]. The enzyme consists of the membrane-embedded sector F_0, responsible for proton translocation, and the extrinsic catalytic part F_1. The architecture of F_1 is very similar in various kinds of cells or organelles. It is composed of five different subunits designated α, β, γ, δ and ε; their molecular stoichiometry is 3:3:1:1:1 [4]. Three catalytic sites are located on the β subunits at the interfaces with the α subunits. Three non-catalytic nucleotide-binding sites are located on the α subunits at the interfaces with the β subunits. The α and β subunits, which have similar three-dimensional structures, alternate in a hexagonal arrangement around a central cavity containing the N-terminal and the C-terminal helices of the γ subunit [5]. The crystal structure of an $\alpha_3\beta_3$ complex from the thermophilic bacterium *Bacillus* PS3 is completely symmetrical [6] but the incorporation of the γ subunit into this complex induces a functional asymmetry between the three catalytic sites [7].

The ATP synthase of chloroplasts (CF_0CF_1) is activated by the proton gradient and modulated by the reduction of a disulphide bridge in the γ subunit (thiol modulation) [8]. In the solubilized chloroplast coupling factor 1 (CF_1), reduction of the disulphide bond elicits the latent ATP-hydrolysing activity. The structural basis for thiol modulation is assigned to a sequence of nine amino acids containing two cysteine residues (Cys^{199} and Cys^{205} in spinach) in the γ subunit [9]. This motif is characteristic for CF_0CF_1 from plants containing chlorophyll $a+b$, i.e. green algae and land plants. Reduction *in vitro* can be achieved by dithiothreitol (DTT) or other dithiols but the preferred natural reductant for thiol modulation is reduced thioredoxin *f* (Trx-*f*) [10]. Introduction of the nine-residue sequence containing the two cysteines into the cyanobacterial γ subunit induced thiol modulation in *Synechocystis* [11,12]. In contrast, replacement of the two cysteine residues by serines in the γ subunit of CF_0CF_1 from the green alga *Chlamydomonas reinhardtii* [13] resulted in a DTT-insensitive enzyme. We have succeeded in reconstituting an $\alpha_3\beta_3\gamma$ complex from the individual recombinant α and β subunits from F_1 of *Bacillus* PS3 (TF_1) and recombinant γ subunit from spinach CF_1 [14]. The resulting chimaeric complex had substantial ATPase activity that was affected by the disulphide/dithiol state of the two regulatory cysteine residues, implying a transfer of redox control to TF_1 by the spinach CF_1-γ subunit. Further studies on this complex indicated the importance of the region around the disulphide bridge for the interaction between the γ subunit and the ε subunit [15]. The segment of approx. 30 residues following the regulatory sequence is also missing from γ subunits of heterotrophic bacteria and mitochondria. Recently we succeeded in introducing this amino acid sequence, including the regulatory region of the CF_1-γ subunit, into the respective position of the TF_1-γ subunit. This mutant TF_1-γ subunit was

Abbreviations used: CF_1, chloroplast coupling factor 1; CF_0CF_1, ATP synthase from chloroplast thylakoid membrane; DTT, dithiothreitol; $\gamma_{(TCT)}$, γ subunit of TF_1 in which 111 residues from Val^{92} to Phe^{202} were replaced by 148 residues of the equivalent region from spinach CF_1-γ; TF_1, F_1 from the plasma membrane of *Bacillus* PS3; Trx, thioredoxin.

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designated $\gamma_{(TCT)}$. The $\alpha_3\beta_3\gamma_{(TCT)}$ complex was successfully expressed and functionally assembled in *Escherichia coli* cells [16]. Most interestingly, the ATPase activity of the complex obtained was clearly regulated by reduction or oxidation of the introduced regulatory region. Moreover, the activity was remarkably inhibited by the CF_1-ε subunit but not by the TF_1-ε subunit, although both subunits could bind to the complex. This inhibition was detectable only when the γ subunit was in its oxidized state. These results suggest that a certain structure of the regulatory region is important for the interaction between the γ subunit and the ε subunit.

The rotation of the γ subunit in the central cavity of the $\alpha_3\beta_3$ hexagon in relation to ATP hydrolysis was demonstrated by indirect methods [17,18] and direct methods [19–21] with the isolated F_1 or $\alpha_3\beta_3\gamma$ subcomplex respectively. Recently we succeeded in directly observing the rotation of the γ subunit with DTT-activated isolated CF_1 [22]. The elicitation of the ATP-hydrolysing activity via reduction of the disulphide bridge of CF_1 can be explained as the unlocking of the arrested γ subunit to allow its rotation within the $\alpha_3\beta_3$ ring. To investigate the molecular mechanism of this regulation of CF_1-ATPase, we prepared CF_1-γ subunits by oligonucleotide-directed mutagenesis carrying various mutations and deletions in the region following Cys^{199} and Cys^{205}. We found several amino acid sequences in this region that are important for the regulation of CF_1.

EXPERIMENTAL

Materials

Restriction endonucleases were obtained from Toyobo (Tokyo, Japan). The Bradford protein assay system was from Bio-Rad (Hercules, CA, U.S.A.). Urea was purchased from Nacalai Tesque (Kyoto, Japan). DTT was from Sigma. Lactate dehydrogenase and pyruvate kinase were from Roche Diagnostics (Mannheim, Germany). Other chemicals were of the highest grade commercially available.

Recombinant protein preparation

Recombinant plasmids carrying the gene for the γ subunit from spinach plastids (*atpC*) were previously constructed [14]. Oligonucleotide-directed mutagenesis was performed as described by Kunkel et al. [23]. The oligonucleotides used to create the mutant γ subunits were determined with the software MOTOJIMAN provided by Fumihiro Motojima (Tokyo Institute of Technology, Tokyo, Japan). Primers used for the mutant γ subunits were as follows: 5′-GTCGACGCAGCAGCTGCAGCGCTCTTCCGTCTCAC-3′ for $\gamma_{EDE/AAA}$, 5′-GGAAAATGTGTCGACGCAGCACTCTTCCGGTTAACAACAAAAGAAGGTAAGC-3′ for $\gamma_{\Delta EDE}$, 5′-GGAAAATGTGTCGACGCAGCAAAAGAAGGTAAGCTTACGGTAGAAAGAGAC-3′ for $\gamma_{\Delta 210E-218T}$, 5′-AAGACGAACTCTTCCGGTTAACAACAGTAGAAAGAGACATGATC-3′ for $\gamma_{\Delta 219K-224T}$ and 5′-TCACAACAAAAGAAGGTAAGCTTACGAAAACCGAAACACCAGC-3′ for $\gamma_{\Delta 225V-230I}$. Each of the genes containing these mutations was transferred to the expression vector pET23d (Novagen) and was transformed into the expression host *E. coli* strain BL21(DE3). Each of the γ subunits was expressed, yielding inclusion bodies, and purified further by methods described previously [14]. The recombinant CF_1-ε subunit was prepared and purified as described previously [14]. The recombinant α and β subunits of TF_1 were expressed in *E. coli* strain DK8 (*bglR*, *thi-1*, *rel-1*, *HfrPO1*, Δγ(*uncB-uncC*)*ilv*::*Tn10*) and purified as described [24,25].

Reconstitution of the chimaeric complex

Reconstitution of the chimaeric subunit complex was performed by the method described previously [14]. In brief, each of the isolated γ subunits, wild-type or mutant, was dissolved in a solution containing 8 M urea, 0.5 mM EDTA, 0.5 mM DTT and 50 mM Tris/HCl, pH 8.0. The dissolved γ subunits were mixed with α and β subunits from TF_1 in the proportions of 1:1:1 (w/v) and the urea concentration was adjusted to 4 M. The solution was dialysed against 50 mM Tris/HCl (pH 8.0)/200 mM NaCl/0.4 mM $MgCl_2$/0.4 mM ATP at 20 °C for 3–6 h. Under these conditions the formation of the $\alpha_3\beta_3$ complex is completely prevented [14]. After dialysis, undissolved γ subunits were removed by centrifugation and the supernatant was used for the measurement of ATPase activity. To purify the complex formed, the protein solution after dialysis was concentrated to 100 μl with a Microcon 100 (Millipore Co. Ltd) and subjected directly to gel-filtration HPLC with on a TSK-$G3000_{XL}$ column (7.5 mm × 300 mm; Tosoh Co., Tokyo, Japan). The peak fraction containing the complex was collected and used for the assay.

Preparation of recombinant chloroplast Trx

Spinach chloroplast Trx-*f* and Trx-*m* were overexpressed in *E. coli* and purified as described [26]. The concentrations of purified Trx-*f* and Trx-*m* were determined by measuring A_{278} and the use of published molar absorption coefficient values of 16830 $M^{-1}\cdot cm^{-1}$ and 20500 $M^{-1}\cdot cm^{-1}$ respectively [27].

Activation and inactivation of the complex

To activate or inactivate the chimaeric $\alpha_3\beta_3\gamma$ complex by thiol modulation, it was incubated with the solution containing 50 mM Tris/HCl, pH 8.0, 0.2 M NaCl, 0.4 mM ATP and 0.4 mM $MgCl_2$ in the presence of the indicated concentrations of DTT and Trx-*f* or Trx-*m* for reduction, or 20 μM $CuCl_2$ for oxidation, for 30 min at 30 °C; the complex solution was then used in the ATPase assay. The activation of ATPase dependent on the Trx concentration was measured as follows: the chimaeric complex was incubated in the presence of 10 μM DTT plus the indicated concentrations of Trx-*f* or Trx-*m* for 30 min at 30 °C, after which the ATPase activity of the complex was measured.

Measurement of ATPase activity

The reaction was initiated by the addition of chimaeric complex (typically 70 μl of 100–300 μg/ml purified complex or 0.67 mg of $\alpha+\beta$/ml of solution containing the mixture of subunits) to the ATPase assay solution (1.93 ml) containing 50 mM Tricine/KOH, pH 8.0, 2 mM ATP, 2 mM $MgCl_2$, 50 mM Na_2SO_3, 50 mM KCl, 5 mM phosphoenolpyruvate, 200 μM NADH, 50 μg/ml lactate dehydrogenase and 50 μg/ml pyruvate kinase. A_{340} was measured every 30 s for 20 min in a U-3100 spectrophotometer (Hitachi, Tokyo, Japan); data were stored in an on-line computer.

RESULTS AND DISCUSSION

Mutations at the regulatory region of the γ subunit of CF_1

If we take the γ subunit of F_1 from bovine heart mitochondria, that of F_1 from plasma membrane of *E. coli* and that of TF_1 as

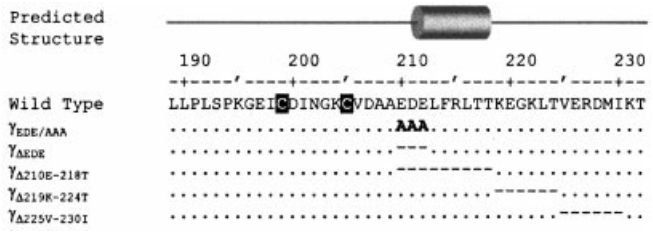

Figure 1 Construction of the mutant CF_1-γ subunit

The secondary structure of the regulatory region of the CF_1-γ subunit was predicted by EMBL-PHD software [28]. The amino acid sequences of five mutants at the region following the regulatory cysteine residues were shown. Substituted sequences are shown with bold letters and deleted sequences with dashes.

a baseline, the γ subunit of CF_1 has an additional stretch of 35–40 residues including Cys^{199} and Cys^{205}. There has so far been no informations on the three-dimensional structure of this region. To estimate its secondary structure, the software PHD provided by EMBL [28] was used. A loop with a short α-helix from Glu^{212} to Thr^{218} was predicted (Figure 1). In the γ subunits of chloroplast ATP synthase from land plants, algae and cyanobacteria, the region from Glu^{210} to Thr^{218} seems to be remarkably conserved, suggesting that this region might also have a certain role in regulation, in addition to the two regulatory cysteine residues.

Five mutant γ subunits shown in Figure 1 were designed and expressed in *E. coli*. The conserved cluster of three acidic amino acids (EDE; single-letter amino acid codes) in positions 210–212 was replaced by three alanine residues ($\gamma_{EDE/AAA}$) or deleted ($\gamma_{\Delta EDE}$); three more deletion mutants, $\gamma_{\Delta 210E-218T}$, $\gamma_{\Delta 219K-224T}$ and $\gamma_{\Delta 225V-230I}$ were prepared. All of them were expressed as inclusion bodies like the wild-type γ subunit of CF_1 [14]. They were purified and reconstituted with a mixture of recombinant α and β subunits from TF_1.

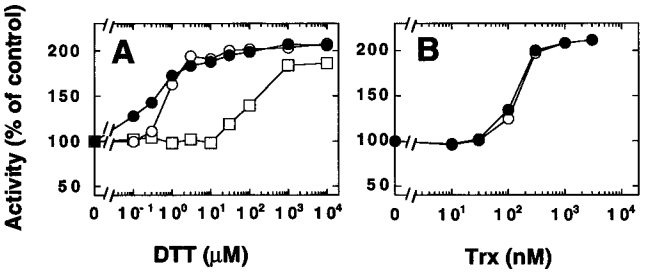

Figure 2 Activation of the ATPase activity of the chimaeric $\alpha_3\beta_3\gamma$ complexes by chloroplast Trx-*f* and Trx-*m*

The complex was reconstituted from 500 μg of wild-type CF_1-γ with 500 μg of TF_1-α subunit and 500 μg of TF_1-β subunit as described in the Experimental section. (**A**) The complex formed was incubated with the indicated concentrations of DTT in the absence (□) or presence of 2 μM Trx-*f* (●) or Trx-*m* (○) for 30 min at 30 °C; ATPase activity was then measured. Because the complex formed was not purified in this assay, the ATPase activity of the complex incubated only with 20 μM $CuCl_2$ was set as 100% and used as control. (**B**) The complex formed was incubated with the indicated concentrations of Trx-*f* (●) or Trx-*m* (○) in the presence of 10 μM DTT for 30 min at 30 °C; ATPase activity was then measured. The complex formed was not purified in this assay, so the ATPase activity of the complex incubated only with 10 μM DTT was set at 100% and used as control.

Activation of the chimaeric complex by reduced chloroplast thioredoxins

The membrane-bound CF_0CF_1 becomes thiol-modulated when chloroplast thylakoids are illuminated in the presence of reduced chloroplast Trx or bacterial Trx. From the kinetic analysis of activation, Schwarz et al. [10] concluded that Trx-*f*, which is 5-fold more efficient than Trx-*m*, should be the natural reductant *in vivo*. Here we investigated the activation of the chimaeric complex by chloroplast thioredoxins (Figure 2). Both Trx-*f* and Trx-*m* assisted in the activation of the chimaeric complex in the presence of DTT but Trx-*f* was slightly more efficient than Trx-*m* at DTT concentrations less than 1 μM. The activation of ATPase in the presence of Trx-*f* or Trx-*m* was saturated when the DTT concentration was more than 1 mM and the resultant activity was higher than that obtained without thioredoxins. We observed no activation by the further addition of DTT, suggesting that the γ subunit in the complex was mostly reduced under our experimental conditions.

The concentration of Trx-*f* or Trx-*m* required for the full activation of the chimaeric complex in the presence of 10 μM DTT was approx. 1 μM (Figure 2B), which was less than that required for the activation of CF_1 (more than 3 μM when 1 mM DTT was used) [26]. At constant Trx concentration, the concentration of DTT required to induce the activation of the enzyme was also lower for our chimaeric complex than for CF_1 [26]. These results suggest that the binding region of the ε subunit with the γ subunit is very close to the regulatory cysteine residues on the γ subunit and that the binding of the ε subunit to the complex affects the accessibility of Trx to the regulatory cysteines. He et al. [29] have also recently pointed out the competition between the ε subunit and Trx on the regulatory region from a kinetic analysis of the activation of membrane-bound CF_0CF_1 by *E. coli* Trx.

Redox regulation of the ATPase activity of the chimaeric complex containing mutant γ subunits

The responses of the chimaeric complexes containing these mutant γ subunits to DTT and Trx incubation were very different from those of the complexes containing the wild-type γ subunit (Figure 3). The complex with $\gamma_{EDE/AAA}$, which lacked the negatively charged amino acid array adjacent to the regulatory cysteine residues, became insensitive to DTT; the addition of Trx-*f* or Trx-*m* did not enhance the ATPase activity of the complex.

Surprisingly, the complex with $\gamma_{\Delta EDE}$ was inactivated rather than activated by the addition of DTT (Figure 3B). This inactivation was even accelerated by chloroplast Trx-*f* and Trx-*m*, implying an inverse regulation mode. Again, Trx-*f* was slightly more effective than Trx-*m* at a DTT concentration of less than 1 μM, suggesting that the conformation of the γ subunit, which is important for Trx binding, was preserved in this mutant complex. The complex with $\gamma_{\Delta 210E-218T}$ did not show significant activation or inactivation by reduction (Figure 3C), like the other two deletion mutant complexes (Figures 3D and 3E). Hence the deletion of sequences following the charged clusters (Glu^{210}–Glu^{212}) makes the complex insensitive to redox regulation.

Change in ATPase activity by the introduction of the mutation into the γ subunit

The mutations in the γ subunit might affect the specific activity and/or the stability of the reconstituted complexes. We therefore purified the complexes by gel-filtration HPLC after reconstitution

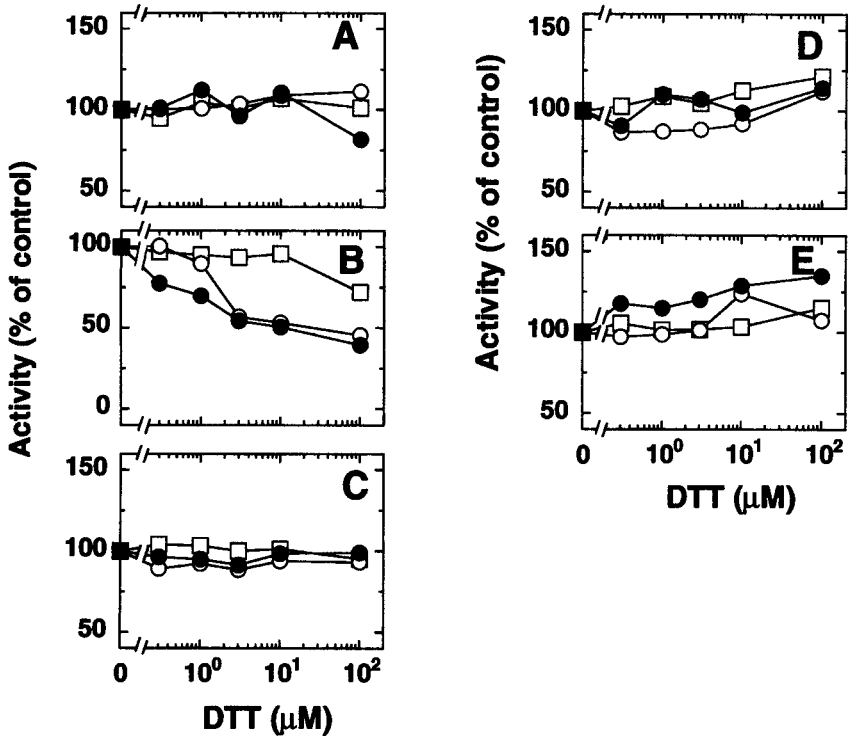

Figure 3 Activation and inactivation of the ATPase activity of the $\alpha_3\beta_3\gamma$ complexes containing mutant γ subunits by incubation with DTT

The complex was reconstituted from 500 μg of $\gamma_{EDE/AAA}$ (**A**), $\gamma_{\Delta EDE}$ (**B**), $\gamma_{\Delta 210E-218T}$ (**C**), $\gamma_{\Delta 219K-224T}$ (**D**) or $\gamma_{\Delta 225V-230I}$ (**E**), with 500 μg of TF_1-α subunit and 500 μg of TF_1-β subunit as described in the Experimental section. The complexes formed were incubated with the indicated concentrations of DTT in the absence (□) or presence of 2 μM Trx-*f* (●) or Trx-*m* (○) for 30 min at 30 °C; ATPase activity was then measured. The ATPase activity of the complex incubated only with 20 μM $CuCl_2$ was set at 100%.

from the individual subunits. Subsequently the γ subunits in the complexes were oxidized with 20 μM $CuCl_2$ or reduced with 1 mM DTT plus 2 μM chloroplast Trx-*m*; ATP hydrolysis activity was measured by the coupled pyruvate kinase/lactate dehydrogenase assay. TF_1 [30], the partial subunit complex $\alpha_3\beta_3\gamma$ [31] and the chimaeric complex $\alpha_3\beta_3\gamma_{(TCT)}$ [16] hydrolysed ATP at a linear rate for more than 10 min. However, under the same conditions the activity of the chimaeric complex decreased gradually during the measurement. This quasi-inactivation might have been the result of instability of the chimaeric complex. The mutations introduced into the γ subunit did not greatly affect this time-dependent decrease. We calculated the specific activity of each of the complexes from the initial slope of the trace for 5 min (Figure 4).

The ATP hydrolysis activities of the complex with wild-type γ subunit were 0.9 (oxidized) and 1.7 μmol of P_i released/min per mg of protein (reduced). The reduced form of the complex with $\gamma_{\Delta EDE}$ showed a similar activity to that of the control wild-type complex in the oxidized state; the activity of the oxidized mutant complex was similar to that of the reduced wild-type complex. This result suggests that the conformation of the $\gamma_{\Delta EDE}$ is not far from that of the wild-type γ subunit but the switching mechanism between the active and inactive forms is opposite.

Interestingly, the complex containing $\gamma_{\Delta 210E-218T}$ showed a 3-fold higher activity than the control complex although, like that with $\gamma_{\Delta EDE}$, this mutant complex also did not possess the array of three negatively charged amino acids. Furthermore, the complex with $\gamma_{\Delta 219K-224T}$ or $\gamma_{\Delta 225V-230I}$ showed similar activities. The ATPase activities of these three mutant complexes were slightly enhanced by reduction (Figure 4). However, we do not know whether the two regulatory cysteine residues in these mutant γ subunits were still able to form a disulphide bridge under oxidized conditions, or whether Trx was able to reduce a possible disulphide bridge formed between these cysteine residues. The amounts of complex obtained for this study were too small to determine the exact number of cysteine residues by biochemical methods. We recently reported that the introduction of regulatory segments of the CF_1-γ subunit, together with the surrounding 100 residues, into the bacterial γ subunit did not change the specific activity of the F_1 complex of bacterial origin [16]. Because the ATPase activity of this modified complex is also regulated by reduction–oxidation of the two regulatory cysteine residues, the whole conformation of the chimaeric γ subunit might not be far from that of the authentic one. To reach a definitive conclusion of how the regulatory segment of the γ subunit controls the activity of the complex, the three-dimensional structure of the regulatory segments of CF_1-γ and the manner of their interaction with the other region of the enzyme complex is necessary.

Interaction between the γ and ε subunits is important for inhibition of the ATPase activity of the complex

The ε subunit is an intrinsic inhibitor of the F_1-ATPase from chloroplasts and bacteria. The inhibitory effect is particularly marked for CF_1 [14,32–34]. In a previous study we found that the ATPase activity was less inhibited by the ε subunit when the chimaeric complex reconstituted with $\alpha_3\beta_3$ from TF_1 and γ subunit from spinach CF_1 was in the oxidized state than when it was in the reduced state (see Figure 4A of [15]). The $\alpha_3\beta_3\gamma_{(TCT)}$

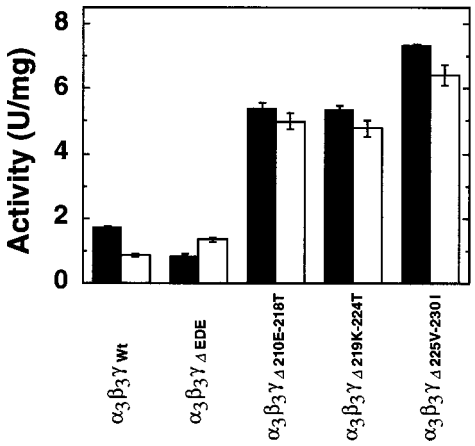

Figure 4 Comparison of the specific ATPase activities of the $\alpha_3\beta_3\gamma$ complexes containing mutant γ subunits

The complex was reconstituted and purified as described in the Experimental section and each of the complexes was incubated with 20 μM $CuCl_2$ (filled bars) or 1 mM DTT plus 2 μM Trx-*m* (open bars) for 30 min at 30 °C. Then 19 μg of purified complex with the wild-type (Wt) γ, 16 μg with $\gamma_{\Delta EDE}$, 8.3 μg with $\gamma_{\Delta 210E-218T}$, 7.9 μg with $\gamma_{\Delta 219K-224T}$ or 7.1 μg with $\gamma_{\Delta 225V-230I}$ was added to the reaction mixture and ATPase activity was calculated from the slope of the decrease in NADH absorption for 5 min after the initiation of the reaction. The results obtained are averages for three independent experiments.

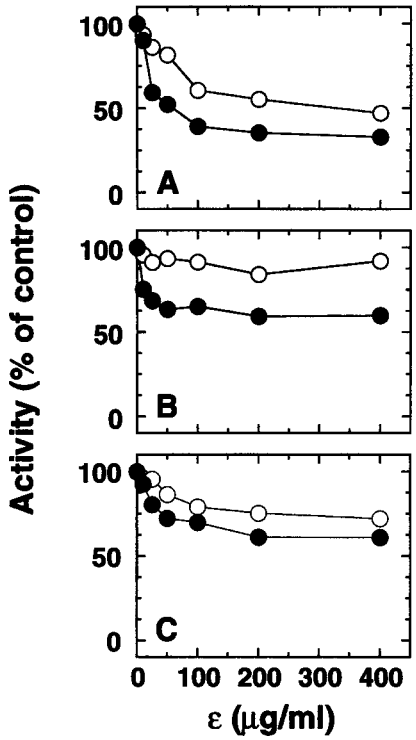

Figure 5 Effects of the ε subunit on the activity of the $\alpha_3\beta_3\gamma$ complexes

Chimaeric complexes were formed with wild-type CF_1-γ (**A**), $\gamma_{\Delta EDE}$ (**B**) and $\gamma_{\Delta 210E-218T}$ (**C**) as described in the legend to Figure 2. The complexes obtained were then incubated with 1 mM DTT plus 2 μM Trx-*m* or 20 μM $CuCl_2$ for 30 min at 30 °C. The obtained reduced (●) or oxidized (○) complexes were incubated with the indicated concentrations of CF_1-ε for 1 h at room temperature. Because the complex formed was not purified in this assay, the ATPase activity of the complex was measured with the solution containing the mixture of subunits and calculated on the basis of the amounts of α and β subunits; each of the activities without CF_1-ε was set at 100%.

complex, containing the regulatory region of CF_1-γ in the TF_1-γ subunit, was also inhibited by the CF_1-ε subunit only when the regulatory cysteine residues were reduced [16]. In contrast, there have been several reports that the ε subunit has a higher affinity for the γ subunit when CF_1 is in the oxidized state [32–34]. The variety of these results suggests that the affinity of the ε subunit for the γ subunit in the complex depends on the conformation of the γ subunit, which is determined by the redox state of two regulatory cysteine residues. Furthermore, the efficiency of reduction of the regulatory cysteine residues by Trx is decreased by the ε subunit on the complex, as mentioned above [29]. These results led us to conclude that the structure of the additional amino acid stretch is important not only for redox regulation but also for the interaction between the γ and ε subunits.

Here we investigated the effect of CF_1-ε subunit on the ATPase activity of the chimaeric complex containing mutated CF_1-γ subunits (Figure 5). As reported previously, our chimaeric complex with the wild-type CF_1-γ subunit was inhibited by the CF_1-ε subunit and the concentration of the ε subunit necessary for the maximal inhibition was lower when the complex was in the reduced state (Figure 5A). However, the complex containing $\gamma_{\Delta EDE}$ was not inhibited by CF_1-ε when the complex was in the oxidized active state (Figure 5B, open circle). Interestingly, the mutant complex containing $\gamma_{\Delta 210E-218T}$ was again inhibited by CF_1-ε, although the maximum inhibition level was much less than that of the wild-type complex (Figure 5C). Because the mutant $\gamma_{\Delta 210E-218T}$ also does not have the three negatively charged amino acids from Glu^{210} to Glu^{212}, we cannot conclude here that this array should alone be the determinant of the sensitivity to CF_1-ε. However, these results show clearly that the conformation of the γ subunit defined by the formation or reduction of the disulphide bond can directly affect the interaction with the ε subunit of CF_1.

Roles of the regulatory region and the following loop region

When the sequences of the γ subunit from different organisms were compared, the regulatory cysteine residues and their surroundings in the CF_1-γ subunit are very specific [9]. This region is predicted to comprise three segments: the two cysteine residues separated by five residues, a short α-helical region predicted by EMBL-PHD [28], and a following loop region. The γ subunit of cyanobacterial F_1 also has the part of this regulatory region that corresponds to the putative α-helical region and the following loop region but the regulatory cysteine residues are missing [11]. In the present study we focused on the segment around these cysteine residues. Because the array of three negatively charged amino acids (Glu^{210} to Glu^{212}) connects the regulatory cysteine region and the following part, the conformational changes induced by the formation or the reduction of the disulphide bridge between Cys^{199} and Cys^{205} must be transmitted directly to the putative α-helical segment plus loop region via these negatively charged amino acids. The deletion of this negatively charged amino acid array might change the geometry of the following region and affect the regulation mode itself. Our findings suggest that the conformational change in the regulatory region itself is responsible for the regulation of the ATPase.

To our knowledge, our complex with $\gamma_{\Delta EDE}$ mutant is the first report on an inverse thiol regulation of a chloroplast F_1-ATPase. Here we found that the negatively charged amino acid array following the regulatory cysteine residues is a determinant for the direction of thiol modulation. The mutant γ subunit, $\gamma_{\Delta EDE}$, should also be a useful tool for understanding the physiological role of thiol modulation of CF_0CF_1 when applied in experiments *in vivo*.

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